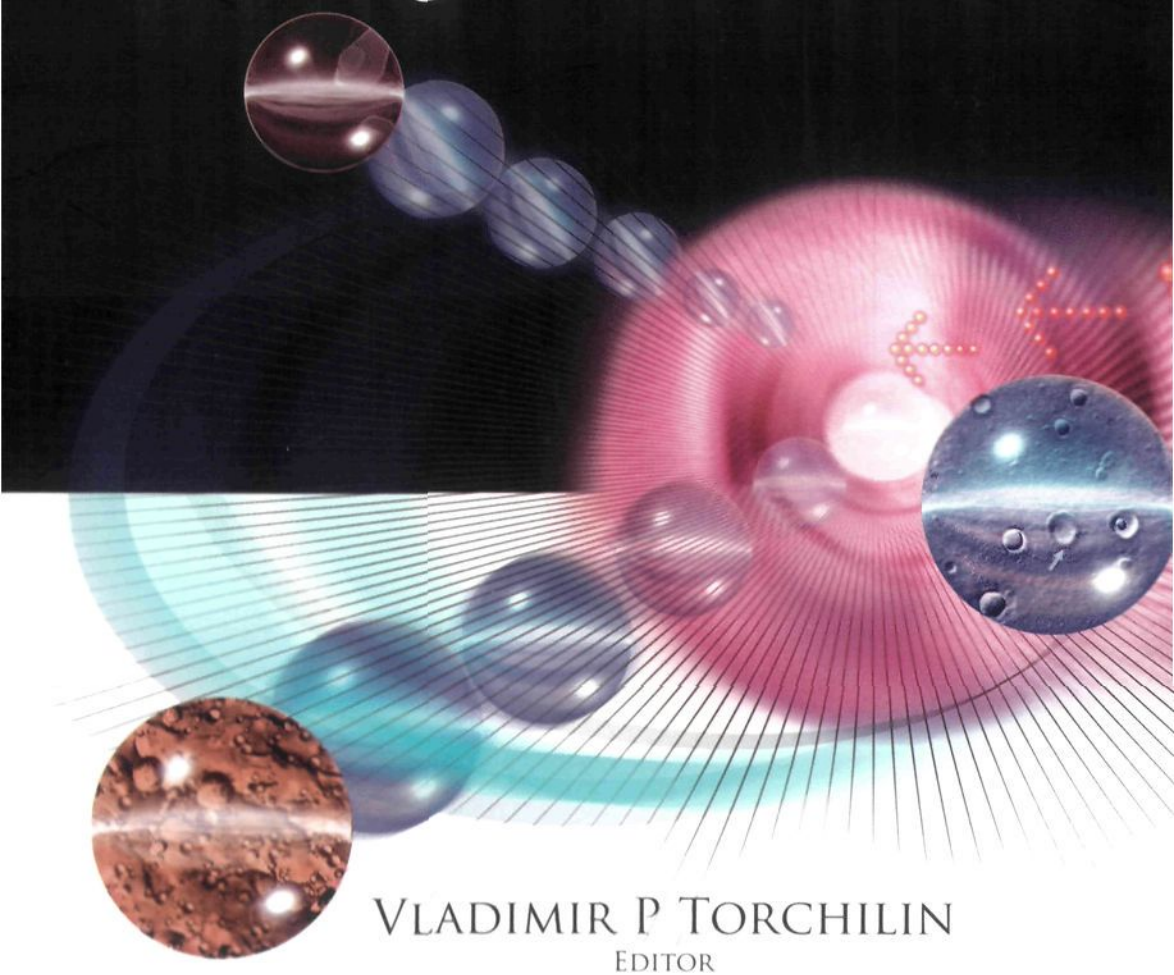
# Nanoparticulates as Drug Carriers



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Imperial College Press

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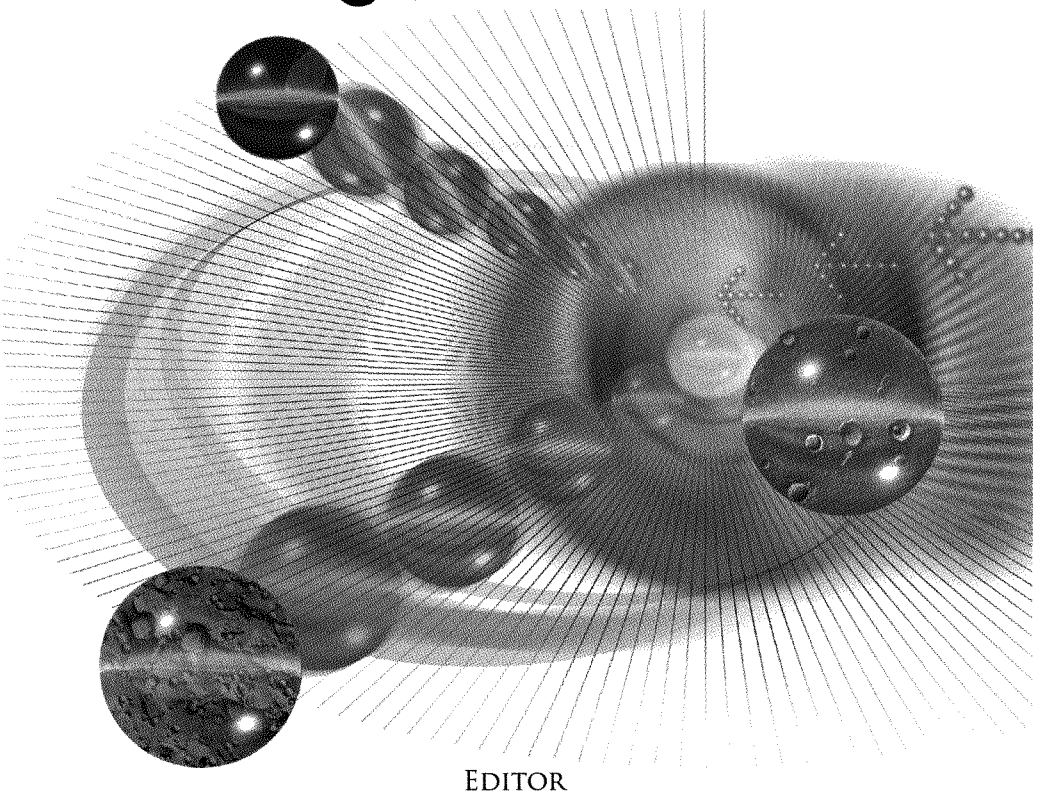

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ICP

Imperial College Press

*Published by*

Imperial College Press
57 Shelton Street
Covent Garden
London WC2H 9HE

*Distributed by*

World Scientific Publishing Co. Pte. Ltd.
5 Toh Tuck Link, Singapore 596224
*USA office:* 27 Warren Street, Suite 401-402, Hackensack, NJ 07601
*UK office:* 57 Shelton Street, Covent Garden, London WC2H 9HE

**British Library Cataloguing-in-Publication Data**
A catalogue record for this book is available from the British Library.

**NANOPARTICULATES AS DRUG CARRIERS**



ISBN 1-86094-630-5

Printed by FuIsland Offset Printing (S) Pte Ltd, Singapore

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# Contents

# 1

# Introduction. Nanocarriers for Drug Delivery: Needs and Requirements

*Vladimir Torchilin*

Fast developing nanotechnology, among other areas, is expected to have a dramatic impact on medicine. The application of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems has recently been determined by the NIH as nanomedicine. Among the approaches for exploiting nanotechnology developments in medicine, various nanoparticulates offer some unique advantages as pharmaceutical delivery systems and image enhancement agents.[1,2] Several varieties of nanoparticles are available[3]: different polymeric and metal nanoparticles, liposomes, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins, and many different nanoassemblies. All of these nanoparticles can play a major role in diagnosis and therapy. This book is attempting to present the broad overview of different nanoparticulate drug delivery systems with all their advantages and limitations, as well as potential areas of their clinical applications.

The paradigm of using nanoparticulate pharmaceutical carriers to enhance the *in vivo* efficiency of many drugs, anti-cancer drugs, first of all, well established itself over the past decade both in pharmaceutical research and clinical setting, and does not need any additional proofs. Numerous nanoparticle-based drug delivery and drug targeting systems are currently developed or under development.[4,5] Their use aims to minimize drug degradation upon administration, prevent undesirable side effects, and increase drug bioavailability and the fraction of the drug accumulated in the pathological area. Pharmaceutical drug carriers, especially the

ones for parenteral administration, are expected to be easy and reasonably cheap to prepare, biodegradable, have small particle size, possess high loading capacity, demonstrate prolonged circulation, and, ideally, specifically or non-specifically accumulate in required pathological sites in the body.[6]

High molecular weight (40 kDa or higher), long-circulating macromolecules, including proteins and peptides, conjugated with water-soluble polymers, are capable of spontaneous accumulations in various pathological sites such as solid tumors, infarcts, and inflammations via the enhanced permeability and retention effect (EPR).[7,8] This effect is based on the fact that pathological (tumor, infarct) vasculature, unlike vasculature of healthy tissues, is "leaky", i.e. penetrable for macromolecules and nanoparticulates which allows for macromolecules to accumulate in the pathological tissue (such as interstitial tumor space). In the case of tumors, such accumulation is also facilitated by the fact that lymphatic system, responsible for the drainage of macromolecules from normal tissues, is virtually not working in case of many tumors as the result of the disease.[8] It has been found that the effective pore size of most peripheral human tumors range from 200 nm to 600 nm in diameter, with a mean of about 400 nm. The EPR effect allows for passive targeting to tumors and other pathological sites based on the cut-off size of the leaky vasculature.[9]

Among particulate drug carriers, liposomes, micelles and polymeric nanoparticles are the most extensively studied and possess the most suitable characteristics for encapsulation of many drugs and diagnostic (imaging) agents. Many other systems meeting certain more specific requirements (and reviewed in this book) are also suggested and currently under development. Making these nanocarriers multifunctional and stimuli-responsive can dramatically enhance the efficiency of various drugs carried by these carriers. These functionalities are expected to provide: (a) prolonged circulation in the blood[10,11] and the ability to accumulate in various pathological areas (such as solid tumors) via the EPR effect (protective polymeric coating with PEG is used for this purpose)[12,13]; (b) ability to specifically recognize and bind target tissues or cells via the surface-attached specific ligand (monoclonal antibodies as well as their Fab fragments and some other molecules are used for this purpose)[14]; (c) ability to respond local stimuli characteristic of the pathological site by, for example, releasing an entrapped drug or specifically acting on cellular membranes under the abnormal pH or temperature in disease sites (this property could be provided by surface-attached pH- or temperature-sensitive coatings); (d) ability to penetrate inside cells bypassing the lysosomal degradation for efficient targeting of intracellular drug targets (for this purpose, the surface of nanocarriers may be decorated by cell-penetrating peptides). Those are just the most evident examples. Some other specific properties can also be listed, such as an attachment of diagnostic moieties. Even the use of individual functionalities is already associated with highly

positive clinical outcome — the success of Doxil®, doxorubicin in long-circulating PEG-coated liposome, represents a good example.[15]

In addition, there are numerous engineered constructs, assemblies, architectures, and particulate systems, whose unifying feature is the nanometer scale size range (from a few to 250 nm). Together with already listed systems, these include cyclodextrins, niosomes, emulsion particles, solid lipid particles, drug nanocrystals, metal and ceramic nanoparticles, protein cage architectures, viral-derived capsid nanoparticles, polyplexes, cochleates, and microbubbles.[4,5,16–19] Therapeutic and diagnostic agents can be encapsulated, covalently attached, or adsorbed on to such nanocarriers. These approaches can easily overcome drug solubility issues, particularly with the view that large proportions of new drug candidates emerging from high-throughput drug screening initiatives are water insoluble. Yet, some carriers have a low capacity to incorporate active compounds (e.g. dendrimers, whose size is in the order of 5–10 nm). There are alternative nanoscale approaches for solubilization of water insoluble drugs too.[20–23] One approach is to mill the substance and then stabilize smaller particles with a coating; this forms nanocrystals in size ranges suitable for oral delivery, as well as for intravenous injection.[24,25] Pharmacokinetic profiles of injectable nanocrystals may vary from rapidly soluble to slowly dissolving in the blood.

In general, the development of drug nanocarriers for poorly soluble pharmaceuticals represents a special task and still faces some unresolved issues. The therapeutic application of hydrophobic, poorly water-soluble agents is associated with some serious problems, since low water-solubility results in poor absorption and low bioavailability.[26] In addition, drug aggregation upon intravenous administration of poorly soluble drugs might lead to such complications as embolism[27] and local toxicity.[28] On the other hand, the hydrophobicity and low solubility in water appear to be intrinsic properties of many drugs,[29] since it helps a drug molecule to penetrate a cell membrane and reach important intracellular targets.[30,31] To overcome the poor solubility of certain drugs, clinically acceptable organic solvents are used in their formulations,[28] as well as liposomes[32] and cyclodextrins.[16] Another alternative is associated with the use of various micelle-forming surfactants in formulations of insoluble drugs.

By virtue of their small size and by functionalizing their surface with synthetic polymers and appropriate ligands, nanoparticulate carriers can be targeted to specific cells and locations within the body after intravenous and subcutaneous routes of injection. Such approaches may enhance detection sensitivity in medical imaging, improve therapeutic effectiveness, and decrease side effects. Some of the carriers can be engineered in such a way that they can be activated by changes in the environmental pH, chemical stimuli, by the application of a rapidly oscillating magnetic field, or by application of an external heat source.[19,33–35] Such modifications

offer control over particle integrity, drug delivery rates, and the location of drug release, for example, within specific organelles. Some are being designed with the focus on multifunctionality; these carriers target cell receptors and delivers drugs and biological sensors simultaneously. Some include the incorporation of one or more nanosystems within other carriers, as in the micellar encapsulation of quantum dots; this delineates their inherent nonspecific adsorption and aggregation in biological environments.[36]

The use of nanoparticulate drug carriers seems to be especially important for developing efficient anticancer therapies. Although significant advances have occurred in our understanding of tumor origin, growth, metastasis, and many different types of pharmacological agents have been developed over the years to treat tumors, the problem of optimum delivery remains a formidable challenge. For any of the drug therapy strategies to be effective, the agent must be able to reach the tumor mass in sufficient concentration, traverse through the tumor microcirculation, diffuse into the interstitium, and remain at the site for the duration to induce tumoricidal effect. As was already mentioned, due to the porosity of the tumor vasculature and the lack of lymphatic drainage, blood-borne macromolecules and nanoparticles are preferentially distributed in the tumor via the EPR effect. However, nanoparticles can also be actively targeted to tumors by modifying their surface with certain cell-specific ligands for receptor-mediated uptake. The use of specific "vector" molecules can further enhance tumor targeting of nanocarries or make them the EPR-effect independent. The latter is especially important for the cases of tumors with immature vasculature, such as tumors in the early stages of their development, and for delocalized tumors. Vector molecules (those having affinity toward ligands characteristic for target tissues), capable of recognizing tumors were found among antibodies, peptides, lectins, saccharides, hormones, transferrin and some low molecular weight compounds (riboflavin, folate). From this list, antibodies and their fragments provide the most universal opportunity to target various for targeting and have the highest potential specificity. Vector molecules can be used for the targeting of nanoreservoir delivery systems as well. PEG-modified long-circulating doxorubicin-containing immunoliposomes targeted with anti-HER-2/neu monoclonal antibody fragments represent a recent example of increased efficiency of targeted delivery systems.[37] In all studied HER2-overexpressing models, immunoliposomes showed potent anticancer activity superior to that of control non-targeted liposomes. In part, this superior activity was attributed to the ability of the immunoliposomes to deliver their load inside the target cells *via* the receptor-mediated endocytosis, which is obviously important if the drug's site of action sites locates inside the cell.

An important problem is associated with the clearance of drug carriers from the circulation. Nanoparticular pharmaceutical carriers administered into the systemic

circulation will be essentially removed within an hour of administration by the macrophages of the reticulo-endothelial system. To prolong the circulation of nanoparticles by evading the macrophages, their surface is modified with water-soluble polymers. Poly(ethylene glycol) (PEG) is very popular for surface modification of nanoparticulate drug delivery systems, since it has a long history of safe usage in biological and pharmaceutical products. Surface-bound PEG chains extend into the aqueous physiological environment and repel proteins, decrease antibody formation, and increase the circulation of the formulation in the plasma for extended period of time by the steric repulsion mechanism.[38]

With rapid advances in molecular biology and genetic engineering, there is an unprecedented opportunity for delivery of drugs and genes to intracellular targets.[39] In the case of cancer, for instance, the effectiveness of many anticancer drugs is limited due to its inability to reach the target site in sufficient concentrations and to exert the pharmacological effect. Current gene delivery systems are classified as being either viral or non-viral in origin. Viruses are efficient in delivery of genes; however, they suffer from poor safety profile. Non-viral gene delivery systems, albeit not as efficient as viruses, have promise of safety and reproducibility in manufacturing. To enhance delivery of drugs to intracellular targets and gene transfection efficiency using non-viral delivery systems, it is necessary to identify ways of overcoming the cellular barriers, for example, by using various cell-penetrating proteins and peptides.[40,41]

Self-assembled nanosystems (nanoassemblies) for targeting subcellular organelles, such as the mitochondria, are also developed.[42] It has become increasingly evident that mitochondrial dysfunction contributes to a variety of human disorders. Moreover, since the middle of 1990s, mitochondria, the "power houses" of the cell, have also become accepted as the cell's "arsenals", which reflects their increasingly acknowledged key role during apoptosis. Based on these recent developments in mitochondrial research, increased pharmacological and pharmaceutical efforts have led to the emergence of "Mitochondrial Medicine" as a whole new field of biomedical research.

Nanoparticulate drug delivery systems are very important for the delivery of peptide and protein drugs and may represent a valid alternative to soluble polymeric carriers used earlier. The use of this type of carriers allows achieving much higher active moiety/carrier material ratio compared with "direct" molecular conjugates. They also provide better protection of protein and peptide drugs against enzymatic degradation and other destructive factors upon parenteral administration, because the carrier wall completely isolates drug molecules from the environment. All nanoparticulate carriers have the size, which excludes a possibility of renal filtration. Among particulate drug carriers, liposomes are the most extensively studied and poses the most suitable characteristics for protein (peptide) encapsulation.

Similar to macromolecules, protein and peptide drug-bearing liposomes are capable of accumulating in tumors of various origins *via* the EPR effect.[6–8] In some cases, however, the liposome size is too large to provide an efficient accumulation via the EPR effect presumably due to relatively small tumor vasculature cut off size.[43,44] In such cases, alternative delivery systems with smaller sizes, such as micelles (prepared, for example, from PEG-phospholipid conjugates) can be used. These particles lack the internal aqueous space and are smaller than liposomes. Protein or peptide pharmaceutical agent can be covalently attached to the surface of these particles or incorporated into them via chemically attached hydrophobic group ("anchor").

In conclusion, even a brief listing of some key problems of nanocarrier-mediated drug delivery shows how broad and intense this area is. In addition to this, nanoscale-based delivery strategies are beginning to make a significant impact on global pharmaceutical planning and marketing. The leading experts in the area of nanparticulate-mediated drug delivery attempted to address these and many other topics in this book. We strongly believe that every reader will find the book useful and stimulating.

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# 2

# Nanoparticle Flow: Implications for Drug Delivery

*Alexander T. Florence*

## 1. Introduction

While the experimental study of nanoparticle flow *in vivo* proves to be difficult, a variety of theoretical and practical techniques are becoming available to allow some understanding of the phenomena involved. These processes include (a) convective flow induced by the flow of blood, lymph or interstitial fluid, (b) the influence of the interaction of nanoparticles with themselves or with biological components and the effect of this on their transport, and (c) the effect of fluid flow and hence shear forces on particle access to, interaction with and removal from receptors. Diffusion and movement of particle suspensions in complex media such as interstitial tissue must also be considered. Much of the theoretical work which is relevant to this exploration of nanoparticle flow has not been directed towards biological endpoints, but this body of knowledge, and the analogous literature on the dynamic behavior of bacteria, erythrocytes and platelets provides the basis of a more rigorous analysis of the factors involved in drug carrier nanoparticle flow.

As discussed in this book, nanoparticles are of value in drug, vaccine and gene delivery because their small dimension compared with microparticles allows them to interact more effectively with cells, be safely injected, and amongst other characteristics, diffuse further into tissues, and into and through individual cells. The flow of nanoparticles in capillaries, lymphatics, tumor vessels, their extravasation[1] and movement in the cytoplasm of cells are all aspects of the topic covered in

this overview, albeit from a phenomenological viewpoint. It is clear that particle diameter is a key parameter in the characterization and behavior of nanoparticle suspensions. In several of the analyses here, it becomes clear that another advantage of nanoparticles may be the relative lack of effect of shear stress, once particles adhere to surfaces as a prelude to uptake; this is contrasted with targeted microspheres whose residence on receptors and surfaces is size dependent, the larger particles being more vulnerable to detachment by shear forces.

This chapter considers questions relating to the flow of nanoparticles *in vivo*, but which has often been simulated *in vitro* by chemical engineers and physicists interested in particle behavior in flow conditions. Spherical particles are the norm, but not all nanosystems are spherical. The influence of asymmetry on the transport of nanoparticles *in vivo* is largely unknown, although the rheological characteristics of asymmetric particle suspensions have been understood for a long time. Flow behavior of nanoparticles in complex networks of narrow capillaries has relevance in the design and operation of microfluidic devices, as well as in drug delivery and targeting, and in toxicology[2]; the extent to which it is relevant for delivery and targeting is explored here. Figure 1 illustrates diagrammatically some of the areas of interest.

In physical terms, the following situations could be considered: (i) particle flow in rapidly flowing blood, including segregation and deposition of particles, and the behavior of particles at bifurcations in the capillary supply; (ii) the effect of shear on adhesion of particles of different size and shape; (iii) particle flow in more static conditions, for example, in the tumor interstitium or the lymphatics; (iv) flow of particles in tissues, including the flow of particles into narrow pores and (v) flow or diffusion within the anisotropic interior of cells. Added complications arise where bioadhesive or ligand-decorated particles are involved. In the latter case, binding and flow are linked.

The enquiry can be divided into a discussion of the flow and movement of nanoparticles as a function of their size and route of administration, the influence of convection and blood or lymph flow, the influence of flow dynamics on the interaction of nanoparticles with target tissues and receptors, and the movement of nanoparticles once they have been absorbed and are making their way through individual cells and tissues towards a target.

The routes of administration where flow is important potentially include all, even the oral route where particle flow and dynamics in the gut lumen and in the vicinity of both villi and microvilli is important. Particles below a critical size are taken up by the M-cells of Peyer's patches and by normal enterocytes, albeit in small quantities and find themselves in the lymph vessels, lymph nodes, blood, liver and spleen.[3,4] If the flow of nanoparticles away from their site of absorption is restricted due to the flow in lymph or blood being slow, this will reduce

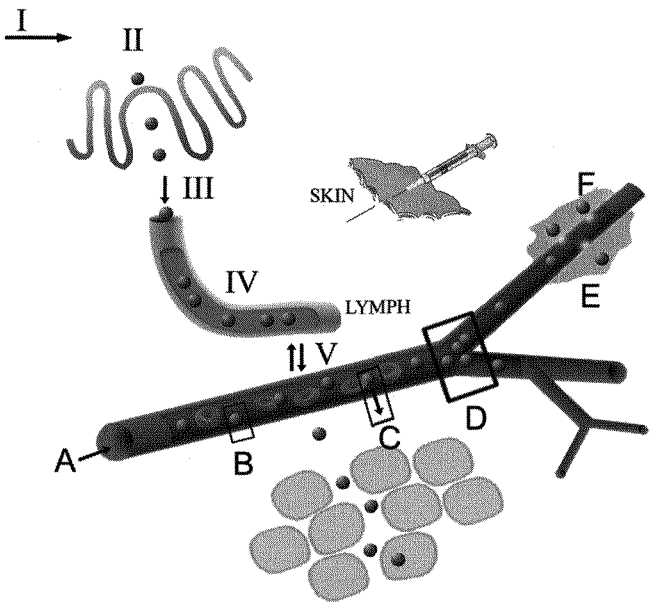


**Fig. 1.** A diagrammatic representation (not to scale) of some of the areas where flow and transport of nanoparticles is key. **I**: flow in the GI tract after oral administration; **II**: access to and adhesion to M-cells of Peyer's patches or to enterocytes; **III**: passage into the mesenteric lymph; **IV**: flow in the lymph vessels and entrapment in the lymph nodes (not shown); **V**: transport between lymph and blood. **A**: blood flow; **B**: adhesion to capillary walls; **C**: extravasation and flow in tissue; **D**: flow and deposition at vessel bifurcations; and **E**: movement into tumours. Each route (the subcutaneous route is also indicated) will involve a complex sequence of nanoparticle pathways, most involving lymph, blood and intestinal fluid.

bioavailability and distribution. Rapid flow provides superior sink conditions and hence the use of everted gut sacs and cell monolayers *in vitro* can give unrealistic results for nanoparticle transit. The influence of flow dynamics on extravasation and perhaps on the enhanced permeability and retention (EPR) effect for nanocarriers has perhaps not been fully addressed. Both involve consideration of particle size, the diffusion and flow of nanoparticles through narrow channels, as well as navigation of tortuous environments. The availability of "extreme" nanoparticles in the form of dendrimers[5] and quantum dots[6] makes this topic a vital one in understanding the fate, toxicity[7] or accumulation of what is metrically a wide range of systems.

## 2. Background

Our own interest in this field has resulted in part from studies on the size dependency of nanoparticle uptake after oral administration, where mesenteric lymphatic transport of 500 nm nanoparticles post absorption is determined by the flow of particles in a single file in the smallest mesenteric lymph vessels (Fig. 2). In addition,

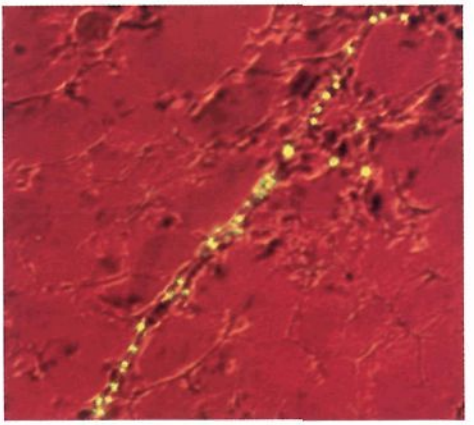

**Fig. 2.** 500 nm polystyrene latex particles flowing in the mesenteric lymph vessels mostly in single-file mode, from Jani *et al.*[10]

studies on the flow of liposomes and niosomes, and the extrusion of flexible vesicles from glass capillaries under pressure, converting polyhedral vesicles into multilayer tubules, led us to consider the influence of stress forces on carrier integrity. Clearly, the elasticity of vesicles is important in their negotiation of capillaries when their diameter exceeds that of the capillary. The flow of particles in fabricated capillaries which have a radius close to the particle radius is a challenge that has been tackled theoretically.[8,9] We have suggested that multi-bilayer tubules (Fig. 3) can act as models for such flow experiments.[11]

Rheological examination of nanoparticle-blood mixtures and nanoparticle suspensions of mixed radius has also illustrated the potential complexity of particle

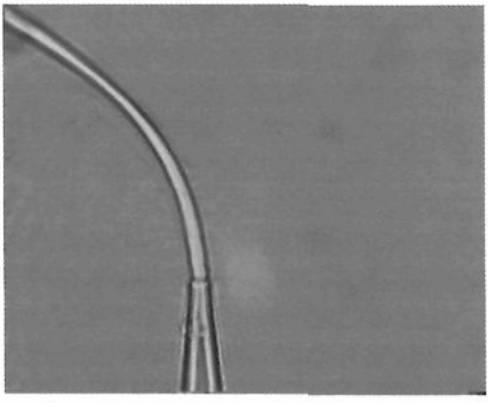

**Fig. 3.** A flexible non-ionic surfactant based multi-bilayer tube, around 1 $\mu$m in diameter, extruded from a suspension of polyhedral niosomes, which might be adapted for use as a model for the study of capillary nanoparticle flow.[11]

movement in blood (unpublished data). In addition, erythrocyte blockage at bifurcations, or narrowing of vessels can lead to slowing down of blood flow and a change in rheology as the haematocrit increases. More recently, investigation of the transport of nanoparticles across cell monolayers[12] and intracellular transport of dendrimers[13] has assisted in defining some of the issues involved in targeting within cells.

## 3. Studies on Nanoparticle Flow

The work of Fokin and colleagues[14] on the transport of viral-sized colloids, following intravenous or intra-lymphatic injection, is relevant to drug delivery even if their objectives were different. 100–200 nm diameter sulphur colloid particles reach the lymph after IV injection in around 25 minutes; after intra-lymphatic injection particles appear in the venous blood only after 4 seconds. Following subcutaneous injection, similar particles[14–16] reach the lymph after 2–9 min, although 95% of the particles remain at the injection site for at least 45 minutes. Here, the nanoparticles are being used as indicators of blood and lymph flow. What is also relevant to drug delivery is the influence of fluid flow on the movement and fate of nanoparticles. Illum *et al.*[17] observed uptake rates of 1.27 $\mu$m and 15.8 $\mu$m polystyrene particles in the lung and liver after IV injection. The sequestration in the lung was size dependent, but possibly affected because the smaller particles were taken up by the Kupffer cells of the liver, leaving the larger particles free to be taken up by the lung tissue. The rapidity of this suggests that, in effect, flow of the microparticles is solely determined by blood flow.

## 4. Convection and Diffusion

Blood flow drives the convective flow of suspended particles. Diffusional transport occurs in static conditions or conditions of low fluid velocity. In a tube of flowing liquid, convective dynamics propel the particles in the direction of flow, but at the walls of the tube, there is the possibility of particle diffusion resulting in deposition. Blood velocity (mm $s^{-1}$) in arterioles and venules is a function of vessel diameter, as shown in Fig. 4. In venules, the maximum velocity according to Jain[18] is approximately 12 mm $s^{-1}$, while in arterioles, it can reach about 30 mm $s^{-1}$.

Fluid velocity in tubes is not constant throughout the diameter of the tube as Fig. 5 indicates, a feature that is important when the interaction of nanoparticles with epithelial cells or capillary walls is considered.

The radial variation of shear is a factor that must be considered in polydisperse nanoparticulate systems and where nanoparticles adhere to erythrocytes, causing two distinct size distributions. If nanoparticles adhere to erythrocytes[20] or other

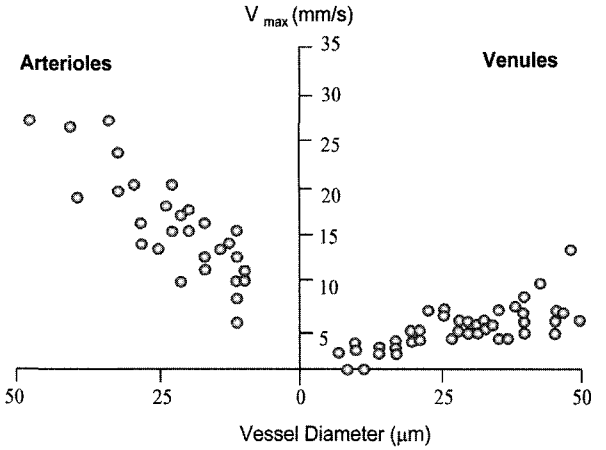


**Fig. 4.** Maximum blood velocity ($mms^{-1}$) in arterioles and venules as a function of vessel diameter, redrawn from R. K. Jain.[18]

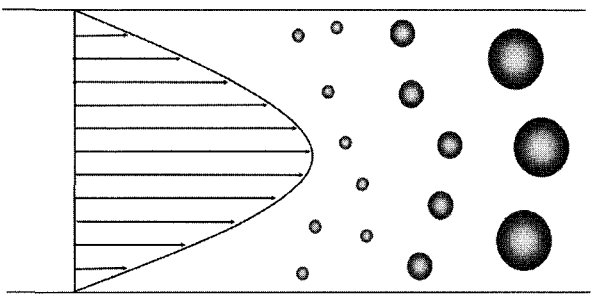

**Fig. 5.** Diagram showing the velocity pattern in a tube of flowing liquid. Particles of different size separate according to their diameter. The large particles, being unable to approach close to the capillary wall, experience the faster fluid streamlines toward the centre; hence, they move more rapidly, as described by Silebi and DosRamos.[18,19] This is the basis of the field flow fractionation.

blood elements, the translocation of the particles is controlled by the particular element to which it adheres. The rheology of suspensions of mixed particles is complex: viscosity reduces first with an increase in the fraction of larger particles in a suspension, and as the volume fraction increases, so does the viscosity.[21]

Ding *et al.*[22] formulated a theoretical model examining particle migration in nanoparticle suspensions flowing through a pipe. "The model considers particle migration due to spatial gradients in viscosity and shear rate as well as Brownian motion. Particle migration due to these effects can result in significant non-uniformity in particle concentration over the cross section of the pipe" in particular for larger particles. Three mechanisms were proposed by the authors for migration in such non-uniform shear flow: (i) shear induced migration where

particles move from regions of higher shear rate to regions of lower shear rate; (ii) viscosity gradient induced migration — particles move from regions of higher viscosity to regions of lower viscosity and (iii) self-diffusion due to Brownian motion.

Diffusion inside microtubules has been studied to understand taxol binding to tubulin structures.[23] The dimension of the tubulin lumen is of the order of 17 nm, approaching macromolecular dimensions, leading to friction between the inner walls and the moving macromolecules. This "hindrance" will also be an issue in the movement of nanoparticles in the smallest capillaries. With dendrimers whose diameters may be as small as 6 nm, the application of hindered theory to their movement could be relevant. No vessels are of this small radius, but the key parameter is the ratio of particle diameter to capillary diameter. The approach may well be important in cellular networks. It is not only capillary vessels that are the conduits of particle movement, but after extravasation, there is passage through cellular networks. The process could be considered to be akin to diffusion in porous networks. Binding of the moving particle (or macromolecule) to the luminal surface of the vessel will also hinder free flow or movement, a positive event in the case of specific ligand targeting of "decorated" systems.

Polydisperse nanosystems can segregate during flow or migrate differentially leading to concentration differences.[22] Particle-image velocimetry (PIV)[24] has been used to track the flow characteristics of microparticles. The effects of flow on adhesion of monocytes to endothelial cells[25] is relevant to the influence of flow and shear in particle interactions and uptake.

The significance of flow can be demonstrated by the use of pharmacological agents which change normal vessel patency, so that by the concomitant use of noradrenalin[26] or angiotensin[26,27] which constrict only normal vessels, the ratio of tumor to normal tissue blood flow can be optimized.

## 5. Bifurcations

Many theoretical studies of nanoparticle flow deal with linear tubes, whereas *in vivo* movement occurs through complex vessel architectures with bifurcations[28,29] (Fig. 6). Behavior at bifurcations in a vascular or capillary system is dependent not only on particle diameter, but also on the rigidity or flexibility of the particle concerned. Colloid transport in a bifurcating structure has been the subject of one recent paper.[30] It is a process which depends on the orientation of the bifurcations, especially with particles whose density is greater than that of the medium, as well as on the different flow rates in the individual branches which are likely to be of different sizes.

If nanoparticles are trapped or associate at bifurcations or indeed other obstacles in capillaries, then it is likely that they might associate more permanently, thereby

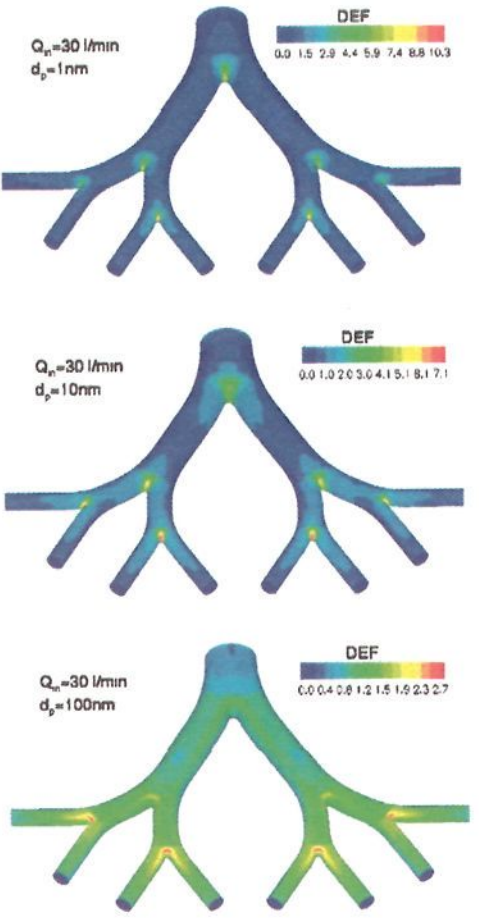


**Fig. 6.** Three-dimensional distributions of nanoparticles in a bifurcation airway model of Zhang *et al.*, *Aerosol Sci.*, 2005, **36**, 211–233. DEF is the deposition enhancement factor, the representations shown here being for a steady inhalation. While these data are for air-flow, not dissimilar patterns of deposition might be estimated to occur in liquid flows. Deposition in these models occurs primarily by Brownian diffusion; deposition efficiencies increase with decreasing nanoparticle size and lower inlet Reynolds numbers.

changing their intrinsic rheological behavior. Flexible particles do not of course suffer the same constraints in movement and progress, but their flexibility can lead to slow negotiation of movement around obstacles (Fig. 7).

## 6. Interaction with Blood Constituents and Endogenous Molecules

Nanoparticles may interact with blood constituents[31]: the adsorption of albumin, IgG and fibrinogen from blood onto hydrophobic particles is well known, but the

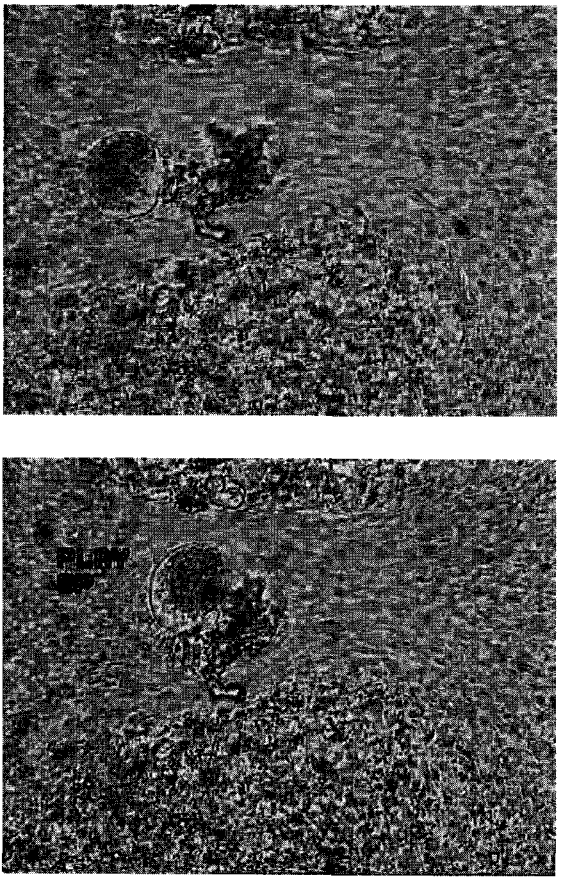

**Fig. 7.** Two captured pictures from a video of a large vesicle moving in a flowing stream of smaller vesicles. The stills show a flexible vesicle approaching an obstacle, and rolling around the obstacle while adhering to it, a process encouraged by its elasticity.

effect of nanoparticles on blood has been less well studied. Kim's[31] data indicate that the interaction of nanoparticles with erythrocytes changes the dynamics of flow of both erythrocytes and particles. Chambers and Mitragotri[20] found that nanoparticles as large as 450 nm adhered to erythrocytes, and thus remained in the circulation for several weeks. The percentage of latex nanospheres in the circulation over a period of 6 hrs was highly dependent on particle size, retention times decreasing with increasing diameter from 220 nm to 1100 nm. These data are difficult to interpret on the basis of flow, as the erythrocytes with attached nanoparticles are eliminated somewhat faster than the native erythrocytes. Gorodetsky and colleagues[32] explored interactions of carboplatin (CPt) nanoparticles (formed by CPt interaction with fibrinogen) with the fibrin mesh caused by the induction of clot formation.

## 7. Nanoparticles with Surface Ligands

There appear to be no rheological studies comparing surface protein decorated nanoparticles with the unadorned forms. Certainly, it is possible that aggregation may be caused by the change in surface properties and that this will in turn change flow patterns and perhaps masking of ligands[33] as posited in Fig. 8. Nanoparticles are of course sensitive to the medium in which they are placed[34] even *in vitro* when cell media can cause significant increases in diameter because of particle flocculation. We have suggested that the interaction with surface receptors of nanoparticles decorated with ligands is more complex than intimated in discussions of targeting generally.[33] Figure 8 represents some of the factors: the aggregation of particles, the masking of ligands by this process, the detachment of ligands and the shear-induced removal of attached particles as discussed above. The instability of plant lectins, frequently used as surface proteins on nanosystems, is discussed by Gabor *et al.*[35] The processes illustrated in Fig. 8 might explain some of the lack of complete success of targeted drug delivery.

## 8. Deposition on Surfaces and Attachment to Receptors in Flow Conditions

Nanoparticles *in vivo* flow in blood, lymph or tissue fluid at greater or lesser velocities, as discussed above. Deposition of particles which might occur in a static situation is itself a complex process, and will depend on the rugosity of the

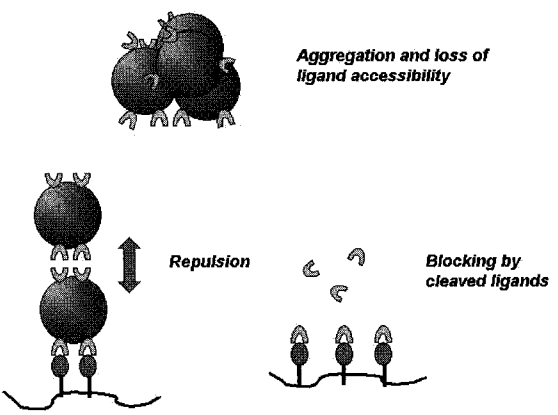


**Fig. 8.** Diagram illustrating variations from the ideal of a single ligand-decorated nanoparticle interacting with receptors spaced at an appropriate distance from the particles. The diagram shows the loss of ligand accessibility which would follow from the aggregation of the particles before interaction with the desired surface, repulsion between a particle attached to the receptor surface, and an approaching particle and blockage of the receptors due to interaction of cleaved ligands with the receptors.

receiving surface.[36] Particle deposition from flowing suspensions has been the subject of research[37] which has considered not only diffusion, convection, geometrical interception and migration under gravity, but also the influence of tangential interactions.

Patil *et al.*[39] examined the rate of attachment of 5, 10, 15 and 20 $\mu$m particles with a reconstituted P-selectin glycoprotein ligand-1 construct 19.ek.Fc. The rate of attachment was not affected by particle diameter. However, the shear stress required to set the adherent particles in motion ($S_c$) decreased with increasing particle diameter, and the rolling velocity of the 19.ek.Fc microspheres increased with increasing diameter. From their data, if we extrapolate the critical shear (a plot of $1/S_c$ is linear with diameter over the range 5–20 $\mu$m), it suggests that particles below one micron in diameter will not be removed by shear forces.

Usually we consider the flow of many particles in collective diffusion. The diffusion coefficient of a single particle and the collective diffusion coefficient coincides at infinite dilution, but can differ at higher concentrations.[40]

Cell adhesion mediated by not one but two receptors has been considered by Bhatia *et al.*[41]; the analysis would also apply to decorated nanoparticles. In their study, the two receptors were selectin and integrin ICAM; "the state diagram" evolved shows the area of firm adhesion as opposed to rolling adhesion for leukocytes as a function of receptor densities and association rate constants. The fate of

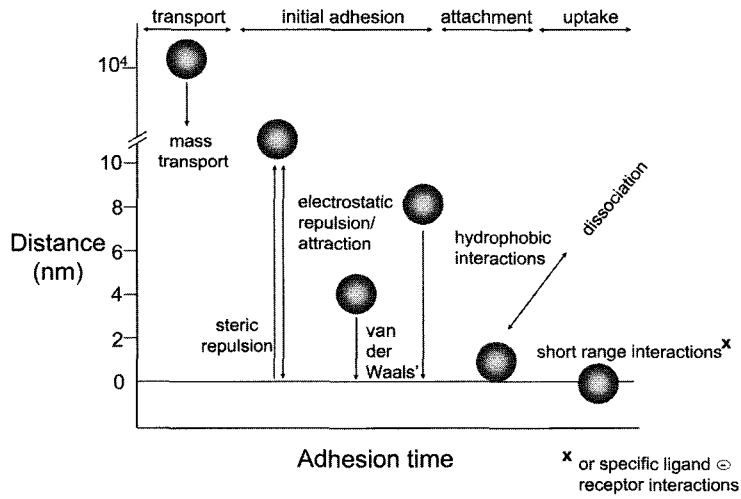


**Fig. 9.** Processes occurring in the deposition of nanoparticles in flow conditions as a function of the range of interaction forces (nm) and adhesion times. At the start, mass transport to the surface occurs, initial adhesion following through electrostatic attraction and van der Waals' forces. Hydrophobic interactions can play their part as well as specific receptor-ligand interactions which are short-range interactions. Drawn after Vacheethasanee and Marchant.[38]

nanoparticles in flowing blood, their adhesion, extravasation and permeation into tumors, thus depends on a complex of factors such as diameter, surface ligand density and orientation, shape, capillary diameter and rugosity, bifurcations, viscosity and flow gradients.

## 9. Does Shape Matter?

Nanosystems can be prepared in a variety of shapes. Nanocrystals[42] are often irregular; there are asymmetric carbon nanotubes, and surfactant and lipid vesicles can be produced as discs, polyhedral structures,[40,43] toroids and tubes.[21,44] The vesicle constructs often have dimensions larger than 500 nm; it must be assumed that vesicles in the nanometer size range will be less affected. In these systems, shape is less important than membrane properties in controlling the release of encapsulated drug, but the flow properties of vesicular suspensions are clearly determined by shape and elasticity. As most particulate delivery vectors have been spherical, little attention has been paid to the influence of shape on fate; yet it is known that the shape of environmental particles and fibres, for example, influences their fate and toxicity.[45]

As discussed above, there are two different but related effects of particle flow: the effect of particle shape and size and characteristics on flow, as well as the effect of flow on flexible particles, as discussed by Bruinsma.[46] With elastic vesicles, we have argued[44] that shape matters because it affects flow and potential fate *in vivo* through extravasation for instance; elasticity also allows vesicles to be transported in vessels which would be blocked by solid particles. The elasticity and visco-elasticity of such systems may be important in differentiating them from solid nanoparticles.

Much of the debate on whether the shape of vesicles matters, is dependent on the knowledge of the nature of the capillary blood supply and the forces exerted on, and the damage done to, vesicles as they move in capillaries.[44] In studies conducted in our laboratories with doxorubicin loaded niosomes, 60% of the drug remained in the vesicles 8 hrs after intravenous administration.[47] The extent to which the drug loss was due to diffusion or to damage is not known, but vesicles subjected to deliberate stress can lose considerable amounts of their payload, simply by extrusion of the vesicles through capillaries of reducing diameter.[48] Reduction in diameter of systems below 1 micron will clearly reduce such stresses and allow flexible systems to retain their loads intact.

Vasanthi *et al.*[49] treated the anisotropic diffusion of oblate spheroids, explaining that because non-spherical molecules rotate as they translate, their motion differs significantly from that of a sphere. For rods, theory predicts that the diffusion coefficient in the direction parallel to the major axis of the rod ($D_{II}$) is twice that in the perpendicular direction ($D_{I}$.).

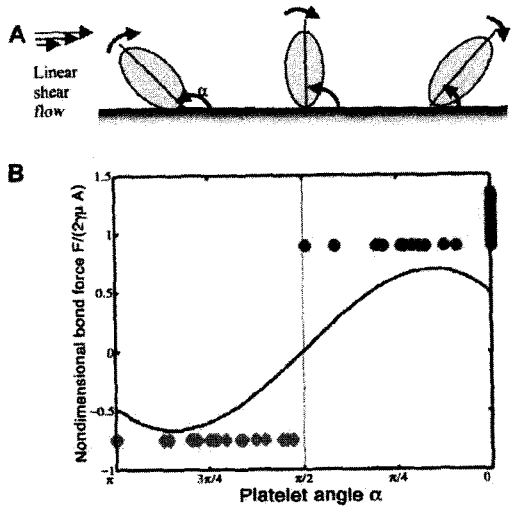


**Fig. 10.** The non-dimensional bond force as a function of the angle of an ellipsoidal platelet passing through zero when the platelet is 90° to the surface. From Mody *et al.*[50]

There are few studies which have considered the motion of ellipsoidal particles near a plane wall, although this is relevant to platelet flow and adhesion to the walls of vessels. Mody and colleagues[50] have addressed the issue, observing the effects of shear stress on platelet adhesion. Platelets, unlike leukocytes, do not roll but display a flipping motion in the direction of flow, due to their flattened ellipsoidal structure. The bond force between the ellipse and the surface is dependent on the platelet angle as defined in Fig. 10.

Flexible systems such as vesicles have been widely studied, while being forced under pressure in capillaries smaller than the vesicle diameter. The elasticity of the membranes can be estimated from the extent of deformation. Vesicle flow in linearly forced motion has been followed. Flexible vesicles adjust their shape to equilibrate the applied force[51]; locally in some cases, two-dimensional flow of lipids in the vesicle membrane occurs,[52] clearly influencing the position of the embedded surface ligands.

There are many nanoparticulates which are produced in non-spherical forms, hence the transport properties of asymmetric particles is important.[53]

## 10. Speculations on Flow and the EPR Effect

Erythrocyte velocity in normal vessels depends on vessel diameter (see Fig. 4 above), but there is no such dependence in tumors (Fig. 11), even though flow may be an order of magnitude slower. According to Jain,[18,52] "to reach cancer cells in a tumor, a blood-borne therapeutic molecule, particle or cell must make its way

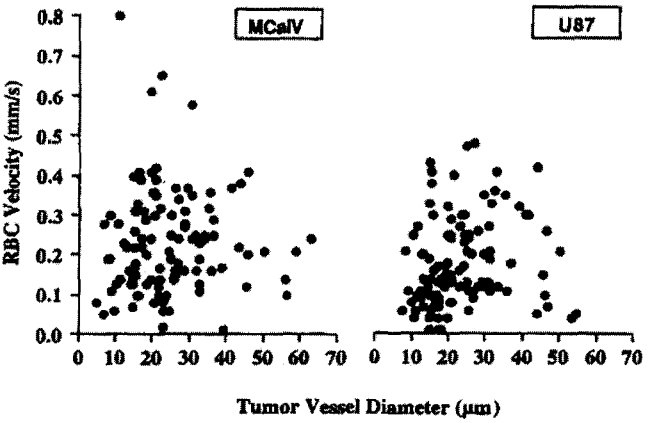


**Fig. 11.** Diagram from Jain[18] showing the lack of a clear relationship between erythrocyte velocity and tumor vessel diameter in two tumor types, MCaIV and U87. The low and variable velocities compared to those shown in Fig. 4 are evident.

into blood vessels of the tumor and cross the vessel wall into the interstitium and finally migrate through the interstitium". While blood flow is reduced in tumor vessels, nonetheless cancer cells have been reported to compress tumor vessels and this will have consequences on fluid flow.[54] This is highly relevant to the enhanced permeation and retention effect (EPR) which allows entry of macromolecules into tumors from spaces in the ill-formed tumor vasculature.[55] Access of nanoparticles to tumors is equally important and must be critically size-dependent.

In convective flow, stable colloidal particles may be captured by the process of hydrodynamic bridging,[52,56] events which may be relevant to the first process in the enhanced permeation and retention (EPR) effect. At high velocities but in the low Re regime, hydrodynamic forces acting on the particles at an entrance to a pore (or a defect in a tumor vessel) may overcome colloidal repulsive forces and result in flocculation of the particles and the plugging of the pore. The effects of velocity, particle concentration, and the ratio of pore size to particle size (the aspect ratio) on retention by hydrodynamic bridging have been studied. The effect of velocity on retention by bridging is opposite to that of retention by deposition. There is a critical flow velocity necessary for particle bridging to occur, a function of the net colloidal interparticle and particle–porous medium repulsion that must be overcome by the hydrodynamic forces for bridging to occur. Figure 12 demonstrates the effect for an aspect ratio of 3.7 (220 nm particles)

## 11. Intra-tumoral Injection

Direct injection of delivery systems into tumors has both been a mode of experimental and clinical drug delivery. Solutions allow the drugs to diffuse or leach out

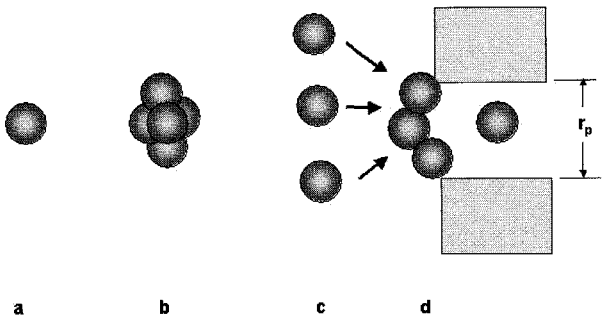


**Fig. 12.** Particle behavior prior to entry to a pore of radius, $r_p$: (a) a discrete nanoparticle, (b) aggregate, (c) individual particles converging on the pore opening demonstrating hydrodynamic bridging, as discussed by Ramachandran.[56] We speculate that events such as bridging might occur during entry of nanoparticles into tumors through fenestrations in the tumor capillary blood supply, aspects of the enhanced permeation and retention effect.

of the tumor, especially through the needle track, whereas suspensions might allow some greater residence time. Viral vectors have been administered by intra-tumoral injection.[57] To decrease the extent of viral dissemination into the systemic circulation, a viscous alginate solution was used as the viral vehicle. However, transgene expression was not increased perhaps because, as the authors speculate, the diffusion of the virus is reduced by the viscous medium once *in situ*. The transport of particles of viral dimensions requires, according to Higuchi *et al.*,[16] convective rather than diffusional transport. "The early transport of colloids into the vascular and lymphatic vessels relies largely on an extracellular pathway which depends on convective transport (i.e. solvent drag)". "Thus the particle uptake in the period immediately after injection is relatively insensitive to particle size; it is expected that viruses will be carried in the tissue towards lymphatics and microvessels with great efficacy leading to enhanced escape compared with the relatively low levels" for 1 and 0.4 $\mu$m particles.[16] The question of how resistance to convective transport in the interstitial space (the interstitial fluid plus the extracellular matrix) has been considered at least for molecules.[58] Clearly, the spacing between the cells or between fibres will be a significant factor in determining the size cut-off for transport.

## 12. Conclusions

This phenomenological survey of possible factors affecting the flow and hence the mass transport of nanoparticles has explored a range of scenarios. It is by no means a comprehensive survey, but there is sufficient in the literature to stimulate further analyses to provide a better overall prediction of the influence of particle characteristics, particularly, diameter and surface nature, shape and flexibility on delivery and targeting to remote sites in the body. Confocal microscopy and other techniques

will allow experimental study of nanoparticles so that their movement and fate can be studied in a variety of tissues. Atomic force microscopy allows measurement of forces of interaction of particles with cells and receptors to aid a more quantitative approach. However, it is wrong to underestimate the challenges ahead if nanoparticulate carriers are to be designed to overcome the various biological barriers and survive transit in the conduits of capillary blood or lymph, extravasation and tissue, and subsequently intracellular transport.[59] One cannot help but conclude that as many properties including flow are dictated by particle diameter, one of the most important strategies is to ensure the maintenance of particle stability *in vivo*.

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# 3

# Polymeric Nanoparticles as Drug Carriers and Controlled Release Implant Devices

*SM Moghimi, E Vega, ML Garcia, OAR Al-Hanbali and KJ Rutt*

## 1. Introduction

Polymeric nanoparticles are submicron size entities, often ranging from 10–1000 nm in diameter, and are assembled from a wide variety of biodegradable (e.g. albumin, chitosan, alginate) and non-biodegradable polymers (Tables 1 and 2). The most active area of research using polymeric nanoparticles is in controlled delivery of pharmaceuticals following parenteral, oral, pulmonary, nasal, and topical routes of administration.[1–6] Indeed, therapeutic agents can be encapsulated, covalently attached, or adsorbed onto such nanocarriers. These approaches can easily overcome drug solubility issues; this is particularly important as a significant proportion of new drug candidates arising from high-throughput screening initiatives are water insoluble. Polymeric nanoparticles, however, differ from nanosuspensions of drugs which are sub-micron colloidal dispersions of pure particles of drug that are stabilized by surfactants.[7] By virtue of their small size and by functionalizing their surface with polymers and appropriate ligands, polymeric nanoparticles can also be targeted to specific cells and locations in the body.[1,3,5,8–10] Thus, polymeric nanoparticles may overcome stability issues for certain drugs and minimize drug-induced side effects. The extent of drug encapsulation/incorporation, as well as

the release profile from polymeric nanocarriers, however, depends on the polymer type and its physicochemical properties, the particle size and its morphology (e.g. solid nanospheres as opposed to polymeric nanocapsules).[4] In addition, depending on the polymer characteristics, polymeric nanocarriers can also be engineered in such a way that they can be activated by changes in the environmental pH, chemical stimuli, or temperature.[11,12] Such modifications offer control over particle integrity, drug delivery rates, and the location of drug release, for example, within specific organelles. For instance, nanoparticles made from poly(lactide-co-glycolide), PLGA, can escape the endo-lysosomal compartment within minutes of internalization in intact cells and reach the cytosol.[12] This is due to the selective reversal of the surface charge of nanoparticles from the anionic to the cationic state in endo-lysosomes, resulting in a local particle-membrane interaction with subsequent cytoplasmic release. This is an excellent approach for channelling antigens into the highly polymorphic MHC class-I molecules of macrophages and dendritic cells for subsequent presentation to $CD8^+$ T lymphocytes. Other applications include cytoplasmic release of plasmid vectors and therapeutic agents (e.g. for combating cytoplasmic infections and for slow cytoplasmic release of drugs that act on nuclear receptors).

Polymeric nanoparticles are also beginning to make a significant impact on global pharmaceutical planning (life-cycle management) and market intelligence. For example, due to imminent expiration of patents, pharmaceutical companies may launch follow-up or nano-formulated versions of a product to minimize generic threats to best-selling medicines. This could lead to an extension of as much as 20 years from a new patent on the nanoparticulate formulation of the drug.

By coalescing certain polymeric nanoparticles carefully from an aqueous suspension, shape retentive hydrogels can be formed to erode partially or completely.[11,13] Drugs and macromolecules may be trapped within interstitial spaces between particles during aggregate formation. Thus, hydrogel nanoparticles have potential as controlled release implant devices following local administration or implantation, and may also serve as tissue engineering scaffolds with concurrent morphogenic protein release.

This article will briefly review some of the most commonly used laboratory scale methods for the production of polymeric nanoparticles and drug encapsulation procedures. The importance of the nanometre scale size range and surface engineering strategies for site-specific targeting of polymeric nanoparticles, following different routes of administration, are also discussed.

## 2. Nanoparticle Engineering

Polymeric nanoparticles are usually prepared either directly from preformed polymers such as aliphatic polyesters (Table 1) and block copolymers (Table 2),

**Table 1** Chemical properties of some commonly used aliphatic polyesters in nanoparticle engineering.

| Polymer Type | Melting Point (°C) | Glass Transition Temperature (°C) | Resorption Time (Months) |
|---|---|---|---|
| DL-PLA | Amorphous | 50–60 | 12–16 |
| PGA | 220–230 | 35–40 | 6–12 |
| DL-PLGA (50/50) | Amorphous | 45–50 | 1–2 |
| DL-PLGA (75/25) | Amorphous | 45–50 | 4–5 |
| PCL | 55–65 | (−65)–(−60) | >24 |

DL-PLA: poly(L-lactide); PGA: poly(glycolide); DL-PLGA: poly(DL-lactide-co-glycolide); PCL: poly-ε-caprolactone.

**Table 2** Selected examples of block copolymers for production of biodegradable nanospheres.

| |
|---|
| PLA-poly(ethyleneglycol), PLA-PEG |
| MonomethoxyPEG-poly(alkylcyanoacrylate) |
| Poly(poly(ethyleneglycol)cyanoacrylate-co-hexadecylcyanoacrylate) |
| Poly(ethyleneoxide-b-sebacic acid) |
| Poly(phosphazene)-poly(ethyleneoxide) |
| poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline) |

or by polymerization of monomers.[4] Commonly used methodologies include the solvent evaporation,[14,15] the spontaneous emulsification/solvent diffusion,[16] nanoprecipitation or solvent displacement[17,18] and emulsion polymerization techniques.[19–21] The method of choice depends on the polymer and the drug type, as well as the required particle size distribution and polydispersity indices. However, some polymers, such as comb-like polyesters, the di-block copolymer poly(ethylene oxide-b-sebacic acid) and tri-block copolymer poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) can spontaneously form stable nanoparticles (core-shell type nanospheres).[22–24]

In the solvent evaporation method, the polymer is simply dissolved together with the drug in an organic solvent and the mixture is then emulsified to form either an oil-in-water nanoemulsion (for encapsulation of hydrophobic drugs) or water-in-oil nanoemulsion (for encapsulation of hydrophilic drugs) using suitable surfactants. Nanoparticles are then obtained following evaporation of the solvent and can be concentrated by filtration, centrifugation or lyophilization. The spontaneous emulsification/solvent diffusion method is a modified version of the solvent evaporation technique, which utilizes a water-soluble solvent (e.g. methanol or acetone) along with a water-insoluble one such as chloroform. As a result of the spontaneous

diffusion of the water-soluble solvent into the water-insoluble phase, an interfacial turbulence is created leading to the formation of nanoparticles. Nanoprecipitation, however, is a versatile and simple method. This is based on spontaneous formation of nanoparticles during phase separation (the Marangoni effect), which is induced by slow addition of the diffusing phase (polymer-drug solution) to the dispersing phase (a non-solvent of the polymers, which is miscible with the solvent that solubilizes the polymer). The dispersing phase may contain surfactants. Depending on the solvent choice and solvent/non-solvent volume ratio, this method is suitable for encapsulation of both water-soluble and hydrophobic drugs, as well as protein-based pharmaceuticals.[17,18]

In emulsion polymerization, the monomer is dispersed into an aqueous phase using an emulsifying agent. The initiator radicals are generated in the aqueous phase and they diffuse into the monomer-swollen micelles. Anionic polymerization in the micelles is then initiated by the hydroxyl ions of water. Chain transfer agents are abundant and termination occurs by radical combination. The size and molecular masses of nanoparticles are dependent on the initial pH of the polymerization medium.[20] Drugs are incorporated during the polymerization step or can be adsorbed into the nanosphere surface afterwards. The addition of cyclodextrins to the polymerization medium can promote the encapsulation of poorly water-soluble drugs.[25] Depending on the monomer used, some drugs can also initiate the polymerization step, resulting in the covalent attachment of drug molecules to the nanospheres. For instance, photosensitizers such as naphthalocyanines, can initiate the polymerization of alkylcyanoacrylates.[26]

A number of specialized approaches (e.g. dialysis, salting-out, supercritical fluid technology, denaturation, ionic interaction, ionic gelation, and interfacial polymerization) have also been described for the preparation of polymeric nanoparticles, based on the choice of the starting material and the biological needs.[4,27–32]

## 2.1. *Drug release mechanisms*

The release profile of drugs from nanoparticles depends on the physicochemical nature of the drug molecules as well as the matrix.[4,16,28,33–36] Factors include mode of drug attachment and/or encapsulation (e.g. surface adsorption, dispersion homogeneity of drug molecules in the polymer matrix, covalent conjugation), the physical state of the drug within the matrix (such as crystal form), and parameters controlling matrix hydration and/or degradation. Generally, rapid release occurs by desorption, where the drug is weakly bound to the nanosphere surface. If the drug is uniformly distributed in the polymer matrix, the release occurs either by diffusion (if the encapsulated drug is in crystalline form, the drug is first dissolved locally

and then diffuses out) or erosion of the matrix, or a combination of both mechanisms. Erosion can be further subdivided into either homogeneous (with uniform degradation rates throughout the matrix) or heterogeneous (where degradation is confined at the surface) processes. Parameters such as polymer molecular weight distribution, crystallinity, hydrophobicity/hydrophilicity, melting and glass transition temperature, polymer blends and prior polymer treatment (e.g. oxygen-plasma treatment) all control the extent of matrix hydration and degradation. For instance, in the case of aliphatic polyesters, their degradation time is shorter for low molecular weight polymers, more hydrophilic polymers, more amorphous polymers and copolymers with high glycolide content (Table 1).

## 3. Site-specific Targeting with Nanoparticles: Importance of Size and Surface Properties

Numerous articles have recently discussed the importance of nanoparticle size and surface characteristics in controlling their biodistribution, following different routes of administration.[1–3,5] Only a brief overview is provided here.

Following intravenous injection, liver (Kupffer cells) and spleen (marginal zone and red pulp) macrophages clear polymeric nanoparticles rapidly from the blood circulation.[1] Opsonization, which is surface deposition of blood opsonic factors such as fibronectin, immunoglobulins, C-reactive and certain complement proteins, often aid particle recognition by these macrophages. Indeed, the propensity of macrophages of the reticuloendothelial system for rapid recognition and clearance of particulate matter has provided a rational approach to macrophage-specific targeting with nanoparticles (e.g. for the treatment of obligate intracellular microorganisms, delivery of toxins for macrophage killing, and diagnostic agents).[1] However, the rapid sequestration of nanoparticles by macrophages in contact with blood is problematic for the efficient targeting of polymeric nanoparticles to non-macrophage sites. Thus, inherent in nanoparticle design is the precision surface manipulation and engineering with synthetic polymers; this affords control over nanoparticle interaction and fate within biological systems. There are numerous examples where the surface of nanocarriers is carefully assembled with projected "macromolecular hairs" made from poly(ethyleneglycol), PEG, or its derivatives (e.g. methoxyPEG-albumin, PLA-PEG) or other related polymers [e.g. block copolymers such as selected poloxamers and poloxamines, poly(phosphazene)-poly(ethyleneoxide)].[3,5] This is achieved either during the particle assembly procedures or polymerization step, or post particle manufacturing. This strategy suppresses macrophage recognition by an array of complex mechanisms, which collectively achieve reduced protein adsorption and surface opsonization. Therefore, such entities, provided that they are below 150 nm in size, exhibit prolonged

residency time in the circulation, and are referred to as "stealth" or "macrophage-evading" nanoparticles.[1,5] The efficiency of the "macrophage-evading" process is dependent on polymer type and its surface stability, reactivity, and physics (e.g. surface density and assumed conformation).[5] Prolonged circulation properties are ideal for slow or controlled release of therapeutic agents in the blood to treat vascular disorders. Long circulating polymeric nanoparticles may have application in vascular imaging too (e.g. detection of vascular bleeding or abnormalities). Long-circulating nanoparticles can also escape from vasculature and this is normally restricted to sites where the capillaries have open fenestration or when the integrity of the endothelial barrier is perturbed by inflammatory processes or by tumor growth.[5] However, extravasated nanoparticles, as in tumour interstitium, distribute heterogeneously in perivascular clusters that do not move significantly; these particles may therefore act as depot systems, particularly for the sustained release of antiangiogenic agents, and to some extent, for drug delivery to multidrug resistant tumors (e.g. by co-encapsulation of both anticancer drugs and the competitive inhibitors of active drug efflux pumps).[1] The surface of long-circulating nanoparticles is also amenable for modification with targeting ligands. Such entities can navigate capillaries and escape routes in search of signature molecules expressed by the target; this process is often referred to as "active targeting".[1,5] For example, certain cancer cells express folate receptors and these receptors have the ability to endocytose stealth nanoparticles that are decorated with folic acid. Delivery of anti-cancer agents to tumor cells by such means could overcome the possibility of multi-drug resistance.[1,37]

Non-deformable "stealth" nanoparticles, however, are prone to splenic filtration at interendothelial cell slits, if their size exceeds that of the width of the cell slits (200–250 nm).[38,39] Indeed, these "splenotropic" vehicles can deliver their cargo efficiently to the red-pulp regions of the sinusoidal spleen. Activated or stimulated macrophages are also known to rapidly phagocytose stealth nanoparticles; stealth nanospheres may therefore have applications as diagnostic/imaging tools for the identification of stimulated or newly recruited hepatic macrophages.[40] Such diagnostic procedures may prove useful for patient selection or for monitoring the progress of treatment with long-circulating nanoparticles carrying anti-cancer agents, thus minimizing damage to hepatic macrophages.[41]

Polymeric nanospheres can also target endothelial cells on the blood-brain barrier. For instance, following intravenous injection polysorbate 80-coated poly(alkylcyanoacrylate), PACA, nanospheres attract apolipoprotein E from the blood, thus mimicking low density lipoprotein (LDL) and become recognizable by LDL receptors expressed by the blood-brain barrier endothelial cells.[10] Another related example is PEG-coated PACA nanoparticles, with the ability to localize mainly in the ependymal cells of the choroid plexus and the epithelial cells of pia

region and the ventricles of the mouse and the rat brain.[42] The molecular basis of this deposition pattern remains to be unravelled.

Others have administered nanoparticles directly to pathological sites for optimal biological performance.[43] One example is intramurally delivered PLGA nanoparticles to an injured artery following angioplasty, using a cardiac infusion catheter. Here, nanoparticles penetrate the dilated arterial wall under pressure and once the pressure is released, the artery returns to its normal state resulting in particle immobilization in the arterial wall, where they may act as a sustained release system for drugs and genetic materials.[43] Again, particle size is an important parameter; the smaller the size, the greater the arterial deposition and cellular entry, as well as lower inflammatory responses.

Polymeric nanospheres also provide intriguing opportunities for lymphatic drug delivery, as well as for diagnostic imaging of the lymphatic vessels and their associated lymph nodes when injected interstitially.[44] The extent of lymphatic delivery and lymph node localization of nanospheres depends on their size and surface characteristics. For instance, hydrophilic nanoparticles, in the size range of 30–100 nm, as opposed to their hydrophobic counterparts, repulse each other and interact poorly with the ground substance of the interstitium and drain rapidly into the initial lymphatics through patent junctions in the lymphatic capillaries.[45,46] The drained particles are conveyed to the nodes via the afferent lymph. Macrophages of medullary sinuses and paracortex are mainly responsible for particle capture from the lymph, but this also depends on nanoparticle surface properties. Larger nanospheres (>150 nm), however, are retained at interstitial sites for prolonged periods of time and may therefore act as sustained release systems for drugs and antigens.[47,48] For example, large-sized PLGA particles can provide antigen release over weeks and months following continuous or pulsatile kinetics. By mixing particle types with different degradation and pulsatile release kinetics, multiple discrete booster doses of encapsulated antigens can be provided after a single administration of the formulation (e.g. 1–2 and 6–12 months).[48] An alternative approach is the use of nanoparticle hydrogels for slow and local antigen release. For example, by controlling the ionic strength of the dispersion medium, monodisperse nanoparticles of poly-2-hydroxyethylmethacrylate, poly(HEMA), and poly[HEMA-co-methacrylic acid] coalesce together to form a shape retentive hydrogel suitable for interstitial implantation.[13] Macromolecules may be trapped between the particle aggregates and their release is controlled by a combination of diffusion (larger particles packed together have larger spaces in the lattice, and this allows for faster diffusion) and erosion (arising from aggregates that contain particles with methacrylic acid).[13] Nanoparticles that erode from the aggregate are drained into the lymphatic system and may be retained by the regional nodes. Similarly, by controlling the inherent physical attractive forces between model polystyrene nanoparticles, ordered lattices

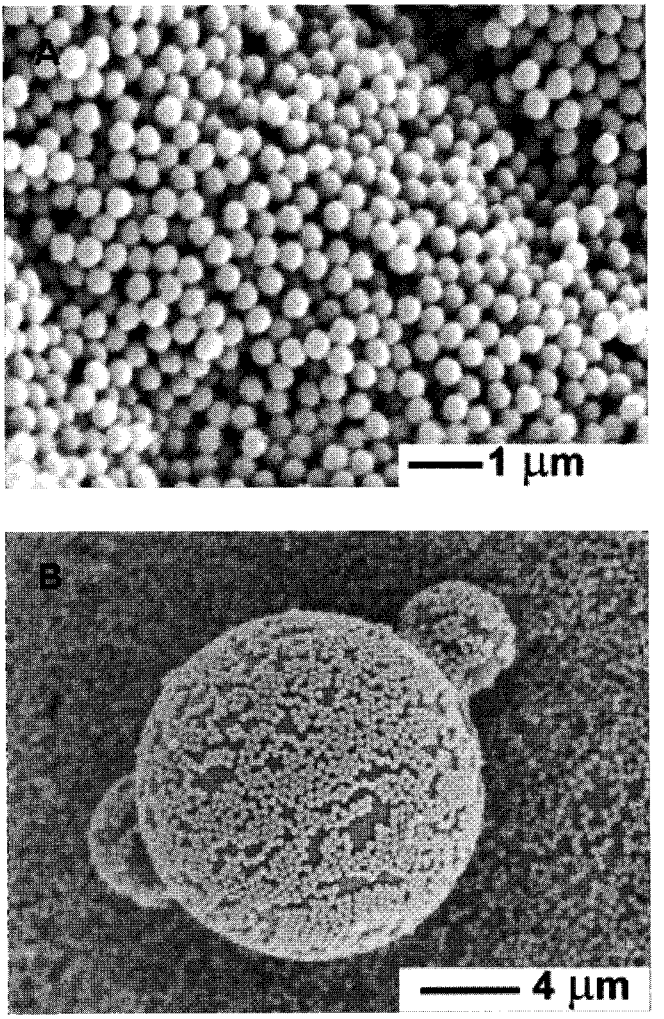


**Fig. 1.** Scanning electron micrographs of uncoated and surface-modified polystyrene nanoparticles. Due to surface hydrophobicity uncoated nanospheres (A), 350 nm in size, tend to aggregate. By controlling the physical attractive forces between the nanoparticles (by surface coating with an appropriate concentration of a block copolymer), ordered structures are formed and these can be deposited onto the surface of large microspheres (B).

can be deposited on the surface of very large microspheres (Fig. 1). Following subcutaneous localization, surface adsorbed nanospheres may gradually detach from the parent microsphere and gain entry into the lumen of the lymphatic capillaries.

Polymeric nanoparticles also have numerous applications following oral delivery. Evidence suggests that the adsorption of particulates in the intestine following oral administration take place at the Peyer's patches.[49,50] The epithelial cell layer overlying the Peyer's patches contains specialized M cells. These cells can

sample particles from the lumen and transport them to the underlying macrophages and dendritic cells. Indeed, numerous studies have confirmed protective immunity induced by mucosal immunization with PACA, PLGA and chitosan based particulate systems.[3,32,48,50–53] Part of the success is due to the encapsulation of antigens in polymeric particulate systems, which provides better protection for the antigen during intestinal transit. The immune outcomes have included mucosal (secretory IgA) and serum antibody (IgG and IgM) responses, as well as systemic cytotoxic T lymphocyte responses in splenocytes. Induction of an appropriate immune response following oral administration depends primarily on factors that affect uptake and particle translocation by M cells. These include particle size, dose, composition, and surface chemistry, as well as the region of the intestine where particles are taken up, membrane recycling from intracellular sources and the species.[50] Tolerance to orally administered microparticulate encapsulated antigens is another potential outcome, but it has received little attention.

The bioavailability of some drugs can be improved after oral administration by means of polymeric nanoparticles.[54–57] This is a reflection of drug protection by the nanoparticle against hostile conditions of the gastrointestinal tract, as well as the mode of nanoparticle interaction with mucosal layers. However, the bioadhesive properties of nanoparticles may vary with their size and surface characteristics (e.g. surface charge, surface polymer density and conformation), as well as the location and type of the mucosal surface in the gastrointestinal tract. Similarly, improved drug bioavailability has also been reported following ocular administration with PLA, PACA, poly(butylcyanoacrylate) and Eudragit nanoparticles.[6,58–61] For example, loading of tamoxifen in PEGylated nanoparticles proved successful in the treatment of autoimmune uveortinitis following intraocular injection.[59] Interaction of surface-modified polymeric nanoparticles with nasal associated lymphoid tissue and their transport across nasal mucosa have also received attention, particularly with respect to peptide-based pharmaceuticals and antigen delivery.[53,62]

## 4. Conclusions

Polymeric nanoparticles are promising vehicles for site-specific and controlled delivery of therapeutic agents, following different routes of administration and these trends seem to continue with advances in materials and polymer chemistry and pharmaceutical nanotechnology. However, nanoparticles do not behave similarly; their encapsulation capacity, drug release profile, biodistribution and stability vary with their chemical makeup, morphology and size. Inherently, nanosphere design and targeting strategies may vary according to physiological and therapeutic needs, as well as in relation to the type, developmental stage and location of

the disease. Attention should also be paid to toxicity issues that may arise from nanoparticle administration and the release of their polymeric contents and degradation products. These issues are discussed elsewhere.[1,63–66]

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# 4

# Genetic Vaccines: A Role for Liposomes

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## 1. Introduction

Prevention of microbial infections by the use of vaccines is a preferred alternative to treatment. Vaccines have been applied successfully, for example, in the eradication of smallpox as well as against tetanus, diphtheria, whooping cough, polio and measles, thus preventing millions of deaths each year. However, vaccines made of attenuated organisms, which mimick natural infections usually without the disease, can be potentially unsafe. For instance, there is a risk of reversion during replication of live viruses or even mutation to a more pathogenic state. Furthermore, with immunocompromised individuals, some of the attenuated viruses may still provoke disease. On the other hand, with killed virus vaccines, their extracellular localization and subsequent phagocytosis by professional antigen presenting cells (APC) or antigen-specific B cells, lead to MHC-II class restricted presentation and to T helper cell and humoural immunity. However, they do not elicit significant cytotoxic T cell (CTL) responses. Moreover, subunit vaccines produced from biological fluids may not be entirely free of infectious agents. Even with subunit and peptide vaccines produced recombinantly or synthetically (and thus considered safe), immune responses are weak and often not of the appropriate kind. The great variety of immunological adjuvants[1,2] that are now available go a long way in rendering subunit and peptide vaccines stronger and more efficient. However, more than seventy years after the introduction of aluminium salts as an adjuvant, only two other adjuvants, liposomes[3] and MF59,[1] have been approved for use in humans.[4] Thus,

inspite of considerable progress, the road to the ideal vaccine appears as elusive as ever, until recently.

Recent developments have led to a novel and exciting concept, namely *de novo* production of the required vaccine antigen by the host's cells *in vivo*, which promises to revolutionize vaccination especially where vaccines are either ineffective or unavailable. The concept entails the direct injection of antigen-encoding plasmid DNA which, on uptake by cells, localizes to some extent into the nucleus where it transfects the cells episomally. The produced antigen is recognized as foreign by the host and is thus subjected to pathways similar to those observed for antigens of internalized viruses (but without their disadvantages), leading to protective humoural and cell mediated immunity.[5–9] A series of publications since 1992 first established the ability of plasmid DNA to induce an immune (antibody) response to the encoded foreign protein[10]; in experiments with DNA encoding influenza nucleoprotein, immunity was both humoural and cell-mediated, and also protective in mice challenged with the virus.[11,12] This was the first demonstration of an experimental DNA vaccine. Another observation was the induction of humoural and cell-mediated immunity against HIV-1 using plasmids encoding the HIV *rev* and *env* proteins.[13] Similar results were obtained with a gene for the hepatitis B surface antigen (HBsAg).[14] DNA immunization was also found to apply in cancer treatment. For instance, injection of plasmids encoding tumor antigens promoted immune responses[15,16] which were protective in an animal model.[6] The concept of DNA immunization has now been adopted by vaccinologists worldwide using an ever increasing number of plasmids encoding immunogens from bacterial, viral and parasitic pathogens, and a variety of tumors.[8,9] In many of these studies, genetic immunization has led to the protection of animals from infection.[5–9] A number of clinical trials for the therapy of, or prophylaxis against, a variety of infections are in progress.[8,9]

## 2. The DNA Vaccine

A plasmid DNA vaccine is usually[6] supercoiled and consists of the gene encoding the vaccine antigen (the section of the target pathogen which elicits protective immunity), a promoter sequence which is often derived from cytomegalovirus (CMV) or Rous sarcoma virus (RSV)), an mRNA stability polyadenylation region at the 3′ end of the insert, and the plasminogen activator gene which controls the secretion of the recombinant product. In addition, there are an origin of replication for the amplification of the plasmid in bacteria, and a gene for antibiotic resistance to select the transformed bacteria.

Immunization procedures with DNA vaccines are carried out by the intramuscular and, to a lesser extent, the intraepidermal route. Other routes include the

oral, nasal, vaginal, intravenous, intraperitoneal and subcutaneous routes.[8,9] Intramuscular injection of DNA vaccines leads to such types of immunity as CTL.[5,9,11,12] This was unexpected because antigen presentation requires the function of professional APC.[17] However, myocytes which were shown[5] to take up the plasmid only to a small extent and with only a fraction of cells participating in the uptake, are not professional APCs. Although myocytes carry MHC class I molecules and can present endogenously produced viral peptides to the $CD8^+$ cells to induce CTLs, they do so inefficiently[18] as they lack vital costimulatory molecules (e.g. the B7-1 molecule). It is thus difficult to accept that antigen presentation, leading to a CTL response, occurs via myocytes. Instead, it was reported[18] that CTL responses occur as a result of the transfer of antigenic material between the myocytes and professional APC to some extent. In parallel, it could also be that plasmid secreted by the myocytes or as such, is taken up directly by APC infiltrating the injected site. Such APC would include dendritic cells which will express and present peptides to $CD8^+$ cells following transport to the lymph nodes or spleen. On the other hand, $CD4^+$ cells may be activated by APCs via MHC class II presentation of antigen secreted by the myocytes (or released from them after their destruction via a Tc response) and captured by the cells. Such events would lead to both cellular (Th 1) and humoural (Th 2) immunity. Indeed, it has been shown[6] that dendritic cells are the essential APC involved in immune responses elicited by intramuscularly given DNA vaccines.

## 3. DNA Vaccination via Liposomes

Vaccination with naked DNA by the intramuscular route is dependent on the ability of myocytes to take up the plasmid. However, some of the DNA may also be engulfed by APC infiltrating the site of injection, or in the lymph nodes following migration of the DNA to the lymphatics. The extent of DNA degradation by extracellular deoxyribonucleases is unknown, but depending on the time of its residence interstitially, degradation could be considerable. Therefore, approaches that protect DNA from the extracellular nucleases and promote DNA uptake by cells more efficiently, or target it to APC, should contribute to the optimal design of DNA vaccines.

It has been suggested[19] that as APC are a preferred alternative to muscle cells for DNA vaccine uptake and expression, liposomes (known[3] to be taken up avidly by APC infiltrating the site of injection or in the lymphatics, an event that has been implicated[3] in their immunoadjuvant activity) would be a suitable means of delivery of entrapped DNA to such cells. Liposomes would also protect[20] their DNA content from deoxyrubonuclease attack. Moreover, the structural versatility[21] of the system would ensure that its tranfection efficiency is further improved

by the judicial choice of its structural characteristics or by the co-entrapment of cytokine genes, other adjuvants (e.g. immunostimulatory sequences), or indeed protein antigens (see later) together with the plasmid vaccine. As a number of injectable liposome-based drug formulations, including vaccines against hepatitis A and influenza, have been already licensed for clinical use,[21] acceptance of the system clinically would be less problematic than with other systems that are still at an experimental stage.

## 3.1. *Procedure for the entrapment of plasmid DNA into liposomes*

A variety[8,22,23] of plasmid DNAs have been quantitatively entrapped into liposomes by a mild dehydration-rehydration procedure.[20,22,23] The procedure (Fig. 1) consists of mixing preformed small unilamellar vesicles (SUV) with a solution of the DNA destined for entrapment, freeze-drying of the mixture, followed by controlled rehydration of the formed powder, and centrifugation to remove non-entrapped material. Formed liposomes are multilamellar.[20] However, when an appropriate amount of sucrose is added to the SUV and DNA mixture prior to dehydration,[24] the resulting liposomes are much smaller (about 100–160 nm in diameter). As expected, DNA incorporation values[8,23,26] were higher (up to 90% of the amount used) when a cationic lipid was present in the bilayers. No apparent relationship was observed between amount of DNA used (10–500 $\mu$g) and the values of incorporation for the compositions and lipid mass used.[8,23,26] The possibility that DNA was not entrapped within the bilayers of cationic liposomes, but was rather complexed with their surface (as suggested by the high

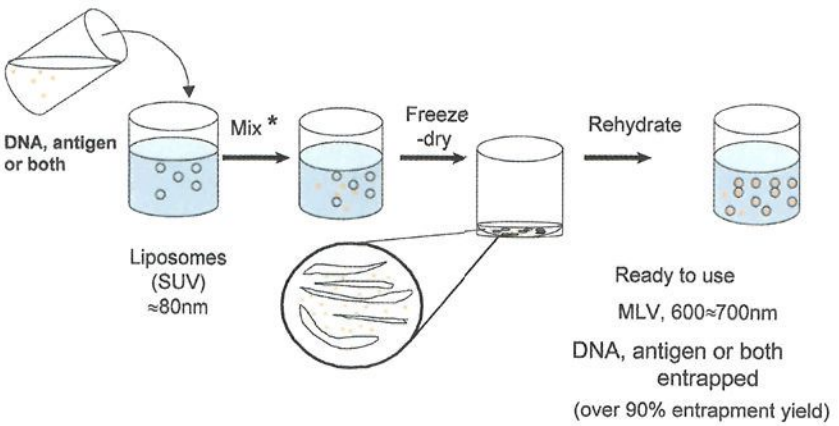


**Fig. 1.** Entrapment of DNA and/or protein into cationic liposomes. The procedure entails mixing up empty SUV with the solute(s) destined for entrapment and subsequent dehydration. On rehydration, most of the solute(s) is recovered entrapped within the generated multilamellar liposomes.

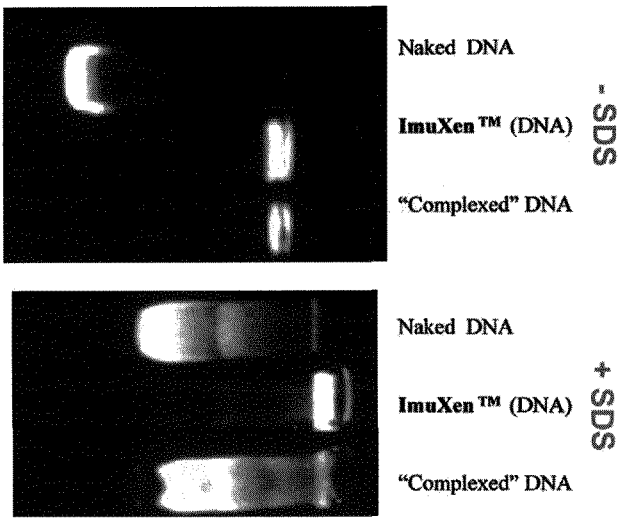


**Fig. 2.** Gel electrophoresis of a mixture of cationic SUV and pRc/CMV HBS before (complexed DNA) and after (entrapped DNA) dehydration-rehydration of the mixture.

"incorporation" values obtained on mixing)[20] was examined by treating liposome-entrapped and liposome-complexed DNA with deoxyribonuclease. Substantially, more liposome-entrapped DNA remained intact than when it was complexed,[20] presumably because of the inability of the enzyme to reach its substrate in the former case. The significant resistance of complexed DNA (despite its accessibility) to the enzyme could be attributed to its condensed state.[25] Additional evidence that the DNA was entrapped within liposomes was obtained by gel electrophoresis of a mixture of cationic SUV and plasmid DNA before (complexed DNA) and after dehydration-rehydration of the mixture (entrapped DNA). When the anionic sodium dodecylsulphate (SDS) was incorporated in the gel, complexed DNA was dissociated from the SUV, presumably because of ionic competition for the cationic charges. As expected, "entrapped" DNA retained its association with the liposomes, suggesting its unavailability to the competing SDS anions[26] (Fig. 2).

## 3.2. *DNA immunization studies*

Previously,[20] liposome-entrapped plasmid found to transfect cells *in vitro* regardless of the vesicle surface charge was tested in immunization experiments,[19,27] using a plasmid (pRc/CMV HBS) encoding the S region of the hepatitis B surface antigen (HBsAg; subtype ayw). Mice (Balb/c) that are repeatedly injected intramuscularly with 5 or 10 $\mu$g plasmid entrapped in cationic liposomes, exhibited at all times much greater (up to 100-fold) antibody ($IgG_1$) responses (Fig. 3) against the

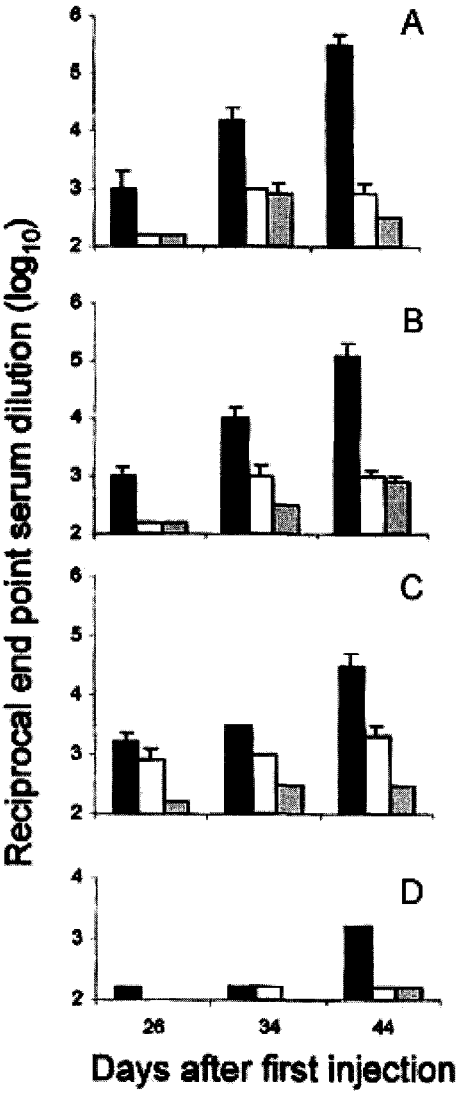


**Fig. 3.** Immune responses in mice injected with naked, or liposome-entrapped pRc/CMV HBS. Balb/c mice were injected intramuscularly on days 0, 10, 20, 27 and 37 with 5 $\mu$g of DNA entrapped in cationic liposomes composed of PC, DOPE and DOTAP (A), DC-Chol (B) or SA (C) (molar ratios 1:0.5:0.25), or in the naked form (D). Animals were bled 7, 15, 26, 34 and 44 days after the first injection and sera tested by ELISA for $IgG_1$ (black bars), $IgG_{2a}$ (white bars) or $IgG_{2b}$ (grey bars) responses against the encoded hepatitis B surface antigen (HBsAg; S region, ayw subtype). Values are means ±SD of $log_{10}$ of reciprocal end point serum dilutions required for OD to reach readings of about 0.2. Sera from untreated mice gave $log_{10}$ values of less than 2.0. $IgG_1$ responses were mounted by all mice injected with liposomal DNA but became measurable only at 26 days. Differences in $log_{10}$ values (all IgG subclasses at all time intervals) in mice immunized with liposomal DNA and mice immunized with naked DNA were statistically significant ($P < 0.0001$–$0.002$). (Reproduced with permission from Ref. 19.)

encoded antigen than animals immunized with the naked plasmid. Values of other subclasses ($IgG_{2a}$ and $IgG_{2b}$) were also greater (up to 10-fold) (Fig. 3). Moreover, $IgG_1$ responses for the liposome-entrapped plasmid DNA were higher (up to 10-fold) than those obtained with DNA complexed with similar cationic liposomes.[19] This was also true for IFN-$\gamma$ and IL-4 levels in the spleens of immunized mice.[19] In other experiments,[8] the effect of the route of injection of the pRc/CMV HBS plasmid was examined with respect to both humoural and cell-mediated immunity, using Balb/c mice and an outbred mouse strain (T.O.). Results[8] comparing responses for liposome-entrapped and naked plasmid DNA showed greater antibody ($IgG_1$) responses for the entrapped DNA, not only by the intramuscular route, but also the subcutaneous and the intravenous routes. As there were no significant differences in the titers between the two strains,[8] it was concluded that immunization with liposomal pRc/CMV HBS is not MHC restricted. Results obtained on the testing of IFN-$\gamma$ and IL-4 in the spleens (not shown) exhibited a similar pattern.

Involvement of muscle cells in the mechanism by which liposomes promote greater immune responses to the encoded antigen than seen with the naked plasmid, is rather unlikely. Although, cationic liposomes could in theory bind to and be taken up by the negatively charged myocytes, the negatively charged proteins present in the interstitial fluid would neutralize[21] the cationic liposomal surface and thus interfere with such binding. In addition, vesicle size (about 600–700 nm average diameter; Ref. 26) would render access to the cells difficult, if not impossible. It is therefore more likely that cationic liposomes are endocytosed by APC, including dendritic cells, in the lymphatics where liposomes are expected to end up.[28] Uptake of liposomal plasmid DNA is supported in studies where mice were injected intramuscularly or subcutaneously with liposomes entrapping the plasmid (pCMV·EFGP), encoding the enhanced fluorescent green protein or with the naked plasmid. Fluorescence microscopy of sections of the lymph nodes draining the injected site revealed (Fig. 4) much more green fluorescence when the plasmid was administered in the liposomal form.[27] It appears[8,19] that the key ingredient of the DNA-containing liposomes as used in Fig. 3, contributing to enhanced immune responses, is the cationic lipid. The mechanism by which liposomal DNA reaches the nucleus for episomal transfection is poorly understood. It is conceivable, however, that some of the endocytosed liposomal DNA escapes the endocytic vacuoles prior to their fusion with lysosomes (in a way similar to that proposed[29] for vesicle-DNA complexes) to enter the cytosol for eventual episomal transfection and presentation of the encoded antigen. It is perhaps at this stage of intracellular trafficking of DNA, spanning its putative escape from endosomes and access to the nucleus, that the cationic lipid, possibly together within the fusogenic phosphatidylethanolamine (PE) component, plays a significant role.

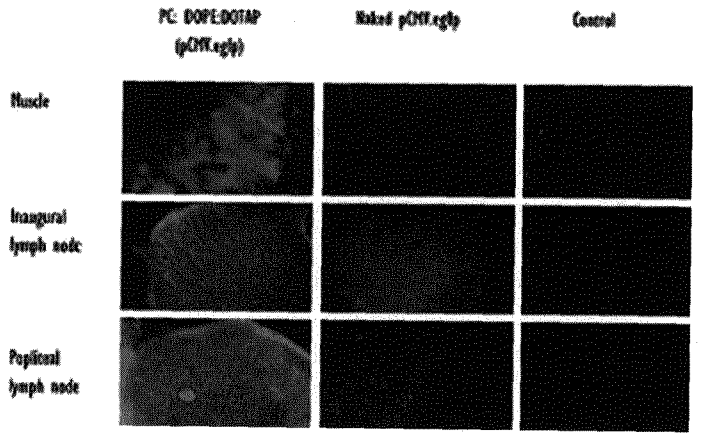


**Fig. 4.** Fluorescence images of muscle and lymph node sections from mice injected intramuscularly with 10 $\mu$g liposome-entrapped or naked pCMV·EGFP and killed 48 h later. Sections from untreated animals were used as controls. (Reproduced with permission from Ref. 27.)

## 3.3. *Induction of a cytotoxic T lymphocyte (CTL) response by liposome-entrapped plasmid DNA*

Immunization studies with liposome-entrapped DNA vaccines were expanded[30] to include the cytotoxic T lymphocyte (CTL) component of the immune response. This was measured by the specific killing of syngeneic target cells pulsed with a recognized CTL epitope peptide derived from the antigen tested. To that end, the type and degree of immune response induced following subcutaneous injection of DNA in cationic liposomes was monitored and compared with that obtained with DNA alone injected by the same route. 6–8 week old, female C57/BL6 (H-$2^d$) mice were injected subcutaneously with one or two doses of 2.5 or 10 $\mu$g ovalbumin (OVA)-encoding plasmid DNA (pCI-OVA), either alone or entrapped in liposomes. Animals immunized subcutaneously with 100 $\mu$g of OVA protein complexed with 1 $\mu$g of cholera toxin (CT) served as a positive control. Blood samples and spleens were collected from all animals one week after the last injection and tested for anti-OVA total IgG (serum), CTL activity and cytokine release (spleen). After a single dose of antigen, only animals immunized with either protein or 10 $\mu$g of liposomal DNA showed significant anti-OVA antibody titres by ELISA. After two doses of antigen, only animals immunized with either protein or liposomal DNA (both 2.5 and 10 $\mu$g DNA) showed significant levels of seroconversion and serum antibody titres against OVA by ELISA.[30] Similarly, no anti-OVA CTL activity was detected in animals immunized with DNA alone. However, animals immunized with two doses of 10 $\mu$g of liposomal DNA displayed a CTL response higher (60% cell killing vs 50%) than that obtained in the positive control group immunized

with OVA protein and adjuvant (CT).[30] Thus, delivery of a small dose of liposomal plasmid DNA subcutaneously, a route of immunization not normally inducing significant plasmid DNA mediated immune activation,[9] results in a strong antigen-specific cellular response which is greater than that achieved by higher doses of a conventional protein antigen together with a powerful adjuvant (CT).

## 4. The Co-delivery Concept

Proteins that are synthesized within a cell (e.g. from plasmid DNA having a mammalian-active promoter) are continuously sampled as peptides by the proteosome/class-I MHC antigen presenting pathway. Conversely, proteins that are acquired exogenously by antigen-presenting cells are sampled in an analogous way by the endosomal/MHC-class-II pathway. It follows that the delivery of both protein and plasmid-DNA-encoded forms of a protein antigen to the same individual antigen-presenting cell would result in the simultaneous presentation of the antigen via both class-I and class-II pathways, thereby providing an opportunity for synergy in the resulting immune response to the antigen. Several appropriate liposomal formulations were designed to test the "co-delivery" hypothesis, exploiting the advantages of the dehydration-rehydration liposome technology that entraps both DNA and protein immunogens efficiently. The formulations, described in Table 1, comprise various test and control permutations of plasmid DNA and protein, either free or entrapped (together or separately) in the liposomal vehicle.

Immunization with DNA encoding the influenza haemagglutinin protein has been explored previously with naked[31] or liposomally formulated DNA.[32] Although immune responses elicited by DNA alone were adequate to achieve protective efficacy against influenza virus challenge in preclinical studies, only weak anti-HA antibody responses were elicited.[31] The present "co-delivery" concept was designed to rectify this deficiency of DNA-based influenza vaccines. In a series of experiments, plasmid DNA encoding the haemagglutinin (HA) antigen [referred to in Table 1 and Fig. 5 as DNA(ha)] of the influenza virus (A/Sichuan/87 or A/PR/8 strains) was co-entrapped with the corresponding whole inactivated virus (referred to as HA) within the same liposomes using the dehydration-rehydration method (for details on lipid composition and method see Refs. 26 and 27). A variety of control preparations including liposomes co-entrapping irrelevant DNA (i.e. plasmid DNA encoding ovalbumin) with HA or irrelevant protein (i.e. ova) with DNA (ha), entrapping DNA(ha) or HA alone, a mixture of the latter two preparations, and a mixture of the naked DNA(ha) and HA were used to immunize mice. Results shown in Fig. 5 demonstrate that the "co-delivery" hypothesis formulation (comprising both HA and its corresponding DNA in the same liposomes), elicited a greater response than all other formulations at each time point in the series, and it

**Table 1** Liposomal formulations of DNA and protein used in immunization experiments.

| Sample | Dose ($\mu$g/animal (0.2 ml S/C)) | | |
|---|---|---|---|
| | Formulation | DNA | Protein |
| 1.1 | Liposomes (co-delivery) | ha (10) | HA (0.6) |
| 2.1 | Liposomes (co-delivery) | ova (11) | HA (0.6) |
| 3.1 | Liposomes (co-delivery) | ha (10) | OVA (0.76) |
| 4.1 | Liposomes | ha (10) | Nil |
| 5.1 | Liposomes | Nil | HA (0.6) |
| 6.1 | Liposomes (samples 4.1 & 5.1) | ha (10) | HA (0.6) |
| 7 | DNA and protein (mixed) | ha (10) | OVA (0.76) |
| 8 | DNA and protein (mixed) | ova (11) | HA (0.6) |
| 9 | DNA and protein (mixed) | ha (10) | HA (0.6) |
| 10 | DNA alone | ha (10) | Nil |
| 11 | Protein alone | Nil | HA (10) |
| 12 | Control (PBS) | Nil | Nil |

Plasmid DNA encoding the HA antigen [DNA(ha)] and the HA antigen (HA) were entrapped in liposomes either together (co-entrapped; sample 1.1) or separately in different formulations (sample 6.1) mixed before injection. In some formulations, DNA(ha) and HA were entrapped alone (samples 4.1 and 5.1 respectively). In others, ovalbumin (OVA) and plasmid DNA encoding ha [DNA(ha)] (sample 7) or HA and plasmid DNA encoding OVA (sample 8) were entrapped separately and then mixed. Mice were injected subcutaneously on days 0 and 28 and blood samples analyzed by ELISA for Ig responses.

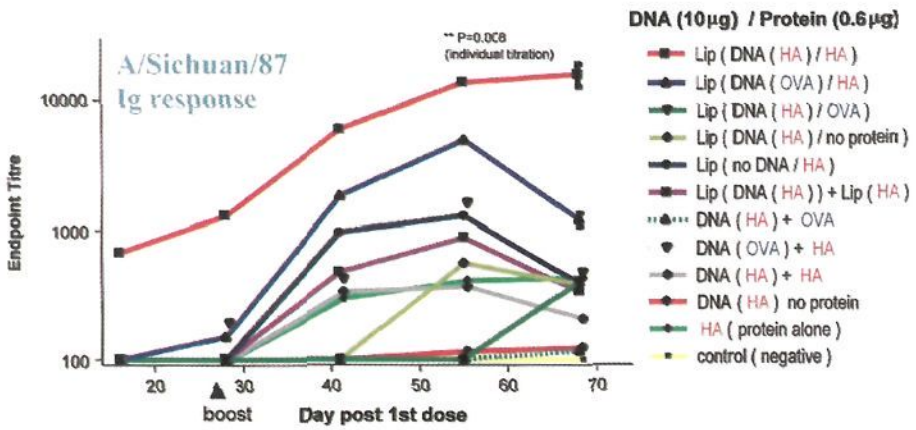


**Fig. 5.** Serum Ig endpoint titres in Balb/c mice immunized on days 0 and 28 with DNA and/or antigen formulations as described in Table 1 and bled at time intervals.

is by far the strongest response after a single dose. Notably, the formulation "Lip (OVA/ha)", which is a control for the CpG adjuvant effect of plasmid DNA,[33] gave a response which was much lower than that of "co-delivery" with the appropriate homologous pair of HA DNA and protein. Likewise, Lip (HA/ova) (an inappropriate pairing according to the hypothesis), gave a markedly weaker response. Figure 5 also demonstrates that separately entrapped HA DNA and protein (in neighbouring vesicles) gave rise to an inferior response, supporting the hypothesis that delivery of both payloads to the same cell (which is best achieved by co-entrapment in the same liposome) is important in achieving the optimal antibody response. It is also remarkable that, inspite the modest DNA dose (10 $\mu$g) and small number (2) of immunizations used, several formulations completely failed to generate an anti-HA response. These included HA DNA alone, and liposomally entrapped HA DNA. These findings serve to emphasize the striking degree of superiority of "co-delivery" over previous methods of DNA-based immunization against influenza virus.

In conclusion, the present studies demonstrate that very small doses of protein as an additive in DNA immunization can dramatically improve the antibody response to the target protein, provided that the protein and DNA are homologous to one-another (i.e. that the DNA can express the protein), and that the payloads are delivered in the same individual liposomal vehicle. The simplest hypothesis to explain our observation is that the synergy observed between the appropriately delivered "homologous pair" of protein and DNA involves delivery of both payloads to the same antigen-presenting cell. The application of the co-delievery concept to alternative delivery systems, e.g. niosomes, dendimers, PLA/PLGA, chitosans, alginates and other microparticles awaits investigation. It is anticipated that the "co-delivery" approach will lead to better DNA-based vaccines for prophylactic and therapeutic use, particularly where vaccines require the elicitation of antibody responses (e.g. influenza vaccines).

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# 5

# Polymer Micelles as Drug Carriers

*Elena V. Batrakova, Tatiana K. Bronich, Joseph A. Vetro and Alexander V. Kabanov*

## 1. Introduction

It has long been recognized that improving one or more of the intrinsic adsorption, distribution, metabolism, and excretion (ADME) properties of a drug is a critical step in developing more effective drug therapies. As early as 1906, Paul Ehrlich proposed altering drug distribution by conjugating toxic drugs to "magic bullets" (antibodies) having high affinity for cancer cell-specific antigens, in order to both improve the therapeutic efficacy of cancer while decreasing its toxicity.[1] Since then, it has become clear that directly improving intrinsic ADME through modifications of the drug is limited or precluded by structural requirements for activity. In other words, low molecular mass drugs are too small and have only limited number of atomic groups that can be altered to improve ADME, which often adversely affects drug pharmacological activity. In turn, the modifications of many low molecular mass drugs, aimed to increase their pharmacological activity, often adversely affect their ADME properties. For example, the potency and specificity of drugs can be improved by the addition of hydrophobic groups.[2] The associated decrease in water solubility, however, increases the likelihood of drug aggregation, leading to poor absorption and bioavailability during oral administration[2] or lowered systemic bioavailability, high local toxicity, and possible pulmonary embolism during intravenous administration.[3]

Although there have been considerable difficulties for improving some existing drugs through chemical modifications, the problem became even more obvious

with the development of high-throughput drug discovery technologies. Almost half of lead drug candidates identified by high-throughput screening have poor solubility in water, and are abandoned before the formulation development stage.[4] In addition, newly synthesized drug candidates often fail due to poor bioavailability, metabolism and/or undesirable side effects, which together decrease the therapeutic index of the molecules. Furthermore, a new generation of biopharmaceuticals and gene therapy agents are emerging based on novel biomacromolecules, such as DNA and proteins. The use of these biotechnology-derived drugs is completely dependent on efficient delivery to the critical site of the action in the body. Therefore, drug delivery research is essential in the translation of newly discovered molecules into potent drug candidates and can significantly improve therapies of existing drugs.

Polymer-based drugs and drug delivery systems emerged from the laboratory bench in the 1990s as a promising therapeutic strategy for the treatment of certain devastating human diseases.[5,6] A number of polymer therapeutics are presently on the market or undergoing clinical evaluation to treat cancer and other diseases. Most of them are low molecular weight drug molecules or therapeutic proteins that are chemically linked to water-soluble polymers to increase drug solubility, drug stability, or enable targeting to tumors.

Recently, as a result of rapid development of novel nanotechnology-derived materials, a new generation of polymer therapeutics has emerged, using materials and devices of nanoscale size for the delivery of drugs, genes, and imaging molecules.[7–12] These materials include polymer micelles, polymer-DNA complexes ("polyplexes"), liposomes, and other nanostructured materials for medical use that are collectively known as nanomedicines. Compared with first generation polymer therapeutics, the new generation nanomedicines are more advanced. They entrap small drugs or biopharmaceutical agents such as therapeutic proteins and DNA, and can be designed to trigger the release of these agents at the target site. Many nanomedicines are constructed using self-assembly principles such as the spontaneous formation of micelles or interpolyelectrolyte complexes, driven by diverse molecular interactions (hydrophobic, electrostatic, etc.). This chapter considers polymeric micelles as an important example of the new generation of nanomedicines, which is also perhaps among the most advanced approach toward clinical applications in diagnostics and the treatment of human diseases.

## 2. Polymer Micelle Structures

### 2.1. *Self-assembled micelles*

Self-assembled polymer micelles are created from amphiphilic polymers that spontaneously form nanosized aggregates when the individual polymer chains

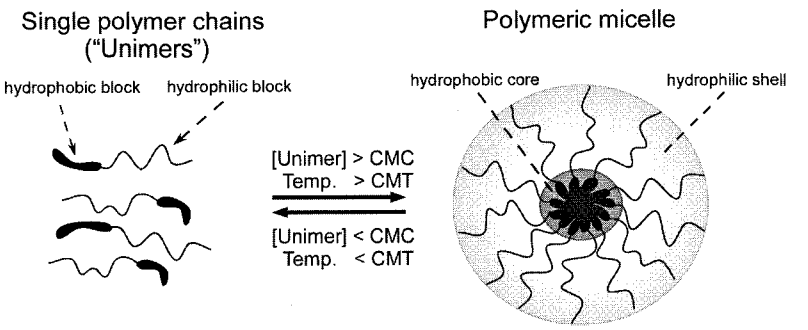


**Fig. 1.** Self-assembly of block copolymer micelles.

("unimers") are directly dissolved in aqueous solution (dissolution method)[13] above a threshold concentration (critical micelle concentration or CMC) and solution temperature (critical micelle temperature or CMT) (Fig. 1). Amphiphilic polymers with very low water solubility can alternatively be dissolved in a volatile organic solvent, then dialyzed against an aqueous buffer (dialysis method).[14]

Amphiphilic di-block (hydrophilic-hydrophobic) or tri-block (hydrophilic-hydrophobic-hydrophilic) copolymers are most commonly used to prepare self-assembled polymer micelles for drug delivery,[9,15,16] although the use of graft copolymers has been reported.[17–19] For drug delivery purposes, the individual unimers are designed to be biodegradable[20,21] and/or have a low enough molecular mass (< ~40 kDa) to be eliminated by renal clearance, in order to avoid polymer buildup within the body that can potentially lead to toxicity.[22] The most developed amphiphilic block copolymers assemble into spherical core-shell micelles approximately 10 to 80 nm in diameter,[23] consisting of a hydrophobic core for drug loading and a hydrophilic shell that acts as a physical ("steric") barrier to both micelle aggregation in solution, and to protein binding and opsonization during systemic administration (Fig. 2).

The most common hydrophilic block used to form the hydrophilic shell is the FDA-approved excipient poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO).[24] PEG or PEO consists of the same repeating monomer subunit $CH_2$-$CH_2$-O, and may have different terminal end groups, depending on the synthesis procedure, e.g. hydroxyl group HO-($CH_2$-$CH_2$-O)$_n$-H; methoxy group $CH_3O$-($CH_2$-$CH_2$-O)$_n$-H, etc. PEG/PEO blocks typically range from 1 to 15 kDa.[16,24]

In addition to its FDA approval, PEG is extremely soluble and has a large excluded volume. This makes it especially suitable for physically interfering with intra-micelle interactions and subsequent micelle aggregation. PEG also blocks protein and cell surface interactions, which greatly decreases nanoparticle uptake by the reticuloendothelial system (RES), and consequently increases the plasma

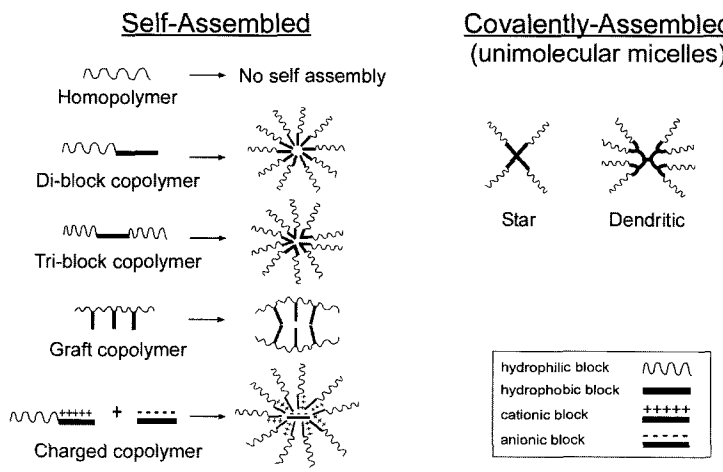


**Fig. 2.** Polymer micelle structures.

half life of the polymer micelle.[25] The degree of steric protection by the hydrophilic shell is a function of both the density and length of the hydrophilic PEG blocks.[25]

Unlike the hydrophilic block, which is typically PEG or PEO, different types of hydrophobic blocks have been sufficiently developed as hydrophobic drug loading cores.[16] Examples of diblock copolymers include (a) poly(L-amino acids), (b) biodegradable poly(esters), which includes poly(glycolic acid), poly(D lactic acid), poly(D,L-lactic acid), copolymers of lactide/glycolide, and poly($\varepsilon$-caprolactone), (c) phospholipids/long chain fatty acids[26]; and for tri-block copolymers, (d) polypropylene oxide (in Pluronics/poloxamers).[9] The choice of hydrophobic block is largely dictated by drug compatibility with the hydrophobic core (when drug is physically loaded, as described later) and the kinetic stability of the micelle.

The self-assembly of amphiphilic copolymers is a thermodynamic and, consequently, a reversible process that is entropically driven by the release of ordered water from hydrophobic blocks; it is either stabilized or destabilized by solvent interactions with the hydrophilic shell. As such, the structural potential of amphiphilic copolymer unimers to form micelles is determined by the mass ratio of hydrophilic to hydrophobic blocks, which also affects the subsequent morphology if aggregates are formed.[14] If the mass of the hydrophilic block is too great, the copolymers exist in aqueous solution as unimers, whereas, if the mass of the hydrophobic block is too great, unimer aggregates with non-micellar morphology are formed.[27] If the mass of the hydrophilic block is similar or slightly greater than the hydrophobic block, then conventional core shell micelles are formed.

An important consideration for drug delivery is the relative thermodynamic (potential for disassembly) and kinetic (rate of disassembly) stability of the polymer

micelle complexes, after intravenous injection and subsequent extreme dilution in the vascular compartment.[28] This is because the polymer micelles must be stable enough to avoid burst release of the drug cargo, as in the case of a physically loaded drug, upon systemic administration and remain as nanoparticles long enough to accumulate in sufficient concentrations at the target site.

The relative thermodynamic stability of polymer micelles (which is inversely related to the CMC) is primarily controled by the length of the hydrophobic block.[13] An increase in the length of the hydrophobic block alone significantly decreases the CMC of the unimer construct (i.e. increases the thermodynamic stability of the polymer micelle), whereas an increase in the hydrophilic block alone slightly increases the CMC (i.e. decrease the thermodynamic stability of a polymer micelle).[14]

Although the CMC indicates the unimer concentration below which polymer micelles will begin to disassemble, the kinetic stability determines the rate at which polymer micelle disassembly occurs. Many diblock copolymer micelles possess good kinetic stability and only slowly dissociate into unimers after extreme dilution.[29] Thus, although polymer micelles are diluted well below typical unimer CMCs[29] ($10^{-6}$–$10^{-7}$ M) after intravenous injection, their relative kinetic stability might still be suitable for drug delivery. The kinetic stability depends on several factors, including the size of a hydrophobic block, the mass ratio of hydrophilic to hydrophobic blocks, and the physical state of the micelle core.[14] The incorporation of hydrophobic drugs may also further enhance micelle stability.

## 2.2. *Unimolecular micelles*

Unimolecular micelles are topologically similar to self-assembled micelles, but consist of single polymer molecules with covalently linked amphiphile chains. For example, copolymers with star-like or dendritic architecture, depending on their structure and composition, can either aggregate into multimolecular micelles,[30–32] or exist as unimolecular micelles.[33] Dendrimers are widely used as building blocks to prepare unimolecular micelles, because they are highly-branched, have well-defined globular shape and controled surface functionality.[34–40] For example, unimolecular micelles were prepared by coupling dendritic hypercores of different generations with PEO chains.[40,41] The dendritic cores can entrap various drug molecules. However, due to the structural limitations involved in the synthesis of dendrimers of higher generation, and relatively compact structure of the dendrimers, the loading capacity of such micelles is limited. Thus, to increase the loading capacity, the dendrimer core can be modified with hydrophobic block, followed by the attachment of the PEO chains. For example, Wang *et al.* recently synthesized an amphiphilic 16-arm star polymer with a polyamidoamine dendrimer core and arms composed of inner lipophilic poly(ε-caprolactone) block and outer PEO

block.[42] These unimolecular micelles were shown to encapsulate a hydrophobic drug, etoposide, with high loading capacity.

Multiarm star-like block copolymers represent another type of unimolecular micelles.[42–46] Star polymers are generally synthesized by either the arm-first or core-first methods. In the arm-first method, monofunctional living linear macromolecules are synthesized and then cross-linked either through propagation, using a bifunctional comonomer,[47] or by adding a multifunctional terminating agent to connect precise number of arms to one center.[45] Conversely, in the core-first method, polymer chains are grown from a multifunctional initiator.[43,44,46,48] One of the first reported examples of unimolecular micelles, suitable for drug delivery, was a three-arm star polymer, composed of mucic acid substituted with fatty acids as a lipophilic inner block and PEO as a hydrophilic outer block.[44] These polymers were directly dispersible in aqueous solutions and formed unimolecular micelles. The size and solubilizing capacity of the micelles were varied by changing the ratio of the hydrophilic and lipophilic moieties. In addition, star-copolymers with polyelectrolyte arms can be prepared to develop pH-sensitive unimolecular micelles as drug carriers.[46]

## 2.3. *Cross-linked micelles*

The multimolecular micelles structure can be reinforced by the formation of cross-links between the polymer chains. These resulting cross-linked micelles are, in essence, single molecules of nanoscale size that are stabile upon dilution, shear forces and environmental variations (e.g. changes in pH, ionic strength, solvents etc.). There are several reports on the stabilization of the polymer micelles by cross-linking either within the core domain[49–53] or throughout the shell layer.[54–56] In these cases, the cross-linked micelles maintained small size and core-shell morphology, while their dissociation was permanently suppressed. Stable nanospheres from the PEO-b-polylactide micelles were prepared by using polymerizable group at the core segment.[49] In addition to stabilization, the core polymerized micelles readily solubilized rather large molecules such as paclitaxel, and retained high loading capacity even upon dilution.[50] Formation of interpenetrating network of a temperature-sensitive polymer (poly-N-isopropylacrilomide) inside the core was also employed for the stabilization of the Pluronic micelles.[53] The resulting micelle structures were stable against dilution, exhibited temperature-responsive swelling behavior, and showed higher drug loading capacity than regular Pluronic micelles.

Recently, a novel type of polymer micelles with cross-linked ionic cores was prepared by using block ionomer complexes as templates.[57] The nanofabrication of these micelles involved condensation of PEO-b-poly(sodium methacrylate) diblock

copolymers by divalent metal cations into spherical micelles of core-shell morphology. The core of the micelle was further chemically cross-linked and cations removed by dialysis. Resulting micelles represent hydrophilic nanospheres of core-shell morphology. The core comprises a network of the cross-linked polyanions and can encapsulate oppositely charged therapeutic and diagnostic agents, while a hydrophilic PEO shell provides for increased solubility. Furthermore, these micelles displayed the pH- and ionic strength-responsive hydrogel-like behavior, due to the effect of the cross-linked ionic core. Such behavior is instrumental for the design of drug carriers with controled loading and release characteristics.

## 3. Drug Loading and Release

In general, there are three major methods for loading drugs into polymer micelle cores: (1) chemical conjugation, (2) physical entrapment or solubilization, and (3) polyionic complexation (e.g. ionic binding).

### 3.1. *Chemical conjugation*

Drug incorporation into polymer micelles via chemical conjugation was first proposed by Ringsdorf's group[58] in 1984. According to this approach, a drug is chemically conjugated to the core-forming block of the copolymer via a carefully designed pH- or enzyme-sensitive linker, that can be cleaved to release a drug in its active form within a cell.[59,60] The polymer-drug conjugate then acts as a polymer prodrug which self assembles into a core-shell structure. The appropriate choice of conjugating bond depends on specific applications.

The nature of the polymer–drug linkage and the stability of the drug conjugate linkage can be controled to influence the rate of drug release, and therefore, the effectiveness of the prodrug.[61–63] For instance, recent work by Kataoka's group proposed pH-sensitive polymer micelles of PEO-b-poly(aspartate hydrazone doxorubicin), in which doxorubicin was conjugated to the hydrophobic segments through acid-sensitive hydrazone linkers that are stable at extracellular pH 7.4, but degrade and release the free drug at acidic pH 5.0 to 6.0 in endosomes and lysosomes.[63,64] The original approach developed by this group used doxorubicin conjugated to the poly(aspartic acid) chain of PEO-b-poly(aspartic acid) block copolymer through an amide bond.[65] Adjusting both the composition of the block copolymer and the concentration of the conjugated doxorubicin, led to improved efficacy, as evidenced by a complete elimination of solid tumors implanted in mice.[66] It was later determined that doxorubicin physically encapsulated within the micellar core was responsible for antitumor activity. This finding led to the use of PEO-b-poly(aspartate doxorubicin) conjugates as nanocontainers for physically entrapped doxorubicin.[67]

## 3.2. *Physical entrapment*

The physical incorporation or solublization of drugs within block copolymer micelles is generally preferred over micelle-forming polymer-drug conjugates, especially for hydrophobic drug molecules. Indeed, many polymers and drug molecules do not contain reactive functional groups for chemical conjugation, and therefore, specific block copolymers have to be designed for a given type of drug. In contrast, a variety of drugs can be physically incorporated into the core of the micelles, by engineering the structure of the core-forming segment. In addition, molecular characteristics (i.e. molecular weight, composition, presence of functional groups for active targeting) within a homologous copolymer series can be designed to optimize the performance of a drug for a given drug delivery situation.[9,14] This concept was introduced by our group in the late 1980s and was initially termed "micellar microcontainer",[68] but is now widely known as a "micellar nanocontainer".[9,10] Haloperidol was encapsulated in Pluronic block copolymer micelles,[68] the micelles were targeted to the brain using brain-specific antibodies or insulin, and enhancement of neuroleptic activity by the solubilized drug was observed. During the last 25 years, a large variety of amphiphilic block copolymers have been explored as nanocontainers for various drugs.

Different loading methods can be used for physical entrapment of the drug into the micelles, including but not limited to dialysis,[69–72] oil in water emulsification,[69] direct dissolution,[42,73,74] or solvent evaporation techniques.[75,76] Depending on the method, drug solubilization may occur during or after micelle assembly. The loading capacity of the polymer micelles, which is frequently expressed in terms of the micelle-water partition coefficient, is influenced by several factors, including both the structure of core-forming block and a drug, molecular characteristics of the copolymer such as composition, molecular weight, and the solution temperature.[13]

Many studies indicate that the most important factor related to the drug solubilization capacity of a polymer micelle is the compatibility between the drug and the core-forming block.[9,14,77–80] For this reason, the choice of the core-forming block is most critical. One parameter that can be used to assess the compatibility between the polymer and a drug is the Flory–Huggins interaction parameter, $\chi_{sp}$, defined as $\chi_{sp} = (\delta_s - \delta_p)^2 V_s / kT$, where $\delta_s$ and $\delta_p$ are Scatchard-Hildebrand solubility parameters, and $V_s$ is the molecular volume of the solubilizate. It was successfully used as a correlation parameter for the solubilization of aliphatic and aromatic hydrocarbons in block copolymer micelles.[80,81] Recently, Allen's group[82] elegantly demonstrated that the calculation and comparison of partial solubility parameters of polymers and drugs could be used as a reliable means to predict polymer-drug compatibility and to guide formulation development. Polymer micelles, possessing core-forming blocks predicted to be compatible with the drug of interest (Ellipticine), were able

to increase the solubility of the drug up to 30,000 times, compared with its saturation solubility in water.[82] The degree of compatibility between the drug and the core-forming block has also been shown to influence the release rate of the drug from the micelles. When the environment within the core of the micelle becomes more compatible with the drug, it results in a considerable decrease in the rate of drug release.

For a given drug, the extent of incorporation is a function of factors that also control the micelle size and/or aggregation number. Such factors include the ratio of hydrophobic to hydrophilic block length and the copolymer molecular weight. For example, the loading capacity of Pluronic micelles was found to increase with the increase in the hydrophobic PPO block length. This effect is attributed to a decrease in CMC, and therefore, an increase in aggregation number and micelle core size. Also, but to a lesser extent, the hydrophilic block length affects the extent of solubilization, such that an increase in percentage of PEO in Pluronic block copolymers results in a decrease in the loading capacity of the micelles.[80,83–85] For a given ratio of PPO-to-PEO, higher molecular weight polymers form larger micelles, and therefore, show a higher drug loading capacity. Therefore, the total amount of loaded drug can be adjusted as a function of the micellar characteristics as clearly was demonstrated by Nagaradjan[83] and Kozlov *et al.*[85] Several studies indicate that both the copolymer concentration as well as the drug to polymer ratio upon loading, have a complex effect on the loading capacity of polymer micelles.[79,84,86] In general, more polymer chains provide more absorption sites. As a result, solubilization is increased with polymer concentration.[82] However, the solubilization capacity was found to reach a saturation level with an increase of polymer concentration.[79] The maximum loading level is largely influenced by the interaction between the solubilizate and core-forming block, and stronger interactions enable saturation to be reached at lower polymer concentration. It was also demonstrated in the studies by Hurter and Hatton[84,86] that the loading capacity of micelles formed from copolymers with high hydrophobic content was independent of the polymer concentration. In addition, the location of the incorporated molecules within polymer micelles (micelle core or the core-shell interface) determines the extent of solubilization, as well as the rate of drug release.[87,88] It has been found that more soluble compounds are localized at the core-shell interface or even in the inner shell, whereas more hydrophobic molecules have a tendency to solubilize in the micelle core.[85,87,88] The release rate of drug localized in the shell or at the interface appears to account for the "burst release" from the micelles.[87] In general, for drugs physically incorporated in polymer micelles, release is controled by the rate of diffusion of the drug from the micellar core, stability of the micelles, and the rate of biodegradation of the copolymer. If the micelle is stable and the rate of polymer biodegradation is slow, the diffusion rate of the drug will be mainly determined by the abovementioned factors,

i.e. the compatibility between the drug and core forming block of copolymer,[69,82] the amount of drug loaded, the molecular volume of drug, and the length of the core forming block.[89] In addition, the physical state of the micelle core and drug has a large influence on release characteristics. It was demonstrated that the diffusion of incorporated molecules from the block copolymer micelles with glassy cores is slower, in comparison to the diffusion out of the cores that are more mobile.[87]

## 3.3. *Polyionic complexation*

Charged therapeutic agents can be incorporated into block copolymer micelles, through electrostatic interactions with an oppositely charged ionic segment of block copolymer. Since it was being proposed independently by Kabanov and Kataoka in 1995,[90,91] this approach is now widely used for the incorporation of various polynucleic acids into block ionomer complexes, for developing non-viral gene delivery systems. Ionic block lengths, charge density, and ionic strength of the solution affect the formation of stable block ionomer complexes, and therefore, control the amount of drug that can be incorporated within the micelles.[8,92] The pH- and salt-sensitivity of such block ionomer micelles provide a unique opportunity to control the triggered release of the active therapeutic agent.[15,63,93–96] Furthermore, block ionomer complexes can participate in the polyion interchange reactions which are believed to account for the release of the therapeutic agent and DNA in an active form inside cells.[7] Several comprehensive reviews can be found in the literature that focus on block ionomer micelles as drug and gene delivery systems.[8,92] In addition, physicochemical aspects of the DNA complexes with cationic block copolymers have also been recently reviewed.[97]

As an example, the metal-complex formation of ionic block copolymer, PEO-b-poly(L-aspartic acid), was explored to prepare polymer micelles incorporating *cis*-dichlorodiamminoplatinum (II) (CDDP);[98,99] a potent chemotherapeutic agent widely used in the treatment of a variety of solid tumors, particularly, testicular, ovarian, head and neck, and lung tumors.[100,101] The CDDP-loaded micelles had a size of approximately 20 nm. These micelles showed remarkable stability upon dilution in distilled water, while in physiological saline, they displayed sustained release of the regenerated Pt complex over 50 hrs, due to inverse ligand exchange from carboxylate to chloride. The release rate was inversely correlated with the chain length of poly(L-aspartic acid) segments in the block copolymer. The stability of CDDP-loaded micelle against salt was shown to be improved by the addition of homopolymer, poly(L-aspartic acid), in the micelles.[102] Recently, CDDP-loaded micelles were newly prepared using another block copolymer, PEO-b-poly(glutamic acid) to improve and optimize the micellar stability, as well

as the drug release profile.[103] The drug loading in the micelles was as high as 39% (w/w), and these micelles released the platinum in physiological saline at 37°C in sustained manner > 150 hrs, without initial burst of the drug.

The principle of polyionic complexation can also be used to design new photosensitizers for photodynamic therapy of cancer. The group of Kataoka reported formation of micelles, as a result of mixing of oppositely charged dendrimer porphirin and block ionomer, based on electrostatic assembly[104] or combination of electrostatic and hydrogen bonding interactions.[95,105] The micelles were stabile at physiological conditions and released the entrapped dendrimers in the acidic pH environment (pH 5.0), suggesting a possibility of pH-triggered drug release in the intracellular endosomal compartments. Overall, the photodynamic efficacy of the dendrimer porphyrins was dramatically improved by inclusion into micelles. This process resulted in more than two orders of magnitude increase in the photocytotoxicity, compared with that of the free dendrimer porphyrins.

In addition, the polyionic complexation has been used to immobilize charged enzymes such as egg white lysozyme[106] or trypsin,[107] which were incorporated in the core of polyion micelles, after mixing with oppositely charged ionic block copolymer. A remarkable enhancement of enzymatic activity was observed in the core of the micelles. Furthermore, the on-off switching of the enzyme activity was achieved through the destabilization of the core domain by applying a pulse electric field.[108] These unique features of the polyion micelles are relevant for their use as smart nanoreactors in the diverse fields of medical and biological engineering.

Last, but not the least, a special class of polyion complexes has been synthesized by reacting block ionomers with surfactants of opposite charge, resulting in the formation of environmentally responsive nanomaterials, which differ in sizes and morphologies, and include micelles and vesicles.[109–113] These materials contain a hydrophobic core formed by the surfactant tail groups, and a hydrophilic shell formed, for example, by PEO chains of the block ionomer. These block ionomer complexes can incorporate charged surfactant drugs such as retinoic acid, as well as other drugs via solubilization in the hydrophobic domains formed by surfactant molecules.[114] They display transitions induced by changes in pH, salt concentration, chemical nature of low molecular mass counterions, as well as temperature. They can also be fine tuned to respond to environmental changes occurring in a very wide range of conditions that could realize during delivery of biological and imaging agents.[94,115] The unique self-assembly behavior, the simplicity of the preparation, and the wide variety of available surfactant components that can easily produce polymer micelles with a very broad range of core properties, make this type of materials extremely promising for developing vehicles for the delivery of diagnostic and therapeutic modalities.

## 4. Pharmacokinetics and Biodistribution

Incorporation of a low molecular mass drug into polymer micelles drastically alters pharmacokinetics and biodistribution of the drug in the body, which is crucial for the drug action. Low molecular mass drugs, after administration in the body, rapidly extravasate to various tissues affecting them almost indiscriminately, and then are rapidly eliminated from the body via renal clearance, often causing toxicity to kidneys.[116] Furthermore, many drugs display low stability and are degraded in the body, often forming toxic metabolites. An example is doxorubicinol, a major metabolite of doxorubicin, which causes cardiac toxicity.[117] These impediments to the therapeutic use of low molecular mass drugs can be mitigated by encapsulating drugs in polymer micelles. Within the micelles, the drug molecules are protected from enzymatic degradation by the micelle shell. The pharmacokinetics and biodistribution of the micelle-incorporated drugs are mainly determined by the surface properties, size, and stability of the micelles, and are less affected by the properties of the loaded drug. The surface properties of the micelles are determined by the micelle shell. The shell from PEO effectively masks drug molecules and prevents interactions with serum proteins and cells, which contributes to prolonged circulation of the micelles in the body.[16] From the size standpoint, polymer micelles fit an ideal range of sizes for systemic drug delivery. On the one hand, micelles are sufficiently large, usually exceeding 10 nm in diameter, which hinders their extravasation in nontarget tissues and prevents renal glomerular excretion. On the other hand, the micelles are not considered large, since their size usually does not exceed 100 nm. As a result, micelles avoid scavenging by the mononuclear phagocytes system (MPS) in the liver and spleen. To this end, "stealth" particles whose surface is decorated with PEO are known to be less visible to macrophages and have prolonged half-lives in the blood.[64,118,119]

The contribution of the micelle stability to pharmacokintetics and biodistribution is much less understood, although it is clear that micelle degradation should result in a decrease of the size and drug release, perhaps, prematurely. Degradation of the micelles, resulting in the formation of block copolymer unimers, could also be a principal route for the removal of the polymer material from the body. The molecular mass of the unimers of most block copolymers is below the renal excretion limit, i.e. less than ~ 20 to 40 kDa,[22,120,121] while the molecular mass of the micelles, which usually contain several dozen or even hundreds of unimers molecules, is above this limit. Thus, the unimers are sufficiently small and can be removed via renal excretion, while the micelles cannot. A recent study by Batrakova *et al.* determined pharmacokinetic parameters of an amphiphilic block copolymer, Pluronic P85, and perhaps provided first evidence that the pharmacokinetic behavior of a block copolymer can be a function of its aggregation state.[119] Specifically, the formation

of micelles increased the half-life of the block copolymer in plasma and decreased the uptake of the block copolymer in the liver. However, it had no effect on the total clearance, indicating that the elimination of Pluronic P85 was controled by the renal tubular transport of unimers, but not by the rate of micelles disposition or disintegration. Furthermore, the values of the clearance suggested that a significant portion of the block copolymer was reabsorbed back into the blood, probably, through the kidney's tubular membranes. Chemical degradation of the polymers comprising the micelles, followed by renal excretion of the relatively low molecular mass products of degradation, may be another route for the removal of the micelle polymer material from the body. This route could be particularly important in the case of the cross-linked or unimolecular micelles, micelles displaying very high stability, and/or micelles composed from very hydrophobic polymer molecules that can bind and retain considerably biological membranes and other cellular components.

The delivery of chemotherapeutic drugs to treat tumors is one of the most advanced areas of research using polymer micelles. Two approaches have been explored to enhance delivery of drug-loaded polymer micelles to the tumor sites: (1) passive targeting and (2) vectorized targeting. The passive targeting involves enhanced permeability and retention (EPR) effect.[122,123] It is based on the fact that solid tumors display increased vascular density and permeability caused by angiogenesis, impaired lymphatic recovery, and lack of a smooth muscle layer in solid tumor vessels. As a result, micellar drugs can penetrate and retain in the sites of tumor lesions. At the same time, extravasation of micellar drugs in normal tissues is decreased, compared with low molecular drug molecules. Among normal organs, spleen and liver can accumulate polymer drugs, but the drugs are eventually cleared via the lymphatic system. The increased circulation time of the micellar drugs should further enhance exposure of the tumors to the micellar drug, compared with the low molecular mass drugs. Along with passive targeting, the delivery of micellar drugs to tumors can potentially be enhanced by the modification of the surface of the polymer micelles with the targeting molecules, vectors that can selectively bind to the surface of the tumor cells. Potential vectors include antibodies, aptamers and peptides, capable of binding tumor-specific antigens and other molecules diplayed at the surfaces of the tumors.[124–126]

Altered biodistribution of a common antineoplastic agent was demonstrated for CDDP encapsulated in polyionic micelles with PEO-b-poly(glutamic acid) block copolymers.[103] Free CDDP is rapidly distributed to each organ, where its levels peak at about one hr after *i.v.* administration. In contrast, in the case of the CDDP-incorporated micelles, due to their remarkably prolonged blood circulation time, the drug level in the liver, spleen and tumor continued to increase up to at least 24 hrs after injection. Consequently, the CDDP-incorporated micelle exhibited 4-, 39-, and 20-fold higher accumulation in the liver, spleen and tumor respectively,

than the free CDDP. At the same time, the encapsulation of CDDP into the micelles significantly decreased drug accumulation in the kidney, especially during first hr after administration. This suggested potential for the decrease of severe nephrotoxicity observed with the free drug, which is excreted through the glomerular filtration, thus affecting the kidney.[127]

Promising results were also demonstrated for doxorubicin incorporated into styrene-maleic acid micelles.[128] In this case, as a result of drug entrapment into micelles, the drug was redirected from the heart to the tumor, and the doxorubicin cardiotoxicity was diminished. Complete blood counts and cardiac histology for the micellar drug showed no serious side effects for *i.v.* doses as high as 100 mg/kg doxorubicin equivalent in mice. Similar results were reported for doxorubicin incorporated in mixed micelles of PEO-b-poly(L-histidine) and PEO-b-poly(L-lactic acid) block copolymers.[129] Tissue levels of doxorubicin administered in the micellar formulation were decreased in the blood and the liver, and considerably increased in the solid tumor, compared with the free drug. Further increase in the tumor delivery was achieved by modifying the surface of the micelles with the folate molecules. The accumulated doxorubicin levels observed using folate-modified micelles was 20 times higher than those for free doxorubicin, and 3 times higher than those for unmodified micelles.

The first micellar formulation of doxorubicin to reach clinical evaluation stage, used the micelles composed of triblock copolymer, PEO-b-poly(propylene oxide)-b-PEO, Pluronic.[130] Analysis of pharmacokinetics and biodistribution of doxorubicin incorporated into mixed micelles of Pluronics L61 and F127, SP1049C, demonstrated more efficient accumulation of the micellar drug in the tumors, compared with the free drug. Specifically, the areas under the curves (AUC) in the Lewis lung carcinoma 3LLM-27 solid tumors in C57Bl/6 mice were increased about two fold using SP1049, compared with the free doxorubicin. Furthermore, this study indicated that the peak levels of doxorubicin formulated with SP1049 in the tumor were delayed and the drug residence time was increased, in comparison with the free doxorubicin.[130]

A clear visualization of drug delivery to the tumor site was shown for doxorubicin covalently incorporated through the pH-sensitive link into polymer micelles of PEO-poly(aspartate hydrazone doxorubicin).[64] A phase-contrast image showed that the tumor blood vessels containing the micelles leaked into extravascular compartments of the tumors, resulting in the infiltration of the micelles into tumor sites. The micelles circulated in the blood for a prolonged time, and the AUC for micellar doxorubicin was 15-fold greater than the AUC for the free doxorubicin. Furthermore, the AUC values of the micellar doxorubicin in the heart and kidney decreased, compared with the free drug. Thus, the selectivity of drug delivery to the tumor, compared with heart and kidney ($AUC_{tumor}/AUC_{organ}$) was increased by 6- and 5-folds respectively. This may result in the reduction of side effects of

doxorubicin such as cardiotoxicity and nephrotoxicity. Moreover, the micellar doxorubicin showed relatively low uptake in the liver and spleen, despite very long residence time in the blood.

Biodistribution of paclitaxel incorporated into biodegradable polymer micelles of monomethoxy-PEO-b-poly(D,L-lactide) block copolymer, Genexol-PM, was compared with the regular formulation of the drug in Cremophor EL.[131] Two to three-fold increases in drug levels were demonstrated in most tissues including liver, spleen, kidneys, lungs, heart and tumor, after *i.v.* administration of Genexol-PM, compared with paclitaxel. Nevertheless, acute dose toxicity of Genexol-PM was about 25 times lower than that of the conventional drug formulation, which appears to be a result of the reformulation avoiding the use of Chremophor EL and dehydrated ethanol that are toxic.

Selective tumor targeting with paclitaxel encapsulated in micelles, modified with tumor-specific antibodies 2C5 ("immunomicelles"), was reported using Lewis lung carcinoma solid tumor model in C57Bl/6J mice.[26] These micelles were prepared from PEO-distearyl phosphatidylethanolamine conjugates with the free PEO end activated with the p-nitrophenylcarbonyl group for the antibody attachment. The amount of micellar drug accumulated in the tumor exceeded that in the non-target tissue (muscles) by more than ten times. It is worth noting that the highest accumulation in the tumor was demonstrated in the micelles containing the longest PEO chains, which also had the longest circulation time in the blood. Furthermore, the immunomicelles displayed the highest amount of tumor-accumulated drug, compared with either free paclitaxel or non-vectorized micelles. It was demonstrated that paclitaxel delivered by plain micelles in the interstitial space of the tumor was eventually cleared after gradual micellar degradation. In contrast, paclitaxel-loaded 2C5 immunomicelles were internalized by cancer cells and the retention of the drug inside the tumor was enhanced.[132]

Unexpected results were found using pH-sensitive polymer micelles of N-isopropylacrylamide and methacrylic acid copolymers randomly or terminally alkylated with octadecyl groups.[64,133] It was demonstrated that aluminium chloride phthalocyanine (AlClPc) incorporated in such micelles was cleared more rapidly and less accumulated in the tumor, than the AlClPc formulated with Cremophor EL. Furthermore, significant accumulation in the liver and spleen (and lungs for most hydrophobic copolymers) was observed, compared with Cremophor EL formulation. The enhanced uptake of such polymer micelles by the cells of mononuclear phagocyte system (MPS) could be due to micelle aggregation in the blood and embolism in the capillaries. Thus, it attempted to reduce the uptake of the micelles in MPS by incorporating water soluble monomers, N-vinyl-2-pyrrolidone in the copolymer structure.[134] The modified formulation displayed same levels of tumor accumulation and somewhat higher antitumor activity than the Cremophor

EL formulation. This work serves as an example reinforcing the need of proper adjustment of the polymer micelle structure, and perhaps the need of using block copolymers to produce a defined protective hydrophilic shell to facilitate evasion of the polymer micelles from MPS.

## 5. Drug Delivery Applications

The studies on the application of polymer micelles in drug delivery have mostly focused on the following areas that are considered below: (1) delivery of anticancer agents to treat tumors; (2) drug delivery to the brain to treat neurodegenerative diseases; (3) delivery of antifungal agents; (4) delivery of imaging agents for diagnostic applications; and (5) delivery of polynucleotide therapeutics.

### 5.1. *Chemotherapy of cancer*

To enhance chemotherapy of tumors using polymer micelles, four major approaches were employed: (1) passive targeting of polymer micelles to tumors due to EPR effect; (2) targeting of polymer micelles to specific antigens overexpressed at the surface of tumor cells; (3) enhanced drug release at the tumor sites having low pH; and (4) sensitization of drug resistant tumors by block copolymers.

A series of pioneering studies by Kataoka's group used polymer micelles for passive targeting of various anticancer agents and chemotherapy of tumors.[102,103,135] One notable recent example reported by this group involves polymer micelles of PEO-b-poly(L-aspartic acid) incorporating CDDP. Evaluation of anticancer activity using murine colon adenocarcinoma C26 as an *in vivo* tumor model, demonstrated that CDDP in polymer micelles had significantly higher activity than the free CDDP, resulting in complete eradication of the tumor.[103] A formulation of paclitaxel in biodegradable polymer micelles of monomethoxy-PEO-b-poly(D,L-lactide) block copolymer, Genexol-PM, also displayed elevated activity *in vivo* against human ovarian carcinoma OVCAR-3 and human breast carcinoma MCF7, compared with a regular formulation of the drug in Cremophor EL.[131] In addition, anthracycline antibiotics, doxorubicin and pirarubicin, incorporated in styrene-maleic acid micelles each revealed potent anticancer effects *in vivo* against mouse sarcoma S-180, resulting in complete eradication of tumors in 100% of tested animals.[128] Notably, animals survived for more than one year, after treatment with the micelle-incorporated pirarubicin at doses as high as 100 mg/kg of pirarubicin equivalent. Complete blood counts, liver function test, and cardiac histology showed no sign of adverse effects for intravenous doses of the micellar formulation. In contrast, animals receiving free pirarubicin had a much reduced survival and showed serious side effects.[136] Collectively, these studies suggested that various micelle-incorporated drugs display improved therapeutic index in solid tumors, which

correlates with enhanced passive targeting of the drug to the tumor sites, as well as decreased side effects, compared with conventional formulations of these drugs.

Tumor-specific targeting of polymer micelles to molecular markers expressed at the surface of the cancer cells has also been explored to eradicate tumor cells. For example, a recent study by Gao's group developed a polymer micelle carrier to deliver doxorubicin to the tumor endothelial cells with overexpressed $\lambda_v\beta_3$ integrins.[137] A cyclic pentapeptide, cRGD was used as a targeting ligand that is capable of selective and high affinity binding to the $\lambda_v\beta_3$ integrin. Micelles of PEO-b-poly($\varepsilon$-caprolactone) loaded with doxorubicin were covalently bound with cRGD. As a result of such modification, the uptake of doxorubicin-containing micelles in *in vitro* human endothelial cell model derived from Kaposi's sarcoma, was profoundly increased. In addition, folate receptor often overexpressed in cancer cells has been evaluated for targeting various drug carriers to tumors.[138] This strategy has also been evaluated to target polymer micelles. For example, mixed micelles of PEO-b-poly(L-histidine) and PEO-b-poly(L-lactic acid) block copolymers with solubilized doxorubicin[129] or micelles of PEO-b-poly(DL-lactic-co-glycolic acid) block copolymer with covalently attached doxorubicin,[139] were each surface modified by conjugating folate molecules to the free PEO ends. In both cases, *in vitro* and *in vivo* studies demonstrated increased antitumor activity of the micelle-incorporated drug resulting from such modification. The enhanced delivery of the micellar drugs through the folate receptor, and the enhanced retention of the modified micelles at the tumor sites are possible explanations for the effects of these folate modifications.

Micelles conjugated with antibodies or antibody fragments capable to recognize tumor antigens were shown to improve therapeutic efficacy *in vivo* over non-modified micelles.[23] This approach can result in high selectivity of binding, internalization, and effective retention of the micelles in the tumor cells. In addition, recent advances in antibody engineering allow for the production of humanized antibody fragments, reducing problems with immune response against mouse antibodies.[140] For example, micelles of PEO-distearyl phosphatidylethanolamine were covalently modified with the monoclonal antibody 2C5 that binds to nucleosomes, displayed at the surface of many tumor cells. The micelles were then used for incorporating various poorly soluble anticancer drugs including tamoxifen, paclitaxel, dequalinium, and chlorine e6 trimethyl ester.[26,132,141] It was shown that paclitaxel-loaded 2C5-immunomicelles could specifically recognize a variety of tumor types. The binding of these immunomicelles was observed for all cancer cell lines tested, i.e. murine Lewis lung carcinoma, T-lymphoma EL4, and human breast adenocarcinomas, BT-20 and MCF7.[141] Moreover, paclitaxel-loaded 2C5 immunomicelles demonstrated highest anticancer activity in Lewis lung carcinoma tumor model in mice, compared with plain paclitaxel-loaded micelles and

the free drug.[132] The increased antitumor effect of immunomicelles *in vivo* correlated with the enhanced retention of the drug delivered with the immunomicelles inside the tumor.

Tumors often display low pH of interstitial fluid, which is mainly attributed to higher rates of aerobic and anaerobic glycolysis in cancer cells than in normal cells.[142,143] This phenomenon has been employed in the design of various pH-sensitive polymer micelle systems for the delivery of anticancer drugs to the tumors. One approach consisted in the chemical conjugation of anticancer drugs to the block copolymers through pH sensitive cleavable links that are stable at neutral pH, but are cleavable and release the drug in the mildly acidic pH. For example, several groups used hydrasone-based linking groups, to covalently attach doxorubicin to PEO-b-poly(DL-lactic-co-glycolic acid) block copolymer,[21,144] PEO-b-block-poly(allyl glycidyl ether)[145] or PEO-b-poly(aspartate hydrazone) block copolymer.[63,64] It was suggested that doxorubicin will remain in the micelles in the blood stream, and will be released at tumor sites at lower pH. For example, *in vitro* and *in vivo* studies using PEO-b-poly(aspartate hydrazone doxorubicin) micelles demonstrated that the micelles display an intracellular pH-triggered drug release capability, tumor-infiltrating permeability, and effective antitumor activity with extremely low toxicity.[63,64] Overall, the animal studies suggested that such polymer micelle drug has a wide therapeutic window due to increased efficacy and decreased toxicity, compared with free doxorubicin.[64]

An alternative mechanism for pH-induced triggering of drug release at the tumor sites consists of using pH sensitive polyacids or polybases as building blocks for polymer micelles.[94,146,147] For example, mixed micelles of PEO-b-poly(L-histidine) and PEO-b-poly(L-lactic acid) block copolymers incorporate pH-sensitive poly-base, poly(L-histidine) in the hydrophobic core.[147] The core can also solubilize hydrophobic drugs such as doxorubicin. The protonation of the poly-base at acidic conditions resulted in the destabilization of the core and triggered release of the drug. This system was also targeted to the tumors through the folate molecules as described earlier and has shown significant *in vivo* antitumor activity and less side effects, compared with the free drug.[129] Notably, it was also effective *in vitro* and *in vivo* against multidrug resistant (MDR) human breast carcinoma MCF7/ADR that overexpresses P-glycoprotein (Pgp). Pgp is a drug efflux transport protein that serves to eliminate drugs from the cancer cells and significantly decreases the anticancer activity of the drugs. The micelle incorporated drug was released inside the cells, and thus avoided the contact with Pgp localized at the cell plasma membrane, which perhaps contributed to the increased activity of pH sensitive doxorubicin micelles in the MDR cells.

A different approach using Pluronic block copolymer micelles to overcome MDR in tumors has been developed by our group.[130,148–151] Studies by Alakhov

*et al.* demonstrated that Pluronic block copolymers can sensitize MDR cells, resulting in an increased cytotoxic activity of doxorubicin, paclitaxel, and other drugs by 2,3 orders of magnitude.[148,149] Remarkably, Pluronic can enhance drug effects in MDR cells through multiple effects including (1) inhibiting drug efflux transporters, such as Pgp[149,152] and multidrug resistance proteins (MRPs),[153,154] (2) abolishing drug sequestration within cytoplasmic vesicles,[149,153] (3) inhibiting the glutathione/glutathione S-transferase detoxification system,[154] and (4) enhancing proapoptotic signaling in MDR cells.[155] Similar effects of Pluronics have also been reported using *in vivo* tumor models.[130,150] In these studies, mice bearing drug-sensitive and drug-resistant tumors were treated with doxorubicin alone and with doxorubicin in Pluronic compositions. The tumor panel included *i.p.* murine leukemias (P388, P388-Dox), *s.c.* murine myelomas (Sp2/0, Sp2/0-Dnr), *i.v.* and *s.c.* Lewis lung carcinoma (3LL-M27), *s.c.* human breast carcinomas (MCF7, MCF7/ADR), and *s.c.* human oral epidermoid carcinoma (KBv).[130] Using the NCI criteria for tumor inhibition and increased lifespan, Pluronic/doxorubicin has met the efficiency criteria in all models (9 of 9), while doxorubicin alone was only effective in selected tumors (2 of 9).[130] Results showed that the tumors were more responsive in the Pluronic/doxorubicin treatment groups than in doxorubicin alone. These studies demonstrated improved treatment of drug resistant cancers with Pluronics.

The mechanisms of effects of Pluronic on Pgp have been studied in great detail.[151] In particular, exposure of MDR cells to Pluronics has resulted in the inhibition of Pgp-mediated efflux,[149] and this overcomes defects in intracellular accumulation of Pgp-dependent drugs,[148,149,152] and abolishes the directionality difference in the flux of these drugs across polarized cell monolayers.[156–158] The lack of changes in membrane permeability with Pluronics to (1) non-Pgp compounds in MDR cells,[158,159] and (2) Pgp probes in non-MDR cells,[149,153] suggested that Pluronic effects were specific to the Pgp efflux system. These effects were observed at Pluronic concentrations less than or equal to the critical micelle concentration (CMC).[152,159] Thus, Pluronic unimers rather than the micelles were responsible for these effects. Specifically, Pluronic molecules displayed a dual function in MDR cells.[160–162] Firstly, they incorporated into the cell membranes and decreased the membrane microviscosity. This was accompanied by the inhibition of Pgp ATPase activity. Secondly, they translocated into cells and reached intracellular compartments. This was accompanied by the inhibition of respiration,[163] presumably due to Pluronic interactions with the mitochondria membranes. As a result, within 15 min after exposure to select Pluronics, intracellular levels of ATP in MDR cells were drastically decreased.[160–162] Remarkably, such ATP depletion was not observed in non-MDR cells, suggesting that the Pluronic was "selective", with respect to the MDR phenotype.[160,164] Combining these two effects, Pgp ATPase inhibition and ATP depletion, resulted in the shut-down of the efflux system in MDR

cells.[160–162] The Pgp remained functionally active when (1) ATP was restored using an ATP supplementation system in the presence of a Pluronic, or (2) when ATP was depleted, but there was no direct contact between the Pluronic and Pgp (and no ATPase inhibition). Overall, these detailed studies which resulted in the development of a micellar formulation of doxorubicin that is evaluated clinically, reinforce the fact that block copolymers, comprising the micelles, can serve as biological response modifying agents that can have beneficial effects in the chemotherapy of tumors.

## 5.2. *Drug delivery to the brain*

By restricting drug transport to the brain, the blood brain barrier (BBB) represents a formidable impediment for the treatment of brain tumors and neurodegenerative diseases such as HIV-associated dementia, stroke, Parkinson's and Alzheimer's diseases. Two strategies using polymer micelles have been evaluated to enhance delivery of biologically active agents to the brain. The first strategy is based on the modification of polymer micelles with antibodies or ligand molecules capable of transcytosis across brain microvessel endothelial cells, comprising the BBB. The second strategy uses Pluronic block copolymers to inhibit drug efflux systems, particularly, Pgp, and selectively increase the permeability of BBB to Pgp substrates.

An earlier study used micelles of Pluronic block copolymers for the delivery of the CNS drugs to the brain.[68,73] These micelles were surface-modified by attaching to the free PEO ends, either polyclonal antibodies against brain-specific antigen, $\alpha_2$-glycoprotein, or insulin to target the receptor at the lumenal side of BBB. The modified micelles were used to solubilize fluorescent dye or neuroleptic drug, haloperidol, and these formulations were administered intravenously in mice. Both the antibody and insulin modification of the micelles resulted in enhanced delivery of the fluorescent dye to the brain and drastic increases in neuroleptic effect of haloperidol in the animals. Subsequent studies using *in vitro* BBB models demonstrated that the micelles, vectorized by insulin, undergo receptor-mediated transport across brain microvessel endothelial cells.[156] Based on one of these observations, one should expect development of novel polymer micelles that target specific receptors at the surface of the BBB to enhance transport of the incorporated drugs to the brain.

The studies by our group have also demonstrated that selected Pluronic block copolymers, such as Pluronic P85, are potent inhibitors of Pgp, and they have the increased entry of the Pgp-substrates to the brain across BBB.[156,158,159,165] Pluronic did not induce toxic effect in BBB, as revealed by the lack of alteration in paracellular permeability of the barrier,[156,158] and in histological studies, using specific markers for brain endothelial cells.[166] Overall, this strategy has potential in developing

novel modalities for the delivery of various drugs to the brain, including selective anti cancer agents to treat metastatic brain tumors, as well as HIV protease inhibitors to eradicate HIV virus in the brain.[167,168]

## 5.3. *Formulations of antifungal agents*

The need for safe and effective modalities for the delivery of chemotherapeutic agents to treat systemic fungal infections in immunocompromised AIDS, surgery, transplant and cancer patients is very high. The challenges to the delivery of antifungal agents include low solubility and sometimes high toxicity of these agents. These agents, such as amphotericin B, have low compatibility with hydrophobic cores of polymer micelles formed by many conventional block copolymers. Thus, to increase solubilization of amphotericin B, the core-forming blocks of methoxy-PEO-b-poly(L-aspartate) were derivatized with stearate side chains.[169–172] The resulting block copolymers formed micelles. Amphotericin B interacted strongly with the stearate side chains in the core of the micelles, resulting in an efficient entrapment of the drug in the micelles, as well as subsequent sustained release in the external environment. As a result of solubilization of amphotericin B in the micelles, the onset of hemolytic activity of this drug toward bovine erythrocytes was delayed, relative to that of the free drug.[171] Using a neutropenic murine model of disseminated candidas, it was shown that micelle-incorporated amphotericin B retained potent *in vivo* activity. Pluronic block copolymers were used by the same group for incapsulation of another poorly soluble antifungal agent, nystatin.[172] This is a commercially available drug that has shown potential for systemic administration, but has never been approved for that purpose, due to toxicity issues. The possibility to use Pluronic block copolymers to overcome resistance to certain antifungal agents has also been demonstrated.[173–176] Overall, one should expect further scientific developments using polymer micelle delivery systems for the treatment of fungal infection.

## 5.4. *Delivery of imaging agents*

Efficient delivery of imaging agents to the site of disease in the body can improve early diagnostics of cancer and other diseases. The studies in this area using polymer micelles as carriers for imaging agents were initiated by Torchilin.[177] For example, micelles of amphiphilic PEO-lipid conjugates were loaded with $^{111}$ In and gadolinium diethylenetriamine pentaacetic acid-phosphatidylethanolamine (Gd-DTPA-PE) and then used for visualization of local lymphatic chain after subcutaneous injection into the rabbit's paw.[178] The images of local lymphatics were acquired using a gamma camera and a magnetic resonance (MR) imager. The

injected micelles stayed within the lymph fluid, thus serving as lymphangiographic agents for indirect MR or gamma lymphography. Another polymer micelle system composed of amphiphilic methoxy-PEO-b-poly[epsilon,N-(triiodobenzoyl)-L-lysine] block copolymers, labeled with iodine, was administered systemically in rabbits and visualized by X-ray computed tomography.[179] The labeled micelles displayed exceptional 24 hrs half-life in the blood, which is likely due to the core-shell architecture of the micelle carriers that protected the iodine-containing core. Notably, small polymer micelles (<20 nm) may be advantageous for bioimaging of tumors, compared with PEG-modified long-circulating liposomes (ca. 100 nm). In particular, the micelles from PEO-distearoyl phosphatidyl ethanolamine conjugates containing $^{111}$In-labeled model protein were more efficacious in the delivery of protein to Lewis lung carcinoma than larger long-circulating liposomes.[180] Overall, polymer micelles loaded with various agents for gamma, magnetic resonance, and computed tomography imaging represent promising modalities for non-invasive diagnostics of various diseases.

## 5.5. *Delivery of polynucleotides*

To improve the stability of polycation-based DNA, delivery complexes in dispersion block and graft copolymers containing segments from polycations and non-ionic water-soluble polymers, such as PEO, were developed.[90,181,182] Binding of these copolymers with DNA results in the formation of micelle-like block ionomer complexes ("polyion complex micelles"), containing hydrophobic sites formed by the polycation-neutralized DNA and hydrophilic sites formed by the PEO chains. Despite neutralization of charge, complexes remain stable in aqueous dispersion due to the effect of the PEO chains.[183] Overall, the PEO modified polycation-DNA complexes form stable dispersions and do not interact with serum proteins.[183,184] These systems were successfully used for intravitreal delivery of an antisense oligonucleotide and the suppression of gene expression in retina in rats.[185] Furthermore, they displayed extended plasma clearance kinetics and were shown to transfect liver and tumor cells, after systemic administration in the body.[186–188] In addition, there is a possibility targeting such polyplexes to the specific receptors at the surface of the cell, for example, by modifying the free ends of PEO chains with specific targeting ligands.[189–191] Alternatively, to increase the binding of the complexes with the cell membrane and the transport of the polynucleotides inside the cells, the polycations were modified with amphiphilic Pluronic molecules.[192,193] One recent study has shown a potential of Pluronic-polyethyleneimine-based micelles for *in vivo* delivery of antisense oligonucleotides to tumors, and have demonstrated sensitization of the tumors to radiotherapy as a result of systemic administration of the oligonucleotide-loaded micelles.[194]

## 6. Clinical Trials

Three polymer micelle formulations of anticancer drugs have been reported to reach clinical trials. The doxorubicin-conjugated polymer micelles developed by Kataoka's group[195] have progressed recently to Phase I clinical trial at the National Cancer Center Hospital, Tokyo, Japan. The micelle carrier NK911 is based on PEO-b-poly(aspartic acid) block copolymers, in which the aspartic acid units were partially (ca. 45%) substituted with doxorubicin to form hydrophobic block. The resulting substituted block copolymer forms micelles that are further noncovalently loaded with free doxorubicin. Preclinical studies in mice demonstrated higher NK911 activity against Colon 26, M5076, and P388, compared with the free drug. Moreover, NK911 has less side effects, resulting in less animal body and toxic death than the free drug.[196]

Clinically, the Pluronic micelle formulation of doxorubicin has been most advanced. Based on the *in vivo* efficacy evaluation, Pluronic L61 was selected for clinical development for the treatment of MDR cancers. The final block copolymer formulation is a mixture of 0.25% Pluronic L61 and 2% Pluronic F127, formulated in isotonic buffered saline.[130] This system contains mixed micelles of L61 and F127, with an effective diameter of ca. 22 to 27 nm and is stable in the serum. Prior to administration, doxorubicin is mixed with this system, which results in spontaneous incorporation of the drug in the micelles. The drug is easily released by diffusion after dilution of the micelles. The formulation of doxorubicin with Pluronic, SP1049C, is safe, following systemic administration based on toxicity studies in animals.[130] A two-site Phase I clinical trial of SP1049C has been completed.[197] Based on its results, the dose-limiting toxicity of SP1049C was myelosuppression, reached at 90 mg/m$^2$ (maximum tolerated dose was 70 mg/m$^2$). Phase II study of this formulation to treat inoperable metastatic adenocarcinoma of the esophagus is near completion as well.[198]

Finally, Phase I studies were reported for Genexol-PM, a Cremophor-free polymer micelle-formulated paclitaxel.[199] Twenty-one patient entered into this study with lung, colorectal, breast, ovary, and esophagus cancers. No hypersensitivity reaction was observed in any patient. Neuropathy and myalgia were the most common toxicities. There were 14% partial responses. The paclitaxel area under the curve and peak of the drug concentration in the blood were increased with the escalating dose, suggesting linear pharmacokinetics for Genexol-PM.[199]

## 7. Conclusions

Approximately two decades have passed since the conception of the polymer micelle conjugates and nanocontainers for drug delivery. During the first decade,

only a few studies were published; however, more recently, the number of publications in this field has increased tremendously. During this period, novel biocompatible and/or biodegradable block copolymer chemistries have been researched, the block ionomer complexes capable of incorporating DNA and other charged molecules have been discovered, the pH and other chemical signal sensitive micelles have been developed. Many studies focused on the use of polymer micelles for delivery of poorly soluble and toxic chemotherapeutic agents to the tumors to treat cancer. There has been considerable advancement in understanding the processes of polymer micelle delivery into the tumors, including passive and vectorized targeting of the polymer micelles. Notable achievements also include the studies demonstrating the possibilities for overcoming multidrug resistance in cancer, and enhancing drug delivery to the brain using block copolymer micelles systems. Overall, it is clear that this area has reached a mature stage, reinforced by the fact that several human clinical trials using polymer micelles for cancer drug delivery have been initiated. At the same time, it is obvious that the possibilities for delivery of the diagnostic and therapeutic agents using polymer micelles are extremely broad, and one should expect further increase in the laboratory and clinical research in this field during the next decade. Targeting polymer micelles to cancer sites within the body will address an urgent need to greatly improve the early diagnosis and treatment of cancer. Capabilities for the discovery and use of targeting molecules will support the development of multifunctional therapeutics that can carry and retain antineoplastic agents within tumors. This will also be instrumental in developing novel biosensing and imaging modalities for the early detection of cancer and other devastating human diseases.

## Acknowledgment

The authors acknowledge the support of the research using polymer micelles by grants from the National Institutes of Health CA89225, NS36229 and EB000551, as well as the National Science Foundation DMR0071682, DMR0513699 and BES-9907281. We also acknowledge financial support of Supratek Pharma, Inc. (Montreal, Canada). AVK and EVB are shareholders and AVK serves as a consultant to this Company.

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# 6

# Vesicles Prepared from Synthetic Amphiphiles — Polymeric Vesicles and Niosomes

*Ijeoma Florence Uchegbu and Andreas G. Schatzlein*

## 1. Introduction

This chapter will examine what is known about vesicles prepared from synthetic amphiphiles and will encompass a review of the data published on polymeric vesicles and non-ionic surfactant vesicles (niosomes). Schematic representations of the molecular arrangements in these systems are as depicted in Fig. 1. Examples of drug delivery applications will also be presented.

Vesicular systems arise when amphiphilic molecules self assemble in aqueous media in an effort to reduce the high energy interaction between the hydrophobic portion of the amphiphile and the aqueous disperse phase, and maximize the low energy interaction between the hydrophilic head group and the disperse phase (Fig. 1). These self assemblies reside in the nanometre to micrometre size domain. Excellent reviews exist on the self assembly of amphiphiles,[16] and hence this topic will not be dealt with in great detail here. Vesicles are important pharmaceutical systems, especially as liposomes, the result of phospholipid self assembly,[19] are licensed for the clinical delivery of anti cancer drugs.[21] It is thus possible that the vesicles described here may be incorporated into licensed medicines at some point in future.

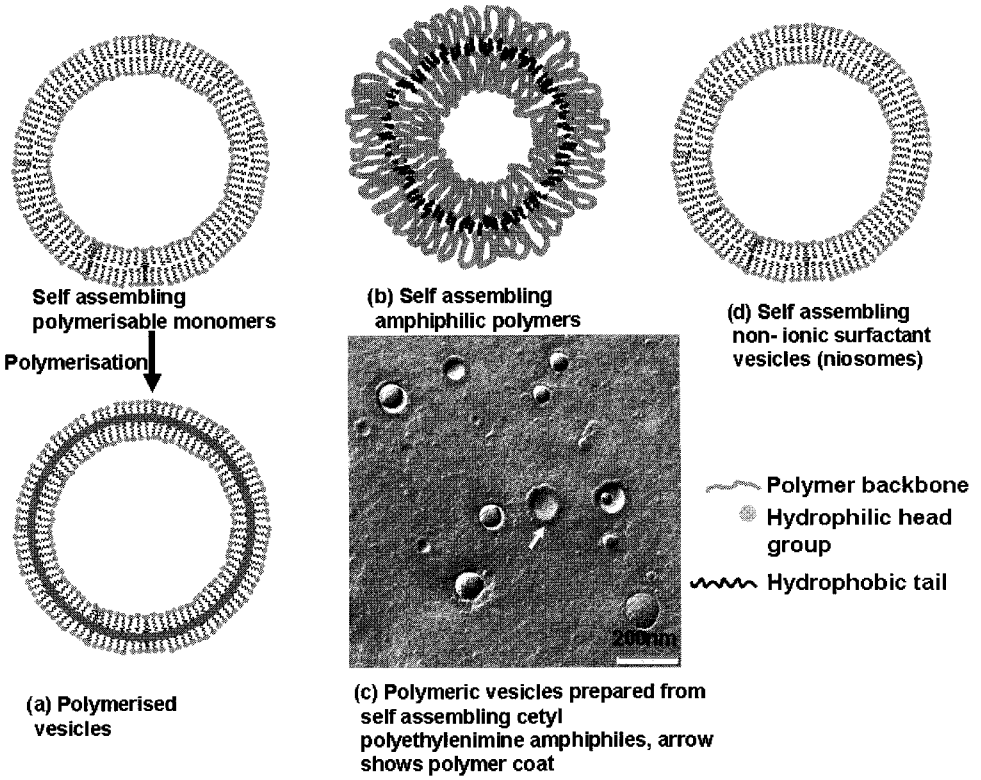


**Fig. 1.** Polymeric vesicles and niosomes. Polymeric vesicles arise from: (a) the self assembly of polymerizable monomers which are subsequently polymerized and (b) and (c) the self assembly of amphiphilic polymers bearing hydrophobic pendant groups. Additionally, both block and random copolymers are able to self assemble into polymeric, vesicles. (d) Niosomes arise from the self assembly of non-ionic surfactants.

## 2. Polymeric Vesicles

Polymeric vesicles were first investigated as a means of stabilizing the metastable self assemblies formed from low molecular weight amphiphiles, with the polymer providing a kinetic trap for the self assembled system.[12,22] A wealth of polymer architectures are now known to assemble into vesicles: namely block copolymers,[23,24] random graft copolymers,[20] polymerized self assembling monomers[22] and polymers bearing lipid pendant groups[10,22,25] (Fig. 2). Additionally, polymeric vesicles, although not normally termed as such, arise from the self assembly of amphiphilic polymers, i.e. poly(oxyethylene) amphiphiles with: (a) lipids[26] to give poly(oxyethylene) coated liposomes, or (b) non-ionic surfactants[4] to give poly(oxyethylene) coated niosomes. A summary of the liposome literature exists in other parts of this volume and hence liposome–polymer interactions or

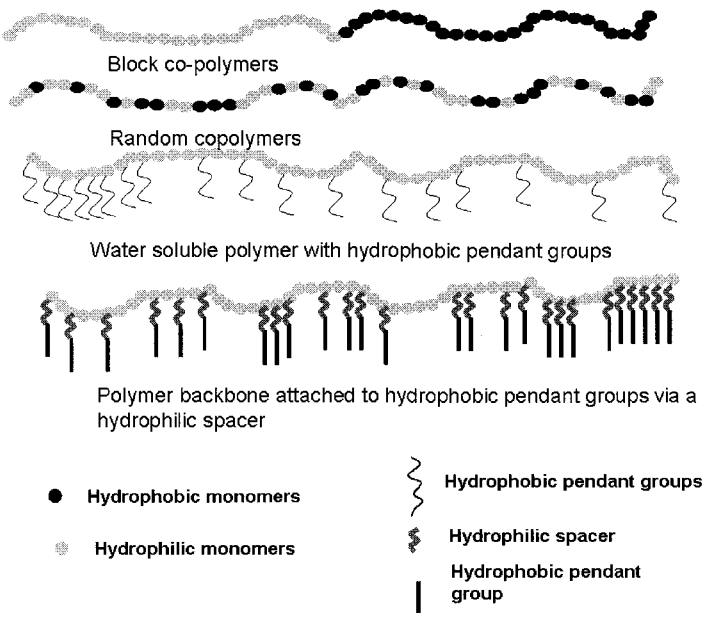


**Fig. 2.** Schematic representation of self assembling vesicle forming polymers.

liposomes incorporating minor amounts of polymers will not be treated in this chapter; while details on niosomes appear later on this chapter. The current section of this chapter will thus focus on the self assembly of block copolymers, random graft copolymers, polymers bearing lipid pendant groups and polymerized vesicles in which polymerization takes place after self assembly.

## 2.1. *Polymer self assembly*

As may be inferred from the list above, there are two main ways in which polymeric vesicles may be formed, either from the self assembly of polymers or from the polymerization of monomers subsequent to self assembly (Fig. 1). Vesicular self assembly is not a spontaneous process and normally requires an input of energy in the form of probe sonication for instance.[20] It is known that vesicle formation from low molecular weight amphiphiles in aqueous media is controlled by two opposing forces, namely the steric or ionic repulsion between hydrophilic head groups which maximizes the interfacial area per molecule and the attractive forces between hydrophobic groups which serve to reduce the interfacial area per molecule.[16] Attaining a minimal interfacial energy is thus served by the formation of a closed spherical bilayer. In general terms, the lesser hydrophobic amphiphiles form micelles, while amphiphiles of intermediate hydrophobicity form vesicles.[16]

The self assembly of polymers into vesicles is governed by similar constraints and as such, the hydrophobic-lipophilic balance of polymers determines whether a polymer will self assemble into vesicles in a similar manner as is found in liposomes and niosomes.[18,24]

There are also some polymer specific factors which impact on vesicle forming ability. For example, the degree of polymerization is critical to vesicle forming ability, and generally very high degrees of polymerization prevent vesicle formation.[13,20] Furthermore, the flexibility of the hydrophobic block in block copolymers determines which self assemblies will be formed; the more flexible hydrophobic portions of the polymer are able to form vesicles, whereas the more rigid polymers are unable to self assemble into three dimensional structures.[24] Polymeric vesicles appear to be largely unilamellar[10,27] and unilamellarity is favored when the molecular weight of the amphiphile increases.[18]

Polymeric vesicles often possess superior mechanical stability,[24] are usually less susceptible to degradation by organic solvents and soluble surfactants,[24] and are frequently less permeable to hydrophilic solutes, compared with vesicles prepared from low molecular weight amphiphiles.[22,28] The widespread exploitation of these fascinating nanocarriers for the development of responsive and biomimetic nanomedicines with superior stability characteristics is what awaits the science.

## 2.2. *Polymers bearing hydrophobic pendant groups*

The first report on the use of preformed polymers to prepare polymeric bilayer vesicles was presented in 1981 by Kunitake and others.[3] In this report, bilayer vesicles were prepared from Compound 1 shown in Fig. 3(a). Compound 1 comprises a hydrophilic polyacrylamide backbone and dialkyl hydrophobic pendant groups separated from the polymer backbone by hydrophilic oligooxyethylene spacers, as shown schematically in Fig. 2. The hydrophilic spacer group between the dialkyl moieties is essential for vesicle formation for these polyacrylamide type polymers.[22] The hydrophilic spacer allows the decoupling of the polymer motion from the ordering of the bilayer.[28]

The introduction of essentially water soluble carbohydrate [e.g. Compound 2[10,11] Fig. 3(a)], polyelectrolyte [e.g. Compounds 3–5,[18] Fig. 3(a)] and polyamino acid [e.g. Compound 6,[20] Fig. 3(a)] polymer backbones bearing hydrophobic pendant groups is a fairly recent development. The bilayer arrangement is as depicted in Fig. 3(b) and the thick polymer coat is clearly visible on micrographs (Fig. 1). For drug delivery applications, it is important to appreciate that the conversion of the poly(L-lysine) into vesicles reduces its cytotoxicity,[29] thus allowing this molecule to be exploited as a pharmaceutical excipient.

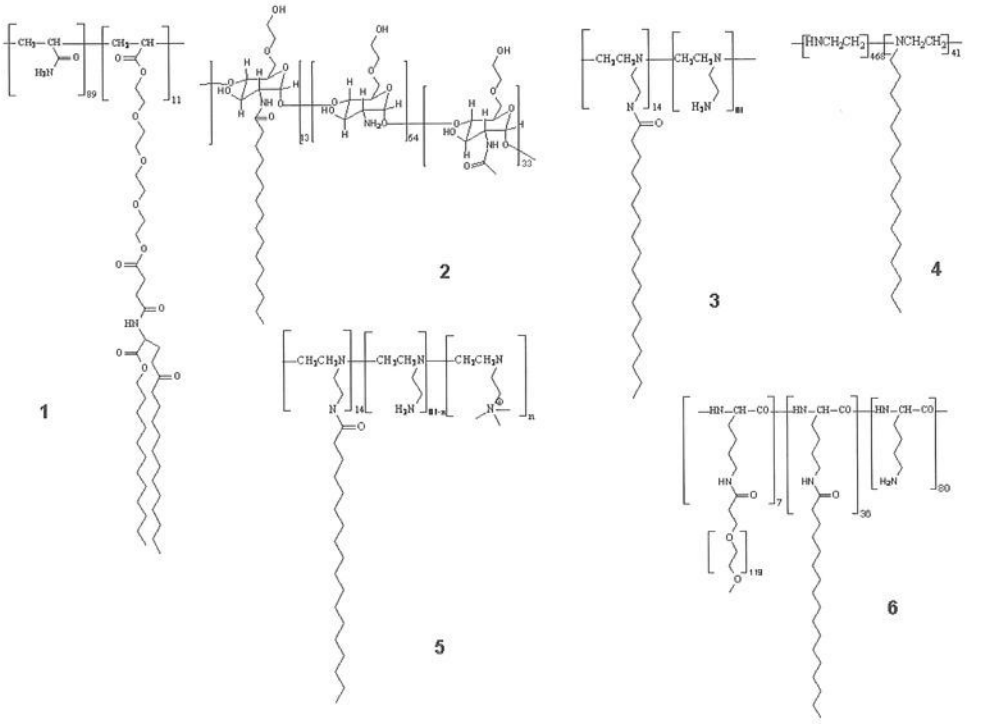


**Fig. 3(a).** Representative vesicle forming polymers from the polyacrylamide (Compound 1),[3] chitosan (Compound 2),[10,11] polyamine (Compounds 3–5),[17,18] and polyamino acid (Compound 6)[20] classes of polymers bearing hydrophobic pendant groups.

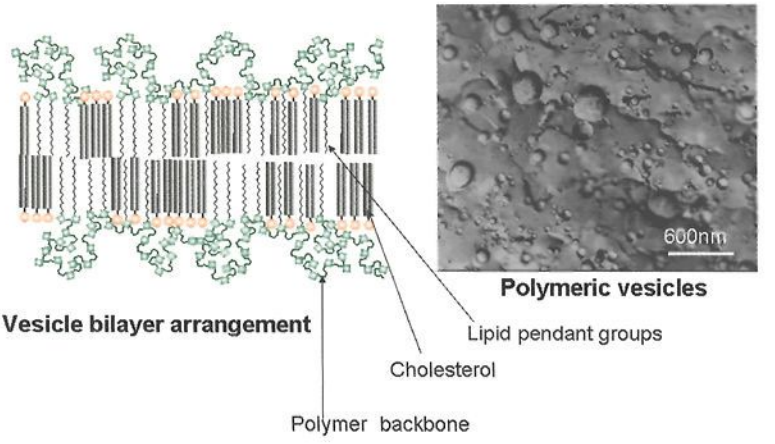


**Fig. 3(b).** The glycol chitosan polymer: i.e. Compound 2, is arranged in the bilayer as shown.

Poly(ethylenimine) [Compound 4, Fig. 3(a)[18]] and poly(L-lysine) [Compound 6, Fig. 3(a)[20,30]] based amphiphiles have been studied in some detail and the formation of vesicles from these amphiphiles is dependent on the level of lipid pendant groups. Hydrophobically modified poly(ethylenimine) for example forms dense nanoparticles, bilayer vesicles or micellar self assemblies, depending on its hydrophobic content.[18] Hence, poly(ethylenimine) amphiphiles with a hydrophobic content of 58% and above favor dense nanoparticle self assemblies, while a hydrophobic content of 43–58% favors bilayer vesicle assemblies, and finally a hydrophobic content of less than 43% favors the formation of micellar assemblies.[18]

A remarkably similar trend has been reported for the poly(oxyethylene)-*block*-poly(lactic acid) system in that a poly(lactic acid) fraction of $\geq$80% favors dense nanoparticles, while a poly(lactic acid) fraction of 58–80% favors bilayer vesicle assemblies, and a poly(lactic acid) fraction of less than 50% favors the production of micellar self assemblies.[31]

The sizes of the vesicle and dense nanoparticle assemblies formed from amphiphilic poly(ethylenimines) are also dependent on polymer levels of hydrophobic modification (mole % cetylation) and the relationships shown in Eqs. (1) and (2) have been developed,[18]

$$d_v = 1.95Ct + 139 \tag{1}$$

$$d_n = 2.31Ct + 5.6 \tag{2}$$

where $d_v$ = vesicle z-average mean hydrodynamic diameter, Ct = mole% cetylation (number of cetyl groups per 100 monomer units), and $d_n$ = nanoparticle z-average mean hydrodynamic diameter.

The molecular weight of the polymer is also an important factor to consider when choosing vesicle forming polymers. The importance of this parameter has been demonstrated with the poly(L-lysine) vesicle system[20] [e.g. Compound 6, Fig. 3(a)]. With these amphiphiles a vesicle formation index (F′) has been computed:

$$F' = \frac{H}{L\sqrt{DP}} \tag{3}$$

where H = mole% unreacted L-lysine units, L = mole% L-lysine units substituted with palmitic acid and DP = the degree of polymerisation of the polymer. An F′ value in excess of 0.168 is necessary for vesicle formation.[20]

Additionally, not only does the molecular weight of the polymer impact on vesicle formation, but it is also a direct controller of the vesicle mean size; the relationship shown in Eq. (4) has been developed for the palmitoyl glycol chitosan system,[11]

$$\sqrt{MW} = 0.782d_v + 107 \tag{4}$$

where MW = polymer molecular weight, and $d_v$ = vesicle z-average mean hydrodynamic diameter.

## 2.3. *Block copolymers*

Block copolymer vesicles, termed "polymersomes" are fairly new discoveries, being first reported in the 1990s.[32] Polymersomes have been prepared from a variety of block copolymers, some examples of which are given in Fig. 4. There is a clear relationship between the hydrophobic content of polymers and self assembly. Low levels of hydrophobicity (less than 50% of the polymer consisting of hydrophobic

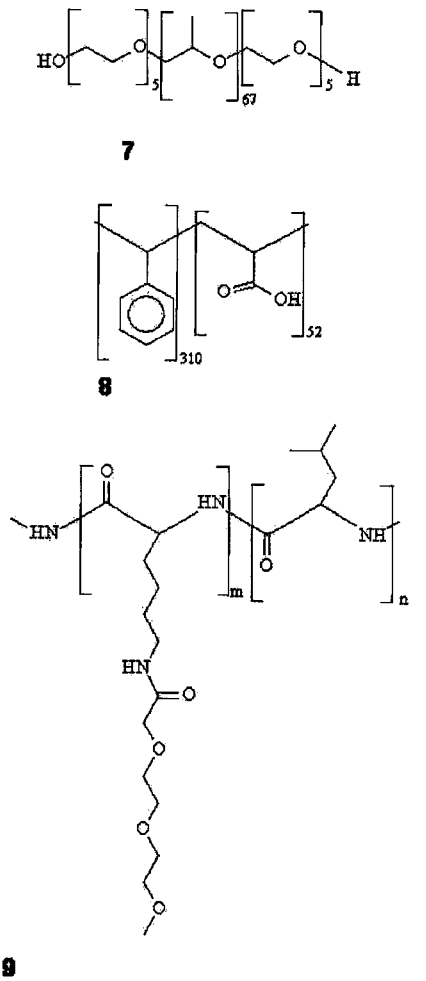


**Fig. 4.** Examples of some vesicle forming block copolymers Compound 7,[1] Compound 8,[7] and Compound 9.[13]

moieties) favors the formation of micelles[33] and intermediate levels of hydrophobicity (50–80%) favors the formation of bilayer vesicles.[31,33,34] For the self assembly of block copolymers, it has been established that generally the critical packing parameter (CPP):

$$\mathbf{CPP} = \frac{\mathbf{v}}{\mathbf{al}} \tag{5}$$

should approach unity for vesicular self assemblies to prevail,[24] where v = volume of the hydrophobic block, l = length of the hydrophobic block and a = the area of the hydrophilic block.

Vesicle sizes are varied and range from tens of nanometres[35] to tens of microns.[36] Polymersome membranes are 8–21 nm thick; 2–5 times thicker than the 4 nm membrane thickness displayed by conventional low molecular weight amphiphiles.[16,27,31,34,35] The thickness of the membrane is determined by the degree of polymerization in the hydrophobic block[34] and these extra thick membranes confer, on the vesicle, exceptional stability to soluble surfactants[24] and mechanical stress.[24,27,37,38] With these vesicles, there is an asymmetric distribution of the polymers in the inner and outer leaflets of the bilayer and polymers with a large hydrophilic chain length are preferentially localized to the exterior leaflet and vice versa.[39] Preferred residence in the outer leaflet is favored by the more hydrophilic polymers, because the greater repulsion between the longer hydrophilic corona molecules on the outer leaflet stabilize the vesicle curvature.[39]

Vesicle stability is a desirable characteristic for pharmaceutical vesicles and as such, a great deal of effort has been expended on producing stable systems. As the drive for nanomedicines (medicines incorporating functional nanoparticles) grows, stability issues will need to be adequately addressed to ensure the widespread adoption of such systems. In actual fact, the early workers in the polymeric vesicle field were primarily driven by this need to produce stable drug carriers. Extremely stable systems are possible on polymerization of block copolymers subsequent to self assembly. Poly(ethylene oxide)-*block*-poly[3-(trimethoxysilyl)propyl methacrylate] copolymer vesicles in water, methanol, triethylamine mixtures produced polymerized polymersomes that are stable for up to one year.[40] Triethylamine hydrolyzes the trimethoxysilyl groups and then catalyzes their polycondensation to yield an extremely stable hydrophobic polysilsesquioxane core.[40,41] Additionally, poly(ethylene oxide)-*block*-poly(butadiene) vesicles on cross linking produce vesicles which are organic solvent resistant.[42]

## 2.4. *Preparing vesicles from self-assembling polymers*

Polymeric vesicles are relatively simple to prepare. The input of energy is achieved in the laboratory by probe sonication of the amphiphilic polymer in the disperse

phase.[11,20] However, clearly the energy required for self assembly is not trivial as vesicles are not easily formed by hand shaking, unlike low molecular weight surfactant formulations.[4] Vesicles once formed are morphologically stable for months[11] and may be loaded with hydrophilic[43–45] and hydrophobic [see Fig. 6(b) below] solutes, by probe sonicating in the presence of such solutes. Commercially, it is envisaged that polymeric vesicles may be fabricated by microfluidization and high pressure homogenization techniques.

## 2.5. *Self assembling polymerizable monomers*

Polymerized vesicles may also be prepared by utilizing self assembling polymerizable amphiphiles, followed by the polymerization of the resulting vesicular self assembly (Fig. 1). Examples of some polymerizable vesicle forming monomers are shown in Fig. 5. This method of producing polymerised vesicles is the oldest form of polymeric vesicle technology.[12,46]

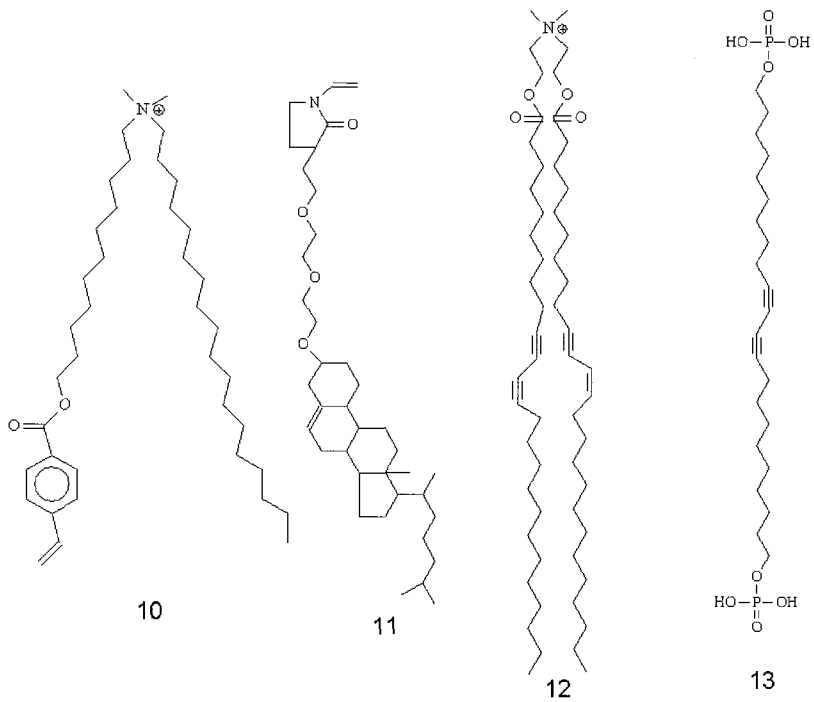


**Fig. 5.** Polymerizable vesicle forming monomers used to make polymerized vesicles by Jung and others (Compound 10),[5] Cho and others (Compound 11),[8] Hub and others (Compound 12)[12] and Bader and others (Compound 13).[15]

Polymerized vesicles prepared using polymerized self assembling monomers are essentially polymer shells and it is unclear how much of the bilayer assembly actually survives the polymerization step. The advantage, however, is that they are extremely stable, resisting degradation by detergents[47–49] or organic solvents.[8,48,50,51] They are also less leaky,[50] thermostable,[52] and because the vesicle forming components are kinetically trapped by the polymerization process, they have improved colloidal stability.[8] A major advantage of these nanosystems is that they may be isolated as dry powders which are readily dispersible in water to give 50–100 nm particles;[48] thus potentially allowing the formulation of solid vesicle dosage forms. Polymerization involves fairly reactive species and hence vesicles are best prepared prior to drug loading, which may be a limitation.

## 3. Polymeric Vesicle Drug Delivery Applications

Polymeric vesicles, which are the focus of this chapter, exist in two main varieties as illustrated in Fig. 1. These technologies are suitable candidates for the development of robust, controllable and responsive nanomedicine drug carriers.

### 3.1. *Drug targeting*

Poly(oxyethylene) amphiphiles, when incorporated into liposomal[26] and niosomal[6] bilayers, prolong vesicle circulation and facilitate tumor targeting,[6,53] due to the leaky nature of the poorly developed tumor vascular endothelium.[54] Only 10 mole % poly(ethylene oxide) — lipid amphiphiles may be incorporated into liposomes[55] or niosomes,[56,57] without a loss of vesicle integrity due to the preferred tendency of the hydrophilic poly(oxyethylene) amphiphiles to form micelles. Polymersomes composed of poly(ethylene oxide)-*block*-polybutadiene or poly(ethylene oxide)-*block*-poly(ethylethylene), in which the entire vesicle surface is covered with the poly(ethylene oxide) coat, have been studied as long circulating nanocarriers for drug delivery.[58] The circulation time of poly(ethylene oxide) polymersomes is directly dependent on the length of the poly(ethylene oxide) block and polymersome half lives of up to 28 hrs have been recorded in rats with a poly(ethylene oxide) degree of polymerization of 50.[58] This half life compares favorably with a half life of 14 hrs recorded for poly(oxyethylene) coated liposomes.[59] It is assumed that the 100% surface coverage of the polymeric vesicles is responsible for the reduced clearance of these polymersomes from the blood.[38] The long half life of these polymersomes makes them excellent candidates for the development of anti tumor medicines.

Furthermore, drug release may be controlled in the polymersomes by controlling the hydrolysis rate of the hydrophobic blocks.[31] This has been demonstrated

with poly(L-lactic acid)–*block*-poly(ethylene glycol) and poly(caprolactone)-*block*-poly(ethylene glycol) vesicles.[31] Hydrolysis of the hydrophobic block causes the polymer to move from a vesicular to a micellar assembly, as the overall level of hydrophobic content diminishes, and this in turn leads to drug release.[31] Hydrolysis rates and implicitly release rates may be controlled by varying the relative level of the hydrophobic blocks.

Carbohydrate polymeric vesicles may also be used as drug targeting agents. Vesicles prepared from glycol chitosan vesicles improve the intracellular delivery of hydrophilic macromolecules[44] and anti cancer drugs,[45] the latter is achieved with the help of a transferrin ligand attached to the surface of the vesicle.

## 3.2. *Gene delivery*

Poly(L-lysine) based vesicles, prepared from Compound 6 [Fig. 3(a)] have been used for gene delivery,[29,60] as these vesicles are less toxic than unmodified poly(L-lysine) and produce higher levels of gene transfer (Table 1).[29] The production of polymeric vesicles and the resultant reduction in cytotoxicity enables poly(L-lysine) to be used in *in vivo* gene, as the unmodified polymer is too toxic for *in vivo* use. When the targeting ligand, galactose, was bound to the distal ends of the poly(oxyethylene) chains, gene expression was increased in HepG2 cells *in vitro*.[60] However, *in vivo* targeting to the liver hepatocytes was not achieved with these systems.[60]

A similar procedure with the poly(ethylenimine) vesicles prepared using Compound 5 [Fig. 3(a)] also resulted in a reduction in the cytotoxicity of the polymer (Table 1),[17] although in this case, the poly(ethylenimine) vesicles were not as efficient gene transfer agents as the free polymer.

**Table 1** Biological Activity of poly(ethylenimine)[17] and poly(L-lysine)[29] Vesicles.

| Polymer | A431 cells | | A549 | |
|---|---|---|---|---|
| | IC50 ($\mu g\ mL^{-1}$) | Gene Transfer Relative to Parent Polymer | IC50 ($\mu g\ mL^{-1}$) | Gene Transfer Relative to Parent Polymer |
| Poly(ethylenimine) | 1.9 | 1 | 5.2 | 1 |
| Polymer 5 (Fig. 6(a)) | 16.9 | 0.2 | 12.6 | 0.08 |
| Polymer 5, cholesterol vesicles 2: 1 ($g\ g^{-1}$) | 15.9 | 0.2 | 11 | 0.08 |
| Poly(L-lysine) | 7 | 1 | 7 | 1 |
| Polymer 6 (Fig. 6(a)) | 74 | 7.8 | 63 | 2.3 |

## 3.3. *Responsive release*

The ultimate goal of all drug delivery efforts is the simple fabrication of responsive systems that are capable of delivering precise quantities of their pay load in response to physiological or more commonly pathological stimuli. Pre-programmable pills, implants and injectables are so far merely the unobtainable ideal, however, polymeric systems have been fabricated with responsive capability and it is possible that in the future, these may be fine tuned to produce truly intelligent and dynamic drug delivery devices or systems.

The various environmental stimuli that may be used to trigger the release of encapsulated drug are outlined below and examples are given of existing developments in the area. However, in addition to the areas covered below, it may be possible in future for pathology specific molecules to interact with polymeric vesicles to trigger release.

### 3.3.1. *pH*

Diblock polypeptides, in which the hydrophilic block consists of ethylene glycol derivatised amino acids (L-lysine), and the hydrophobic block consists of poly (L-leucine), form pH responsive vesicles which disaggregate at low pH, providing the level of L-leucine and polymer chain length is maintained within defined limits of about 12–25 mole% and the polymer has a degree of polymerization of less than 200.[13] These L-lysine based systems may be applied to facilitate endosome specific release.

### 3.3.2. *Enzymatic*

Vesicles which release their contents in the presence of an enzyme may be formed by loading polymeric vesicles with an enzyme activated prodrug (Fig. 6). The particulate nature of the drug delivery system should allow the drug to accumulate in tumors, for example, where it may then be activated by an externally applied enzyme in a similar manner to the antibody directed enzyme prodrug therapeutic strategy. The antibody directed enzyme prodrug therapeutic strategy enables an enzyme to be homed to tumors using antibodies followed by the application of an enzyme activated prodrug.[61] Alternatively, a membrane bound enzyme may be used to control and ultimately prolong the activity of either an entrapped hydrophilic drug (entrapped in the vesicle aqueous core) or an entrapped hydrophobic drug (entrapped in the vesicle membrane) as illustrated in Fig. 6. It is possible that the enzyme may be chosen such that it is activated in the presence of pathology specific molecules, thus achieving pathology responsive and localized drug activity.

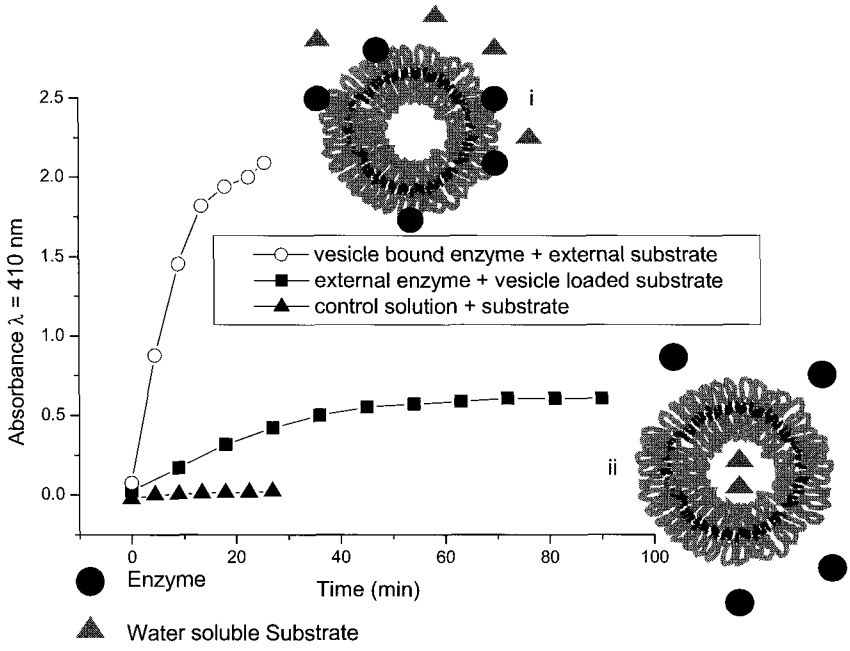


**Fig. 6(a).** Enzyme activated polymeric vesicles. Vesicles bearing membrane bound enzyme (i) were formed by probe sonicating Compound 2 [Fig. 3(a)], cholesterol, N-biotinylated dipalmitoyl phosphatidyl ethanolamine (8: 4: 1 g $g^{-1}$) in neutral phosphate buffer (2 mL), isolation of the vesicles by ultracentrifugation (150,000 g), redispersion in a similar volume of neutral phosphate buffer and incubation of the vesicles with β-galactosidase streptavidin (3 U). Membrane bound enzyme (0.2 mL) was then incubated with o-nitrophenyl-β-D-galactoside (2.1 mM, 2 mL) and the absorbance monitored (λ = 410 nm). The control solution contained similar levels of substrate (o-nitrophenyl-β-D-galactoside) but no enzyme. Vesicles encapsulating 0-nitrophenyl-β-D-galactoside (ii) were prepared by probe sonicating Compound 2, cholesterol (8: 4 g $g^{-1}$) in the presence of o-nitrophenyl-β-D-galactoside solution (34 mM, 2 mL) and isolation of the vesicles by ultracentrifugation and redispersion in neutral phosphate buffer. These latter vesicles (0.4 mL) were then incubated with β-D-galactosidase (2 U $mL^{-1}$, 0.1 mL) and the absorbance once again monitored.

### 3.3.3. *Magnetic*

Magnetically responsive polymerized liposomes composed of 1,2-di (2,4-octadecadienoyl)-sn-glycerol-3-phosphorylcholine, loaded with ferric oxide and subsequently polymerized may be localized by an external magnetic field to the small intestine, and specifically the Payer's patches.[47] These polymerized vesicles are stable to the degradative influence of solubilizing surfactants such as triton-X 100,[47] and hence should not suffer excessive bile salt mediated degradation during gut transit. These magnetically responsive polymeric vesicles improve the absorption of drugs via the oral route.[47]

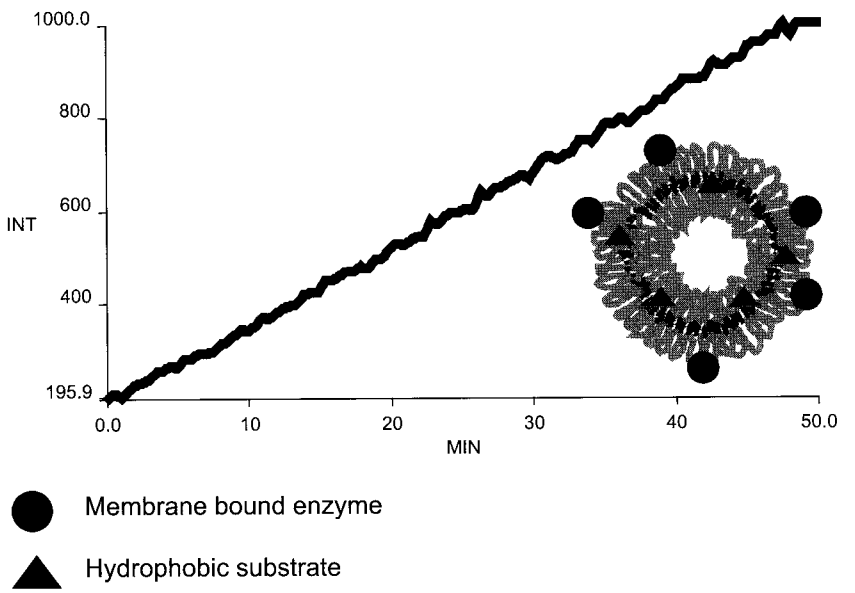


**Fig. 6(b).** Enzyme activated polymeric vesicles. Vesicles bearing membrane bound enzyme and containing the hydrophobic substrate fluorescein di-$\beta$-D-galactospyranoside were formed by probe sonicating Compound 2 [Fig. 3(a)], cholesterol, N-biotinylated dipalmitoyl phosphatidyl ethanolamine, fluorescein di-$\beta$-D-galactospyranoside (8: 4: 1: 0.0005 g $g^{-1}$) in neutral phosphate buffer (2 mL) and incubation of the resulting vesicles with b-galactosidase streptavidin (0.3 U). The fluorescence of the enzyme hydrolysed substrate was then monitored (Excitation wavelength = 490 nm, Emission wavelength = 514 nm).

### 3.3.4. *Oxygen*

Block copolymer vesicles which are destabilized by oxidative mechanisms have been constructed from poly(oxyethylene)-*block*-poly(propylene sulphide)-*block*-poly(oxyethylene) ABA block copolymers.[62] These polymeric vesicles are destabilized on the oxidation of the central sulphide block to give sulphoxides and ultimately sulphones.[62] On oxidation, vesicles are transformed to worm-like micelles and finally to spherical micelles, eventually releasing their contents.

## 4. Non-ionic Surfactant Vesicles (Niosomes)

### 4.1. *Self assembly*

The self assembly of non-ionic surfactants into niosomes is dependent on the hydrophilic — hydrophobic balance of the surfactant and a CPP (Eq. 1) of between 0.5–$10^{16}$ enables niosomal self assembly. Some examples of niosome forming molecules are given in Fig. 7. Further molecular specifics that govern niosome

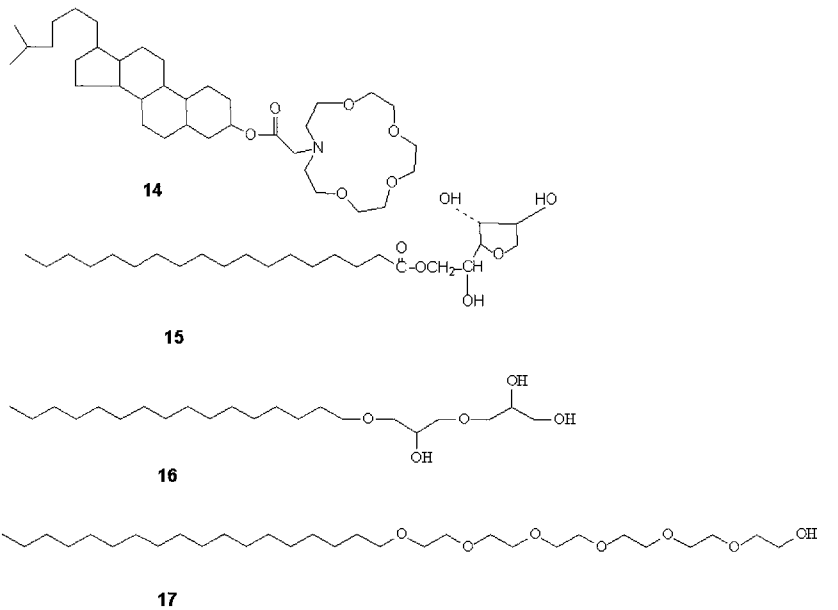


**Fig. 7(a).** Examples of some niosome forming surfactants: Compound 14,[2] Compound 15,[6] Compound 16,[9] and Compound 17.[14]

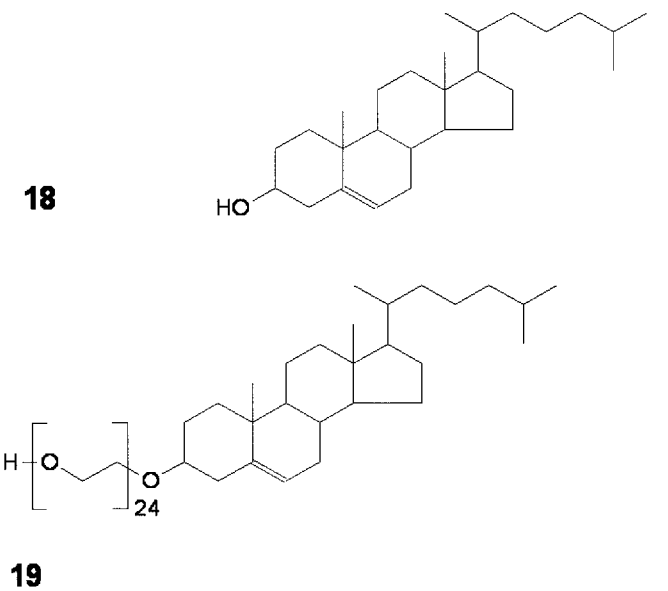


**Fig. 7(b).** Niosomal membrane additives, Compound 18 = cholesterol, Compound 19 = Solulan C24.[4]

formation by non-ionic surfactants may be found in published reviews.[4,63] Compounds such as Compounds 15 (from the sorbitan surfactant class) are established pharmaceutical excipients,[64] and hence formulation scientists looking to prepare a niosome formulation for speedy transition to the clinic will do well looking at this class of molecules for exploitable materials. Most niosomes will not only contain the non-ionic surfactant, but will also contain other molecules such as the membrane stabilizer cholesterol [Fig. 7(b)].[4]

The bilayer membrane is an ordered structure which may exist in the gel or liquid crystal state. Essentially, molecules are more mobile in the liquid crystalline state, enjoying lateral diffusion within the bilayer that is denied them in the gel state. For any system, the liquid crystal state exists at a higher temperature (T) than the gel state. An increase in temperature favors the transition from the gel to the liquid state because of the entropy gain ($\Delta S$) associated with this transition, ultimately leading to a lowering of the free energy ($\Delta G$) of the system. Cholesterol abolishes this membrane phase transition, thus fluidizing the gel state.[65]

Niosomes are 30 nm to 120 $\mu$m in size[4] and often their surfaces must be stabilized against aggregation. Molecules such as the cholesteryl poly(oxyethylene ether) — Solulan C24[6] (Compound 19, Fig. 7b) or the ionic molecule dicetyl phosphate[66] have been used to confer steric and electrostatic stabilization on these vesicles respectively. The reader should be aware that the inclusion of minor quantities (<10% by actual weight or molar content) of ionic surfactant does not prevent these structures from being discussed in this chapter under the niosome heading. Niosomes are often formulated with minor quantities of cationic and other surfactants.[4]

It can be said that the formulation of liposomes with poly(ethylene oxide) amphiphiles such as distearolyphosphatidylethanolamine–poly(ethylene glycol)[26] was the crucial step that allowed liposomes to become clinically relevant drug delivery systems. The resulting liposomes possess a hydrophilic polymer surface, which prevents recognition and clearance of the particles from the blood by the liver and spleen macrophages,[26,67] thus increasing the liposomes' circulation time and allowing tumor targeting.[68] Niosomes (non-ionic surfactant vesicles), when formulated with a water soluble poly(oxyethylene) cholesteryl ether — (Solulan C24), also circulate for prolonged periods in the blood, accumulate in the tumor tissue and improve tumoricidal activity.[6] As well as stabilizing vesicles in the blood, poly(oxyethylene) amphiphiles also stabilize vesicles against aggregation, thus promoting vesicle colloidal stability.[56]

Poly(oxyethylene) amphiphiles, such as Solulan C24, have a large hydrophilic head group [Fig. 7(b)], and are thus more hydrophilic than the vesicle forming amphiphiles, and hence the level of the former must be kept low to avoid solubilization of the membrane and the formation of mixed micelles.[57] In actual fact,

unusual morphologies[57] result from the incorporation of non-micellizing quantities of Solulan C24 in vesicles as discussed below.

## 4.2. *Polyhedral vesicles and giant vesicles (Discomes)*

A series of unusual morphologies have been isolated from the hexadecyl diglycerol ether, Solulan C24, cholesterol phase diagram [Fig. 8(a)]. The addition of Solulan C24 to hexadecyl diglycerol ether [Compound 16, Fig. 7(a)] niosomes eventually results in the formation of mixed micelles.[57] At sub-micellar concentrations of Solulan C24 (20–40 mole%), however, giant vesicles (discomes) of 25–100 $\mu$m in size are formed.[57] Discomes are thermoresponsive vesicles, which become more leaky as the temperature is increased from room temperature to 37°C.[63] These vesicles may thus be used to construct thermoresponsive controlled release systems.

In cholesterol low regions of the hexadecyl diglycerol ether, cholesterol, Solulan C24 phase diagram, polyhedral vesicles [Figs. 8(a) and 8(b)] are found.[9] These polyhedral vesicles are able to entrap water soluble solutes and the membrane, which is in the gel state contains areas of high and low curvature as shown in

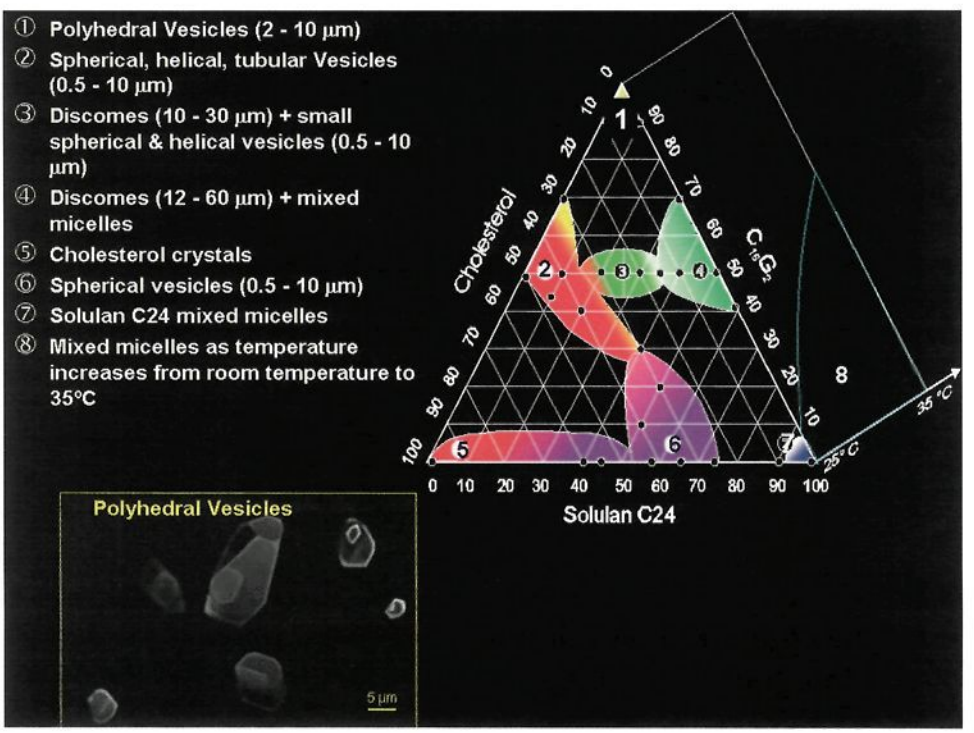


**Fig. 8(a).** Polyhedral vesicles are found in cholesterol poor regions of the hexadecyl diglycerol ether, cholesterol, Solulan C24 phase diagram.

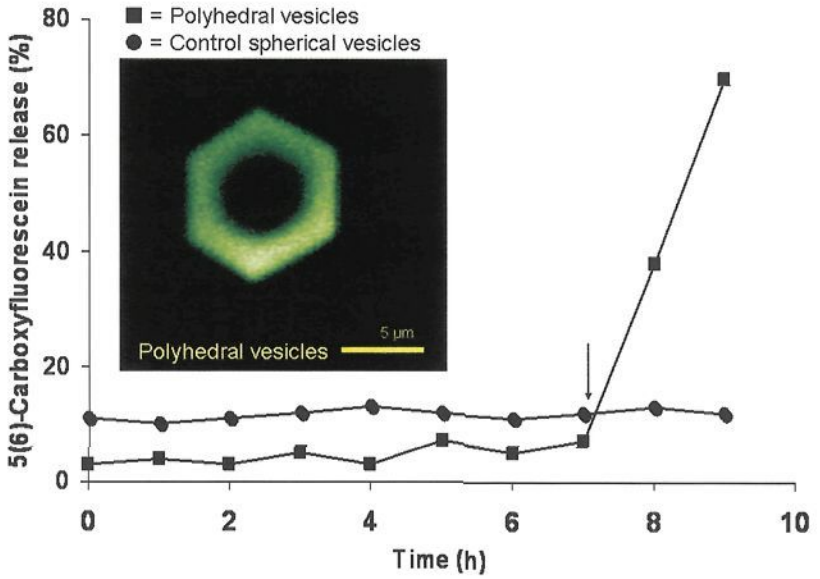


**Fig. 8(b).** The release of 5(6)-carboxyfluorescein from thermoresponsive polyhedral vesicles: arrow indicates an increase in the temperature from 25°C to 37°C.

Fig. 8. The high curvature areas are believed to be rich in the hydrophilic Solulan C24 molecules, while the low curvature areas are rich in the less hydrophilic hexadecyl diglycerol ether. This separation of molecules is believed to be stabilized by favorable bonding interactions between like molecules. The gel to liquid phase transition occurs at 43°C–45°C and as these polyhedral vesicles are heated to this temperature, they adopt a spherical morphology, and on cooling, do not completely revert to the polyhedral morphology.[63,69] The spherical morphology presumably results from the increased mobility of molecules in the bilayer. Conversion to spheres reduces the viscosity of the niosomes.[69]

Polyhedral vesicles are thermoresponsive [Fig. 8(b)] and become leakier when heated to a temperature just below their phase transition temperature (37°C).[9] The polyhedral morphology is retained at 37°C and polyhedral vesicles devoid of Solulan C24 are not thermoresponsive. The thermoresponsive behaviour arises from a diminished hydrogen bonding between the poly(oxyethylene) chain and the solvent on heating as measured by viscometric means.[69] This diminished hydration of the hydrophilic amphiphile — Solulan C24 as the temperature rises,[69] leads to defects in the membrane and an increase in membrane permeability. Hydration values for Solulan C24 micelles are $3.8\,g\,g^{-1}$ and $2.3\,g\,g^{-1}$ at 25°C and 35°C respectively.[69]

Recently, polyhedral vesicle morphologies have been identified when other surfactants are formulated in the absence of cholesterol and the presence of

Solulan C24, including examples such as poly(oxyethylene)-5-cetyl ether and poly(oxyethylene)-5-stearyl ethers.[70] These poly(oxyethylene) polyhedral vesicles fuse to form multilamellar tubes of ~80 $\mu$m in length, when extruded under pressure, thus offering the possibility of making entirely new biomimetic materials.[70]

### 4.3. *Vesicle preparation*

Vesicle formation requires an input of energy either in the form of gentle agitation or probe sonication.[63] Usually, the surfactant/lipid mixtures are hydrated in the presence of the drug in aqueous solution and the unentrapped drug separated by either ultracentrifugation, low pressure gel filtration chromatography or exhaustive dialysis.[63,71] Since vesicle size is an important determinant of drug biodistribution, vesicle size may be subsequently reduced by extruding via nucleopore filters, microfluidization or high pressure homogenization.[63] Due to the non-abundance of reactive groups within synthetic surfactants, these molecules are stable on storage and vesicles prepared from surfactants have been observed to remain morphologically stable for up to seven years![63]

## 5. Niosome Delivery Applications

A variety of non-ionic surfactant vesicles have been employed as drug, vaccine and imaging agent delivery systems (Table 2). The most popular surfactants used are the sorbitan amphiphiles (Span 20, Span 40, Span 60 and Span 80) which incidentally are approved excipients.[64] Details follow on how researchers have sought to exploit the ability of niosomes to control the distribution of drug within the body for drug delivery, vaccine delivery and diagnostic imaging.

### 5.1. *Drug targeting*

#### 5.1.1. *Anti cancer drugs*

Anti cancer drugs such as the model drug doxorubicin, when encapsulated in sorbitan monostearate poly(oxyethylene) coated (coated with Solulan C24) niosomes, circulate for prolonged periods.[6] The area under the plasma level time curve is increased six-fold by the niosomes when compared with the drug in solution, tumor levels are increased by 50% and tumoricidal activity is doubled.[6] These particles circulate for prolonged periods due to the poly(oxyethylene) coating, which prevents particle recognition[59] and the uptake by the liver and spleen.[53] However, while a poly(oxyethylene) coat may improve the delivery of drugs to tumors by achieving long blood circulation times, niosomes devoid of poly(oxyethylene) coatings are also able to improve the tumoricidal activity of drugs such as doxorubicin,[72]

**Table 2** Examples of niosomal delivery applications.

| Therapeutic/ Prophylactic/ Diagnostic Area | Formulation and Route of Administration | Advantage of Niosomal Formulation | Reference |
|---|---|---|---|
| Cancer | Intravenous sorbitan monostearate doxorubicin niosomes | 50% increase in tumor drug levels and twice as active against mouse MAC 15A murine tumors as the drug in solution | 6 |
| | Intravenous hexadecyl triglycerol ether doxorubicin niosomes | Increase in tumor drug levels and twice as active against S180 murine tumors as the drug in solution | 72 |
| | Intravenous sorbitan monostearate methotrexate niosomes | A 23 fold increase in plasma levels when compared to the drug in solution and improvements in tumouricidal activity against murine S180 tumors | 73, 98 |
| Anti-infectives | Intravenous hexadecyl triglycerol ether niosomes | Liver peak levels of antimony are twice those of the drug in solution and niosomal formulations are 10 times as active in reducing the parasite burden as the drug in solution | 76 |
| Glaucoma | Topically applied timolol maleate sorbitan monostearate niosomes coated with chitosan | A sustained lowering of intraocular pressure with formulations being twice as active as a commercial gel formulation | 88 |
| Neurological disorders | Intravenous administration of N-palmitoyl glucosamine vasoactive intestinal peptide (VIP) niosomes | Delivery of VIP to the brain when encapsulated in niosomes but not when administered as the drug in solution | 80 |
| Vaccination | Intramuscular palmitoyl glycerol herpes simplex virus type 1 niosomes | Higher levels of neutralising antibodies than the antigen in phosphate buffered saline and a higher survival rate after challenge with the virus (25% of animals still surviving after 10 days compared to a 0% of animals dosed with antigen in phosphate buffered saline surviving after 10 days) | 95 |

**Table 2** (*Continued*)

| Therapeutic/ Prophylactic/ Diagnostic Area | Formulation and Route of Administration | Advantage of Niosomal Formulation | Reference |
|---|---|---|---|
| Delivery of Diagnostic imaging agents | Intravenous N-palmitoyl glucosamine gadolinium niosomes and N-palmitoyl glucosamine gadolinium niosomes coated with poly(oxyethylene) | Higher tumor (PC3 tumor cells) to muscle ratio of contrast agent 24 h after dosing with glucose or poly(oxyethylene) bearing niosomes when compared to sorbitan monostearate niosomes | 97 |

methotrexate[73] and vincristine,[74] principally by altering drug biodistribution following intravenous administration such that the drug is targeted to some extent to the tumor tissue. The disparate nature of the tumor vasculature[75] is responsible for trapping particulate matter within tumors.

### 5.1.2. *Anti infectives*

The targeting of anti-leishmanial drugs to the liver, the site of pathology, is achievable with niosomal formulations.[76–78] Hexadecyl triglycerol sodium stibogluconate niosomes are rapidly taken up by the liver producing peak levels of antimony that are twice that achieved with the drug in solution.[76] The niosomes are thought to be taken up by macrophages in the liver. The anti-parasitic activity of sodium stibogluconate is increased 10-fold by encapsulation into niosomes.[76] However, it must be noted that splenic and bone marrow parasites are more difficult to reach and eradicate.[79]

### 5.1.3. *Delivery to the brain*

Delivery of peptides to areas beyond the blood brain barrier is a major challenge, however, there is evidence that glucose coated niosomes may be able to achieve brain delivery of hydrophilic peptides.[80] These vesicles are believed to exploit the glucose transporter at the blood brain barrier, possibly by initially concentrating drug at the barrier, and have been shown to deliver intact vasoactive intestinal peptide to the posterior and anterior parts of the brain.[80]

## 5.2. *Topical use of niosomes*

### 5.2.1. *Transdermal*

The topical application of niosome encapsulated drugs results in the enhanced delivery of drugs through the stratum corneum,[81–84] and delivery is specifically enhanced when hydrophilic surfactants such as poly(oxyethylene)-7-dodecyl ether[83] or poly(oxyethylene)-8-lauryl ester[85] are used to produce flexible or "elastic" vesicles, which have similar flexible bilayer properties to the phospholipid transfersomes.[86,87]

### 5.2.2. *Ocular*

Niosomal formulations for the topical treatment of glaucoma have emerged in the form of Carbopol 934P coated sorbitan monostearate acetazolamide niosomes,[88] both chitosan and Carbopol 934P coated sorbitan monostearate timolol maleate niosomes[89] and sorbitan monopalmitate timolol maleate discomes.[90]

Discome formulations with a particle size of 16 $\mu$m produce a sustained lowering of intraocular pressure when compared with normal niosomes.[90] The large particle size of the discomes is believed to limit ocular clearance. Polymer coatings are used to promote bioadhesion and to prolong the drug residence time within the eye and the result is a prolonged lowering of intraocular pressure.[88] Chitosan is a superior bioadhesive than Carbopol 934 in these ocular formulations.[89] The acetazolamide formulations showed comparable activity to a marketed formulation — dorzolamide (Dorzox) and activity was sustained for up to 6 hrs.[88] The timolol maleate sorbitan monostearate niosome formulation on the other hand was twice as active as a marketed gel formulation.[89] All these topical niosome formulations are able to better localize drug activity to the eye, when compared with the drug in solution, thus minimizing deleterious systemic effects.[89,90]

## 5.3. *Niosomal vaccines*

The niosomal encapsulation of both antigens[91] and DNA encoding for antigens[92] results in an enhancement of the humoral[92] and cellular[91] immune response to the said antigens. Although surfactants have immunostimulatory properties,[93] the adjuvancy is attributed to the actual encapsulation of the antigen[94] and its presentation as a particle. Enhanced protection against an infectious challenge has also been demonstrated in mice that are vaccinated against herpes simplex virus type 1.[95]

### 5.4. *Niosomes as imaging agents*

Targeting tumor glucose receptors is a viable method of imaging tumors.[96,97] N-palmitoyl glucosamine niosomes coated with poly(oxyethylene) and encapsulating gadolinium salts target PC3 tumor cells on tail vein injection.[97] Delivery to tumors is sustained as targeting was still observed 24 hrs after dosing with the niosomes bearing both glucose and poly(oxyethylene) units on their surface.[97] Tumor levels were higher with the use of glycosylated, poly(oxyethylene) coated niosomes when compared with plain sorbitan monostearate niosomes, and it is thus concluded that the presence of either glucose or poly(oxyethylene) on the niosome surface enables tumor targeting of the contrast agent.

## 6. Conclusions

The past three decades have witnessed an explosion in the study of synthetic amphiphile vesicles, prepared either from polymers (polymeric vesicles) or from low molecular weight non-ionic surfactants (niosomes). These nanosystems/microsystems are able to encapsulate drugs and other bioactives and control the distribution of the drug for pharmacological and ultimately clinical benefit. A number of disease areas (cancer, infectious diseases, glaucoma, etc.) are likely to benefit from the exploitation of these systems, as they are capable of delivering therapeutics, prophylactics and diagnostic agents. In the case of the niosomes, a large number of the materials used to fabricate the vesicles are already established excipients, and hence the development times of new medicines are likely to be shortened. Polymeric vesicles, by virtue of the tunable nature of the polymeric constituents, could give rise to more complex systems that are responsive to the pharmacological requirements of the pathology. An exciting future awaits these formulation materials.

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# 7

# Recent Advances in Microemulsions as Drug Delivery Vehicles

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## 1. Definition

The term microemulsion (originally introduced by Schulman *et al.*[60] is used to denote a thermodynamically stable, fluid, transparent (or translucent) dispersion of oil, and water, stabilized by an interfacial film of amphiphilic molecules (i.e. molecules that possess within their structure a part that has an affinity for oil and a part that has affinity for water).[45] The amphiphilic molecules used to stabilize the dispersion may be a pure surfactant, a polymeric surfactant, or a mixture of surfactants frequently in conjunction with a weakly amphiphilic molecule or cosurfactant, typically a medium chain alcohol. Although at first glance, a seemingly simple definition, that causes much confusion in the literature, and in particular, the pharmaceutical literature, as to what exactly constitutes a microemulsion such that every article on the subject always devotes some time to precisely defining what the authors mean by the term. This present article is no different.

### 1.1. *Microemulsion versus an emulsion*

Much of the confusion stems from the apparent relationship between a microemulsion and an emulsion. In fact, the two systems have little in common other than the fact that they are both dispersions of oil and water stabilized by an interfacial film of amphiphilic molecules. The most significant difference between the two systems, is

that a microemulsion forms spontaneously, and unlike an emulsion, it requires no mechanical work for its formation. A microemulsion is therefore thermodynamically stable. An emulsion, in contrast, generally requires considerable energy input for its preparation, and while it may possess (some) kinetic stability, it does not possess thermodynamic stability. This is an important distinction between the two systems, and one which has important consequences for their preparation and use. For example, complex equipment and considerable cost in terms of energy input are required for the formation of an emulsion, whereas a microemulsion can be prepared using simple mixing equipment.

The requirement for energy input into a system for its formation provides a simple means of differentiating between a microemulsion and an emulsion. However, as it is virtually impossible to mix ingredients together without the input of some energy, the distinguishing feature is the energy level used, such that a microemulsion could be quite reasonably prepared using gentle heating or stirring to speed up its formation, whereas an emulsion would require the input of considerably more energy, such as that supplied by high pressure homogenization or ultrasonication for its preparation. It is worth noting, however, that a number of papers (e.g. Refs. 108 and 178) incorrectly describe systems as being microemulsions, despite the fact that a considerable input of energy (e.g. homogenization) is required for the formation of droplets, which in some instances are even outside the generally accepted size range for a microemulsion.[76]

Other differences between a microemulsion and an emulsion include the size of the regions of disperse phase; in a microemulsion, the domains of disperse phase are typically less than 140 nm and relatively uniform in size, whereas an emulsion possess droplets of varying size in the general region of 1–10 $\mu$m. As a consequence of their small domain size, microemulsions are transparent (or translucent) while emulsions are usually cloudy. It is worth noting however that it is possible to obtain an optically clear emulsion if the refractive index of the two immiscible phases are of comparable values.[32,75] In addition, although it is frequently stated that one of the immiscible phases is water, it is possible to prepare either a microemulsion or an emulsion using either a non-aqueous polar phase such as ethylene glycol, gylcerol[14] or ethanol,[187] or two immiscible oils such as a fluorocarbon and a hydrocarbon or castor oil and silicone oil.[82] One important outcome of the small domain size (and large interfacial area) of a microemulsion is the large amount of surfactant required to stabilize the system, in the order of 20 wt%, as opposed to a 1 to 2 wt% generally needed to stabilize an emulsion.

## 1.2. *Microemulsion versus a nanoemulsion*

The confusion as to what differentiates a microemulsion and an emulsion has been further complicated by the emergence in the literature of another oil and water

dispersion, known as a nanoemulsion,[60,169] although the terms mini-[51,177] or submicron emulsion[112] have also been used. The distinction between a microemulsion and a nanoemulsion is even more blurred because the description of a nanoemulsion is very similar to that of a microemulsion in that they are both oil-in-water dispersions of small droplet diameter (for nanoemulsions a range of 20–200 nm is typically quoted) and of narrow droplet size distribution.[60,131,169] Although the physical appearance of a nanoemulsion resembles that of a microemulsion, in that both systems are transparent (or translucent) and of low viscosity, there is an essential difference between the two systems, namely that a nanoemulsion (i.e. an emulsion) is, at best, kinetically stable, while a microemulsion is therniodynamically stable. As a consequence, many of the nanoemulsions reported in the literature do not possess long-term stability.[80,81,173] Some nanoemulsions have however exhibited sufficiently high levels of stability for them to be proposed as vehicles for drug delivery.[113,156] It is worth commenting that, while the distinction between a nanoemulsion and an emulsion, in terms of their size, is rather arbitary, nanoemulsions because of their small droplet size, possess a higher stability against sedimentation or creaming than an emulsion.

One supposed advantage of a nanoemulsion over a microemulsion is that it requires a lower surfactant concentration for its formation. For example, nanoemulsion droplets of radius 60–70 nm and containing 20 wt% isohexadecane, using only 4 wt% of a mixture of polyoxyethylene 4-dodecyl ether and polyoxyethylene 6-dodecyl ether surfactants.[81] When comparing this surfactant concentration with the 20 wt% surfactant typically needed to prepare a microemulsion containing a comparable amount of oil, one should realize that the droplet size of a microemulsion thus produced would typically be ~10 nm. Consequently, in order to produce nanoemulsion droplets of the comparable size, the amount of surfactant required would increase (the surface area of the droplet varies with that of the square of the droplet radius) to a comparable value. The pertinent question (in terms of drug delivery) is what is most beneficial or the optimal size of the droplets. Recent results suggest that small may not always be better, especially because of the need for large amounts of surfactant which, under certain circumstances, actually hinder drug absorption.[145]

Nanoemulsions, as a consequence of their relatively high kinetic stability, low viscosity, and transparency/translucency, are very attractive for a range of industrial applications, including the pharmaceutical field where they have been explored as drug delivery systems.[10,23,28] It is worth noting however that the most stable nanoemulsions are generally, although not exclusively, prepared using expensive, high energy input methods such as microfluidization, ultrasonication, which makes their production expensive.

## 1.3. *Microemulsions*

Figure 1 shows a hypothetical ternary phase diagram with the possible extent of the existence of a microemulsion marked (temperature and pressure are constant). If a surfactant or a mixture of surfactants, and a cosurfactant are used to prepare the microemulsion, then it is usual to keep the ratio of the two amphiphiles constant and to plot them as a "single" component, thereby allowing the production of a (pseudo-)ternary phase diagram. As can be seen in Fig. 1, it is possible to prepare microemulsion over a wide range of surfactant concentrations and oil-to-water ratios, although in most systems, a microemulsion exists only over a restricted range of concentrations. Indeed, a microemulsion is only one of a number of possible oil, water and surfactant association structures that can form, depending upon the chemical nature and concentration of each of the components, as well as the temperature and pressure (other structures include gels and various mesomorphic phases). Regardless of whether a microemulsion is formed or not, generally, at very low surfactant concentrations, a large multiphase region is seen, while at very high surfactant concentrations, liquid crystalline phases are frequently formed.

It is clear from Fig. 1 that the microstructure of the microemulsion must vary over the range of possible microemulsion compositions. At low oil or water volume

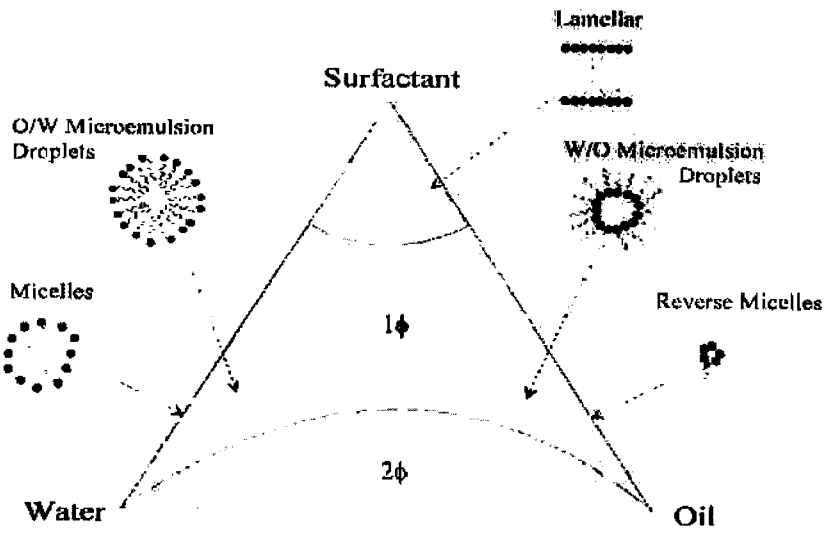


**Fig. 1.** A hypothetical pseudo-ternary phase diagram of an oil/surfactant/water system with emphasis on microemulsion and emulsion phases. Within the phase diagram, existence fields are shown where micelles, reverse micelles or water-in-oil (w/o) microemulsions and oil-in-water microemulsions are formed along with the bicontinuous microemulsions. At very low surfactant concentrations two phase systems are observed (taken from Ref. 107).

fractions, microemulsions are generally considered to be a dispersion of either oil or water droplets stabilized by an interfacial film of surfactant and where appropriate, cosurfactant. These droplet structures are probably the most commonly encountered type of microemulsion microstructure. It is worth noting that both an emulsion and a nanoemulsion can only occur in the form of a droplet, either as an oil-in-water or water-in-oil droplet.

At intermediate oil and water compositions, it is obviously not possible for the microstructure to be composed of droplets of one phase dispersed in the other. In these cases, it is thought that a bicontinuous structure exists, in which the water and oil domains are separated by a regular or topologically chaotic continuous amphiphile-rich interfacial layer. A bicontinuous microemulsion is often the intermediate microstructure between an oil-in-water and a water-in-oil microemulsion, although a number of other microstructures such as cylinders and worm-like microemulsions have been reported to exist.

In terms of its microstructure, a microemulsion is therefore a very complex system, and in instances where a microemulsion exists over a wide range of compositions, several different types of microstructure may be present.[73] It is also important to remember that whatever the microstructure, a microemulsion is a dynamic system in which the interface is continuously and spontaneously fluctuating.[104] For this reason, microemulsions stabilized by polymeric surfactants may be the most long lived.

## 1.4. *Microemulsions, swollen micelles, micelles*

There is much debate in the literature as to what exactly differentiates a microemulsion from a micelle at low volume fractions of disperse phase. Some investigators have perceived a difference between microemulsions and micellar systems containing solubilized oil or water, and have used the terms "swollen" micellar solutions or solubilized micellar solutions to describe such systems. These investigators argue that the term microemulsion should be restricted to systems in which the droplets are of large enough size such that the physical properties of the dispersed oil or water phase are indistinguishable from those of the corresponding oil or water phase, thereby theoretically making it possible to distinguish between oil-in-water (or water-in-oil) microemulsions and micellar solutions containing small amounts of solubilized oil (water). However, in most cases, the transformation between micelles progressively swollen with oil (water) and a microemulsion containing an isotropic core of oil (water) appears to be gradual with no obvious transition point. As a consequence, there is no simple method available for determining the oil (water) content at which the core of the swollen micelle becomes identical to that of a bulk phase. Many researchers therefore use the term microemulsion to include

swollen micelles, but not micelles containing no oil (or water).[34,107] In biotechnological applications, water-in-oil microemulsions are frequently known as reverse micelles and or even as nonaqueous media.

### 1.5. *Microemulsions and cosolvent systems*

The above broad definition does not require a microemulsion to contain any microstructure. In other words, it includes systems that are co-solvents, i.e. systems in which the constituent components are molecularly dispersed. Most researchers in the field agree, however, that for a microemulsion to be formed, it is important that the system contains some definite microstructure. In other words, there is a definite boundary between the oil and water phases, and at which the amphiphilic molecules are located and that a co-solvent is not a type of microemulsion. The only way to distinguish a microemulsion from a co-solvent unambiguously is to perform either a scattering study (light, X-rays or neutrons) or PFG-NMR measurements. Regions of co-solvent formation generally appear at low concentrations of oil or water.

## 2. Microemulsions as Drug Delivery Systems

It is clear from its description that microemulsions possess a number of properties that make their use as drug delivery vehicles particularly attractive. Indeed, microemulsions were first studied with the view of using them as potential vehicles for poorly-water soluble drugs, in the mid 1970s by Elworthy and Attwood.[17] However, it was not until the mid to late 1980s that they were widely investigated as drug delivery systems; this interest being largely the result of the arrival on the market of the cyclosporin A microemulsion preconcentrate, Neoral.

Among the physical properties that make microemulsions attractive as drug delivery vehicle is their transparent nature, which means that the product is not only aesthetically pleasing, but allows easy visualization of any contamination. The small size of the domains present means that a microemulsion can be sterilized by terminal filtration.[84] Furthermore, depending on the composition of the microemulsion, it may be possible to heat sterilize the microemulsions.[39] Since oil-in-water microemulsions are able to incorporate lipophilic substances, they can be used to facilitate the administration of water-insoluble drugs.[24] Significantly, the small droplet size provides a large interfacial area for rapid drug release, and so the drug should exhibit an enhanced bioavailability, enabling a reduction in dose, more consistent temporal profiles of drug absorption, and the protection of drug(s) from the hostile environment of the body. In addition to increasing the rate of drug release, microemulsions can also be used as a reservoir and actually slow the release of drug and prolong its effect, thereby avoiding high concentrations in

the blood.[64,142] Whether a drug is rapidly or slowly released from a microemulsion depends very much on the affinity of the drug for the microemulsion. Since microemulsions contain surfactants (cosurfactants) and other excipients, they may serve to increase the membrane penetration of drug.[163,189]

A number of reviews have been presented, describing the pharmaceutical use of microemulsions.[16,19,50,105–107,176] Since the last major review in the area was writen in 2001, the present review will mainly deal with developments henceforth, although important work prior to this will be discussed when appropriate.

## 2.1. *Self-emulsifying drug delivery systems (SEDDS)*

Before discussing how microemulsions are being exploited in drug delivery, it is worth making one more distinction, namely the difference between a self-emulsifying drug delivery system (SEDDS) and a microemulsion. A SEDDS is a mixture of oil(s), and surfactant(s), ideally isotropic, sometimes containing cosolvent(s), which when introduced into aqueous phase under gentle agitation, spontaneously emulsifies to produce a fine oil-in-water dispersion.[36,146] Typically, the size of the droplets produced by dilution of a SEDDS is in the range of 100 and 300 nm, while, upon dispersal in water, a SMEDDS formulation (a sub-group of the SEDDS) forms a transparent microemulsion with particle sizes <100 nm. A SMEEDS is also known as a pre-microemulsion concentrate.[97] It is worth noticing that this method of producing a fine oil-in-water emulsion using a S(M)EEDS is identical to the low energy emulsification method for producing oil-in water nanoemulsions.[173] It is therefore likely that a diluted S(M)EDDS and nanoemulsion are identically the same.

The technique of low-energy or self-emulsification has been commercially exploited for many years in the agrochemical industry, in the form of emulsifiable concentrates of lipophilic herbicides and pesticides.[146] However, it has only recently been introduced in the pharmaceutical industry as a tool to improve the delivery of lipophilic drugs by incorporating the drug into a S(M)EDDS formulation which is then filled into capsules.[65] Once the capsule has been swallowed and its contents come into contact with the GI fluid, the drug containing (micro)emulsion should be spontaneously formed. Once the drug containing (micro)emulsion is formed, there should be little difference between the fate of the drug thus administered and the same drug administered in a (pre-formulated) microemulsion, although the droplets formed from the S(M)EDDS tend to be of a larger size. One advantage of administering a drug in a SMEEDS as opposed to a pre-formulated microemulsion, is its relatively small volume which can be incorporated into soft or hard gelatin capsules, convenient for oral delivery.

To date, there has been a good amount of commercial success for the first self-microemulsifying drug delivery systems (SMEDDS) on the market, namely Neoral (cyclosporin A). In addition, the recent commercialization of two self-emulsifying

formulations, namely Norvir (ritonavir) and Fortovase (saquinavir), has undoubtedly increased the interest in SEDDS and other emulsion-based delivery systems to improve the delivery of a range of drugs of varying physico-chemical properties.

However, there are a number of reasons why S(M)EDDS are not in greater widespread use, but the main reason is probably the stability of the diluted SEDDS, which is in fact a thermodynamically unstable emulsion (although it may exhibit some limited kinetic or "meta" stability). It should be noted however that as a SEDDS is either diluted just prior to administration or else in the body, the required droplet stability is less than 6 hrs (i.e. the transit time of materials down the small intestine).

Although most studies of SEEDS have utilized isotropic liquids, the earliest reports of these self-emulsifying systems using pharmaceutical materials are in fact related to pastes based on waxy polyoxyethylene *n*-alkyl ethers.[67] In the context of drug delivery via self-emulsifying systems, isotropic liquids are generally preferred to waxy pastes because if one or more excipient(s) crystallize(s) on cooling to form a waxy mixture, it is very difficult to determine the morphology of the materials. Despite this, there is currently a general move towards formulating semi-solid SEDDS. For example, attempts have been made to transform SEDDS into solid dosage forms by addition of large amounts of solidifying excipients such as adsorbents and polymers[15,134] Unfortunately, as the ratio of SEDDS to solidifying excipients required for this approach is very high, this leads to problems in formulating drugs having limited solubility in the oil phase. Recent attempts have been made to reduce the amount of solidifying excipients by gelling the SEDDS with colloidal silicon dioxide.[141]

Khoo *et al.*[93] have recently reported the preparation of a halofantrine-containing lipid-based solid self-emulsifying system using either Vitamin E TPGS or a blend of Gelucire 44:14:Vitamin E TPGS as the base. Upon dispersal, these systems produced dispersions that the authors described as microemulsions. Studies in fasted dogs showed that these solid dispersions exhibited a five- to seven-fold improvement in absolute oral bioavailability, when compared with the commercially available tablet formulation.

In a different approach, Nazzal *et al.*[132] have determined the potential of a reversibly induced re-crystallized semi-solid self-nanoemulsifying drug delivery system, based on a eutectic interaction between the drug and the carrying agent, as an alternative to a conventional SEDDS. In these eutectic-based self-nanoemulsified systems, the melting point depression method allows the oil phase containing the drug itself to melt at body temperature from its semisolid consistency, and disperse to form emulsion droplets in the nanometer size range. Emulsion systems based on a eutectic mixture of lidocaine–prilocaine,[135] and lidocaine–menthol[87] have been

used in the preparation of topical formulations. However, little is known of the use of eutectic mixtures for the preparation of self-(micro) emulsified formulations.

## 2.2. *Related systems*

There are a number of other putative delivery systems that are closely related to, or are prepared from, a microemulsion. These systems include a variety of gel formulations (including microemulsion-based gels, ringing gels, microemulsion gels) and double microemulsions.

### 2.2.1. *Microemulsion gels*

Oil-in-water microemulsions can be readily gelled or thickened by the addition of a non-interacting, water-soluble polymer such as polyHEMA,[158] Carbopol 940[44] or carrageenan[179] to form clear "microemulsion gels". In these cases, it is the external aqueous phase that is gelled, while the microemulsion droplets are unperturbed. The structure of the resulting "microemulsion gel" is quite different, if it is prepared using an interacting polymer, such as stearate-polyethylene oxide-stearate. In this instance, the hydrophilic mid-block of the polymer is located in the continuous aqueous phase, while the hydrophobic end blocks are dissolved in the oil droplets, thereby connecting the various microemulsion droplets and resulting in the formation of a transient gel network.[159] Clear, "microemulsion gels" are also sometimes obtained at surfactant and/or oil concentrations just outside the oil-in-water microemulsion region.[180] Sometimes, the resultant gel "rings" or vibrates when tapped.[180] The ringing is due to the resonance of shear modes within the gel body.[167] Neither of these "microemulsion gels", which are water continuous, are true microemulsions, which are fluid by definition.

Clear gels can also be formed in oil continuous systems. For example, a gel can be formed when water is added to reverse micellar solutions of lecithin-in-oil.[5,12,161] Here, the water causes the worm-like lecithin reverse micelles to intertwine and form a gel. In addition, gels, widely known as microemulsion-based gels, can be formed from water-in-oil microemulsions stabilized (predominately) by the di-chain surfactant sodium bis (2-ethylhexyl) sulfosuccinate (AOT), when gelatin, the natural amphiphilic polymer is added.[70,148] Microemulsion-based gels have now been prepared in systems in which a large amount of the AOT has been replaced by nonionic surfactant;[88,89] or more recently, using in place of AOT, the single chain surfactant, cetyltrimethylammonium bromide in combination with pentanol.[116] In these gels, the gelatin is thought to form water-continuous channels between the microemulsion-droplets. These microemulsion-based gels are very unusual in that, although they are oil continuous, they are electrically conducting. In addition, the

continuous oil behaves as if it were still a fluid, even though placing a gel in a solution of the oil does not dissolve it.

All of these "microemulsion gels" have potential, or are being explored for use as drug delivery systems. Of particular interest is the fact that the gels possess the properties of being transparent, infinitely stable and readily prepared using only the mildest of mixing. In addition the wide range of microemulsion gels available means that it is possible to select the gel of the required consistency for application to large areas of skin, the nasal membrane, vaginal and buccal membranes and for permeation enhancement. Microemulsion-based gels have been explored as vehicles for the iontophorectic delivery of drugs.[88]

### 2.2.2. *Double or multiple microemulsions*

Double (or multiple) *emulsions* have attracted much interest as potential drug delivery vehicles. For example, adding a water-soluble drug into the internal aqueous phase of a water-in-oil-in-water emulsion may allow the sustained release of the water-soluble drug.[59] A double microemulsion should offer similar advantages over the rate of drug release of entrapped solutes. Double emulsions are notoriously difficult to formulate due to the requirement to have one surfactant (or mixture of surfactants) to stabilize the first (internal) emulsion and a second surfactant (or mixture of surfactants) of quite different physico-chemical properties to stabilize the second emulsion. Although a few papers have detailed the production of nanoparticles from systems they described as double microemulsions,[35,56,184] the term "double microemulsion" in this context is very misleading, as it refers to the mixing of two water-in-oil microemulsions of comparable composition, but containing different solute in the aqueous phase.

There are however two papers which describe the preparation of double (oil-in-water-in-oil) microemulsions. In the first, Castro *et al.*,[30] report spectroscopic studies of nifedipine in Brij 96 based oil/water/oil multiple microemulsions. In the second, Carli *et al.*,[29] detail the preparation of an oil-in-water-in-oil microemulsion from an oily phase of either polyglycolized glycerides or a mixture of mono-, di- and tri-glycerides, which is microemulsified using a mixture of water and surfactant (soy lecithin and Tween 80). The resultant o/w microemulsion is subsequently redispersed in an oily phase to produce the double (o/w/o) microemulsion.[29]

## 2.3. ***Processed microemulsion formulations***

### 2.3.1. *Solid state or dry emulsions*

In practical terms, a solid dosage form is preferable to a liquid dosage form in respect of convenience, ease of handling and accurate dosing. Consequently, a number of

researchers have attempted to develop powdered, re-dispersible emulsion-derived formulations, known as solid state or dry emulsions. Such solid-state emulsions can be used to modulate the release rates of emulsified compound.[128] Dry emulsions have been variously prepared by removing water from an oil-in-water emulsion, using water-soluble[182] or -insoluble[150] solid carriers or indeed a mixture of both water-soluble and water-insoluble carriers,[143] by rotary evaporation,[128] lyophilization,[115] or spray drying.[55,127]

Attempts have also been recently made to prepare dry microemulsions using similar methodology. For example, Moreno *et al.*[126] have lyophilized an amphotericin B-containing lecithin-based oil-in-water microemulsion in the presence of 5 wt% mannitol. The lyophilized product was an oily cake from which the microemulsion could be easily reconstituted over several months. The rationale for developing the water-free formulation was to avoid the hydrolysis of lecithin, which occurs upon its dispersal in water, thereby preventing any deterioration of the formulation upon storage. Overall, the lyophilized lecithin based oil-in-water microemulsions appear to be valuable systems for the delivery of amphotericin B, with regard to ease and low-cost of manufacturing and their stability and safety, compared with other formulations already in the market.

In a recent paper, Carli *et al.*[29] reported an alternative approach to prepare a "dry" formulation known as a nanoemulsified composite of the coenzyme Q10, ubidecarenone. This composite is prepared by incorporating the ubidecarenone into the inner phase of the double microemulsion, which is then deposited onto a solid microporous carrier such as cross-linked polyvinylpyrrolidone. Among the advantages offered by this approach are good processing and storage properties, easy re-dispersibility in water, high bioavailability and maintenance of the submicron size of the released droplets.

Kim *et al.*,[97] have prepared entric-coated solid state premicroemulsion concentrates by first preparing a pre-microemulsion concentrate containing 10 wt% of the drug cyclosporin A, 18.5 wt% of a medium chain triglyceride, 51 wt% of surfactant and 20 wt% of cosurfactant. The pre-microemulsion concentrates were then enteric-coated as films using polymers, such as sodium alginate, Eudragit L 100 and cellulose acetate phthalate, and the resulting films were pulverized to produce powdered, dry, enteric coated premicroemulsion concentrates. Using this approach, the authors successfully prepared a once-a-day oral dose form of cyclosporin A.

## 3. Formulation

Microemulsions are far more difficult to formulate than emulsions because their formation is a highly specific process involving spontaneous interactions among their constituent molecules. In addition, in a number of cases, effects due to the order of

the mixing of the component molecules have been observed. Since no adequate theory currently exists to predict from which molecules microemulsions can be formed, mainly because of the requirement to determine a number of unknown parameters, microemulsion formulations are generally developed empirically, although some useful practical guidance as to the choice of the constituent components can be found in the literature.[105,107]

A recognized and classical approach to microemulsion formulation is to undertake a systematic study of the phase behavior of the systems understudying utilizing of phase diagrams. A major drawback of this approach is the considerable time it takes to develop the phase diagram, especially considering the combination of possible oil, surfactant and cosurfactant, and the fact that time may be necessary for a system to equilibrate. Heat and sonication are therefore often used, particularly with systems containing nonionic surfactants, to speed up the formation process. While there are now commercially available automated systems to prepare phase diagrams,[78] the chief drawback of these systems is their cost.

A number of attempts have been recently made to use modeling to predict microemulsion formation, thereby aiding in the formulation of microemulsions. A range of modeling techniques have been used including artificial neural networks,[3,4,8,149] genetic algorithms[3,174] and a combination of data mining, computer-aided molecular modeling, descriptor calculation and multiple linear regression techniques.[174,175] Unfortunately, however, all of these techniques require a considerable amount of work prior to prediction, thereby restricting their potential usefulness. Furthermore, the amount of work required for the predictions increases as the number of components of the microemulsion increase; microemulsions formulated from five components (i.e. oil, water, surfactant, cosurfactant, electrolyte and drug) are not uncommon in pharmaceutical use. To the authors' knowledge, to date, no work has been performed predicting how much drug can be incorporated into a microemulsion and whether the presence of drug has any effect on microemulsion phase behavior. This is an important ommision as microemulsions cannot be considered to be inert, since the presence of drug in some instances (greatly) influences phase behavior (see for example Ref. 138).

## 3.1. *Surfactants and cosurfactants*

The selection of components for the preparation of microemulsions suitable for pharmaceutical use involves a consideration of their toxicity, and if the systems are to be used topically, their irritancy and sensitizing properties as well. There are a number of surfactant and cosurfactants that are considered acceptable for use as excipients in pharmaceutical formulation.[153] Strickley[172] has recently reviewed

those surfactants and cosurfactants currently used in commercially available oral and intravenous formulations.

In the general scientific literature, by far the most widely used surfactant to prepare a microemulsion is the double chain, ionic surfactant, sodium bis (2-ethylhexyl) sulfosuccinate (AOT), although a large number of studies have used the single chain, nonionic surfactants of the type $C_iE_j$, where i is the number of carbons in the alkyl chain, C, and j is the number of ethylene oxide units in the polyoxyethylene chain, E. Both AOT and the $C_iE_j$ surfactants possess the important advantage of being able to form microemulsions in the absence of a cosurfactant,[121,185] unlike most other types of surfactant such as the widely studied single chain, ionic surfactant, sodium dodecyl sulphate (SDS), which will only form microemulsions in the presence of an alcohol cosurfactant. Neither AOT nor SDS would be considered to be apprpropriate for the preparation of pharmaceutically acceptable microemulsions, even though they are listed in the Pharmaceutical Excipient Handbook, Rowe *et al.*[154]

As a general rule, nonionic and zwitterionic surfactants tend to be less toxic than ionic surfactants and are therefore more widely used as pharmaceutical excipients.[154] Assuming that the surfactants do not degrade into toxic materials, surfactants that posses biodegradable/chemically unstable linkers tend to exhibit less chronic toxicity than those that are chemically stable. For example, as a group, the polyoxyethylene *n*-acyl surfactants exhibit ~ten times less chronic toxicity than their *n*-alkyl counterparts, mainly due to their quicker degradation time of days as opposed to weeks. When it comes to comparing acute toxicity, the two groups of surfactants exhibit comparable toxicity.

Perhaps the most widely used nonionic surfactants in pharmaceutical formulations are the polyoxyethylene sorbitan *n*-acyl esters, i.e. the Tweens and in particular, Tween 20 and Tween 80, both of which are used parenterally and orally. In addition, polyoxyethylene derivatives of the triglyceride, castor oil have acceptability for intravenous administration. Other pharmaceutically acceptable surfactants are the polyoxyethylene *n*-alkyl ethers and *n*-acyl esters, although both these groups of surfactant tend to be restricted for topical use.[154] Other nonionic surfactants that are currently attracting much pharmaceutical interest, although they do not yet have acceptability, are the polyglycerol *n*-acyl esters, the *n*-alkyl amine *N*-oxides and the *n*-alkyl polyglycosides (or sugar surfactants). The *n*-alkyl polyglycosides have attracted much pharmaceutical interest, not because of their excellent biodegradability, but because they can be manufactured from renewable resources. All of the aforementioned surfactants have been used to prepare microemulsions, generally as sole surfactant, the only exception being the *n*-alkyl polyglycosides, which tend to require the presence of a cosurfactant.

Pluronics (or poloxamers) of the type poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) are another class of pharmaceutically

attractive surfactant. Interestingly, most reports detailing the use of polymeric surfactants to stabilize a microemulsion describe the preparation of water-in-oil, generally in conjunction with a second surfactant.[96,181] Siebenbrodt and Keipert[162] have reported the formation of a triacetin-in-water microemulsion using Pluronic L 64 as sole surfactant. Lettow *et al.*,[111] have used Pluronic PI23 as sole surfactant to prepare oil-in-water microemulsions, incorporating a 1:1 oil:P123 weight ratio of either 1,3,5-trimethylbenzene or 1,2-dichlorobenzene.

Finally, the pharmaceutically acceptable zwitterionic lecithin has been extensively used as a surfactant, however, with very few exceptions, it is not possible to prepare a microemulsion using lecithin as sole surfactant. Generally, lecithin is combined with another surfactant such as Tween 80, or a cosurfactant such as ethanol, when formulating microemulsions.

Although ethanol is considered to be pharmaceutically acceptable, typical cosurfactants such as propanol and butanol are not. In addition to toxicity issues, the use of such cosurfactants, which may possess partial oil and water-solubility, can lead to problems with the dilutability of the microemulsion. This is a particular issue if the microemulsion is to be administered orally or parenterally. Consequently, a number of researchers have explored the use of a second surfactant as cosurfactant when formulating a microemulsion. Microemulsions thus prepared tend to be very stable against dilution, as the "cosurfactant" generally has little solubility in either the oil or aqueous phase. Alternately (pharmaceutically acceptable), short chain mono- and di-glycerides have been used in place of a short chain alcohol to successfully prepare microemulsions. In a number of instances, short chain fatty acids such as sodium caprylate have been used as cosurfactants, primarily for the formation of microemulsions for oral delivery; sodium caprylate is known to enhance absorption of drugs across the gastrointestinal tract. A number of researchers have also used cosolvents such as the polyhydric alcohols, sorbitol, glycerol and propylene glycol to aid microemulsion formation. In a number of instances, these materials have been described as "cosurfactants", which quite clearly do not sit in the interfacial surfactant monolayer. Rather, they tend to exert their effect by altering the solvent properties of the polar phase.

## 3.2. *Oils*

Most reports in the chemical literature detail the preparation of microemulsions using aromatic oils such as benzene and short chain alkanes such as hexane. "Pharmaceutical" oils, unlike those used in the chemical and agrochemical industries, tend to be large in terms of molecular weight and therefore volume, and are relatively polar. Both of these properties tend to work against the oil when it comes to formulating it in a microemulsion, as it is well established that smaller molecular volume oils are easier to solubilize and are solubilized to a greater extent than larger

oils.[2] Although there are reports that in some systems, particularly those containing surfactants with long, unsaturated hydrophobes such as polyoxyethylene (10) oleyl ether, the largest molecular volume oil is solubilized to a greater extent than some of the smaller molecular volume oils.[122] The most commonly used "pharmaceutical" oils are medium and long chain triglycerides, and esters of fatty acids such as ethyl oleate, isopropyl myristate are popular.

It has become common practice for researchers to screen the solubility of drug in the various components of the microemulsion, in order to predict the optimal composition of the final formulation. However, extreme care has to be exercised when using this approach, as very often, the solubility in the final microemulsion formulation does not correlate well with that seen in the various components.

### 3.3. *Characterization*

It is noticeable that in contrast to their ease of preparation, it is very difficult to establish the microstructure of a microemulsion. Yet, such information is important as it may influence the drug behavior of the microemulsion in use. For example, it is known that the microstructure of the microemulsion may alter the release rate of any incorporated solute.[1,95]

Currently, a range of physico-chemical techniques are used to characterize microemulsions. These techniques are often used in tandem to obtain a better picture of the system, as it is unlikely that any one technique alone will give sufficient information.[144] Scattering techniques (light, neutron and X-ray) and pulsed field gradient NMR are generally used to determine the microstructure of the microemulsion. One serious limitation with characterizing microemulsions is that most techniques rely on the concentration of disperse phase being low enough to avoid particle-particle interactions, as an estimated volume fraction of 1 vol% is suitable.[123] The requirement is a particular problem with microemulsions that contain cosurfactants that partition between the oil and water phases, because these systems frequently undergo a change upon dilution.

## 4. Routes of Administration

Although most of the original work exploring microemulsions as drug delivery vehicles examined their potential for oral drug delivery, microemulsions have now been explored as vehicles for most routes of administration. Currently, they are probably most widely studied for their potential as transdermal delivery vehicles.

### 4.1. *Oral*

Microemulsions (and SMEDDS) have been widely studied as oral drug delivery vehicles. Indeed, the first commercially available "microemulsion" formulation was

a premicroemulsion concentrate of the lipophilic peptide, cyclosporin A. This formulation, known commercially as Neoral, was introduced onto the market in the late 1980s and immediately attracted much attention, mainly because of the high and reproducible bioavailability it produced, but also because developments in biotechnology at that time meant that it had never been easier to produce on a large scale therapeutically-relevant protein and peptides. Unfortunately, because of their physico-chemical properties, in particular their large size and poor stability, proteins and peptides are very difficult to formulate. Microemulsions offered an attractive solution to this problem, and consequently, most of the original exploratory studies on microemulsions as drug delivery vehicles were spent developing oral protein/peptide microemulsion formulations.

### 4.1.1. *Proteins and peptides*

As the majority of therapeutic proteins and peptides are hydrophilic and water-soluble, most studies utilizing microemulsions as vehicles for such molecules have exploited water-in-oil microemulsions. After cyclosporin A, which is unusually highly lipophilic, for a therapeutic peptide, the most widely studied peptide is insulin, with much of the early work in this area being performed by Ritschel.[152] For example, Kraeling and Ritschel[101] compared the peroral microemulsion formulation of insulin and capsule forms and determined that the microemulsion formulation increased the bioavailability of the insulin. Recently, more complex microemulsion-based systems have been developed in an attempt to improve the extent of insulin absorption. For example, a recent study performed by Natnasirichaiku *et al.*[186] showed a significant improvement in the oral bioavailability of insulin (in diabetic rats) when administered in nanocapsules dispersed in a water-in-oil microemulsion. Santiago *et al.*[155] have developed a new, enteric oral dosage form of insulin, in which an association of insulin and cyclodextrin contained within a microemulsion is processed into granules. In the most recent study aimed at developing an oral formulation of insulin, Iek *et al.*[77] used a conventional lecithin-based water-in-oil microemulsion formulation prepared from 21.6 wt% water, 37.6 wt% Labrafil M 1944 CS as oil and stabilized by 40.8 wt% of a 1:1 weight ratio of lecithin (Phospholipon 90G) and ethanol. In addition to insulin (21.6IU/g water), some of the microemulsions contained the enzyme inhibitor aprotinin (2500 KIU/g water). Although it is the first time that a microemulsion formulation has contained both a protein/peptide and an enzyme inhibitor, the concept of adding an enzyme inhibitor, to a formulation containing a peptide in an attempt to reduce its degradation is not new.[188] The plasma glucose and insulin levels of the rats after intragastric administration of the formulations to both diabetic and non-diabetic rats were significantly different from those obtained after oral

administration of an aqueous insulin solution. Although the addition of aprotinin to the microemulsion containing insulin increased bioavailability when compared with those not containing it, the difference was insignificant.

Other peptides formulated as water-in-oil microemulsions in an attempt to improve their oral absorption include RGB peptides,[37,38] and more recently, *N*-acetylglucosaminyl-*N*-acetylmuramyl dipeptide (GMDP).[119] The poor bioavailability of GMDP has been attributed to both its poor stability in the lumen of the gastrointestinal tract and its poor intestinal permeability. When GMDP was administered intraduodenally in a water-in-medium-chain trigylceride microemulsion, a ten-fold increase in bioavailability was observed, i.e. a bioavailability of 80.2% was achieved as opposed to 8.4%, seen after administration of an aqueous solution of GMDP. This increase in bioavailability is consistent with the work of Constantinides *et al.*[37,38] who utilized a similar medium chain triglyceride based microemulsion to increase the oral bioavailability of the water-soluble peptide SK&F 106760, after intraduodenal administration to rats.

Ke *et al.*[92] have recently reported an attempt to develop water-in-oil microemulsions suitable for the incorporation of therapeutic proteins and peptides using a medium chain triglyceride, water and tocopheryl polyethylene glycol 1000 succinate (TPGS) as the primary surfactant. However, as TPGS could not form microemulsions when used as sole surfactant, it was mixed with a second surfactant, either Tween 20, 40, 60 or 80, at a weight ratio in the range of 4:1 to 1:4. A range of glycols and polyols were examined as cosurfactants. Although stable, transparent microemulsion and gel regions were identified, the extent of these regions was influenced by the precise nature and the amount of the secondary surfactant and cosurfactant. For example, Tween 80, which is an ester of the *unsaturated* C18 fatty acid, oleic acid, was more effective in forming a microemulsion than Tween 60, which is an ester of the *saturated* C18 fatty acid, stearic acid. In this study, although the microemulsions were ultimately intended for use as delivery vehicles for protein or peptide drugs, they were not examined for this purpose.

### 4.1.2. *Other hydrophilic molecules*

Other water-soluble therapeutic molecules that have been administered in microemulsions include the aminoglycoside antibiotic, gentamicin[74] and the biologically active polysaccharide, heparin.[99] In common with all aminoglycosides, gentamicin is highly polar and is therefore considered unlikely to be absorbed from the gastrointestinal tract via simple diffusion. In order to facilitate the transmucosal delivery of the drug, Hu *et al.*[74] prepared a SMEDDS formulation of gentamicin using a range of surfactants. When Labrasol was used as surfactant, a 54.2% bioavailability of gentamicin was obtained, compared with only 8.4 and 3.4% when

Tween 80 and Transcutol P were respectively used. Labrasol was also found to inhibit intestinal secretory transport from the intestinal enterocytes, providing the formulation with the additional benefit of inhibiting the efflux of gentamicin out of the enterocytes into the GI lumen.

Due to its low bioavailability, heparin is generally administered by injection. In an attempt to formulate an orally active version of heparin, Kim *et al.*[99] synthesized a low molecular weight heparin (LMWH)-deoxycholic acid (DOCA) conjugate (termed LMWH-DOCA) and formulated it in a water-in-oil microemulsion using as oil, the medium chain trigylceride, tricaprylin, a mixture of Tween 80 and Span 20 surfactants, LMWH-DOCA and water (volume ratios of 5:3:1:1 respectively). Oral administration of LMWH-DOCA in the water-in-tricaprylin microemulsion to mice resulted in a bioavailability of 1.5%. Toxicity studies suggested that the enhancement in bioavailability, observed with the DOCA-conjugated LMWH, was administered in a microemulsion not due any local toxicity such as disruption or damaging of the intestinal membrane.

### 4.1.3. *Hydrophobic drugs*

A number of poorly water-soluble, low molecular weight, lipophilic drugs have also been formulated in microemulsions (or SMEEDS) for oral delivery including nitrendipine,[90] danzol[145] halofantrine[94] and biphenyl dimethyl dicarboxylate.[98] These studies serve as an illustration of how important it is to understand the influence on microemulsion formation of the various formulation components. It is worth commenting that the main use of SMEEDS formulations is for the oral administration of lipophilic drugs.

Formulating nitrendipine in a SMEEDS formulation, composed of a 1:1 (w/w) mixture of glycerol monocaprylic ester (MCG) and propyleneglycol dicaprylic ester (DCPG) and nonionic surfactant (various), was observed to significantly enhance its absorption when compared with a suspension or an oil solution,[90,91] and served to reduce the effect of the presence of food on its absorption. However, the absorption profile of nitrendipine was seen to vary with the type of surfactant used; absorption was rapid from the Tween 80-stabilized formulation, while the HCO-60-based formulation gave a prolonged plasma concentration profile. No absorption of nitrendipine was observed from the formulation containing BL-9EX (polyoxyethylene alkyl ether, $C_{12}E_9$). Damage to the gastrointestinal mucosa also differed with the type of surfactant employed. HCO-60 and Tween 80-based formulations were mild to the organs, while BL-9EX-based formulation caused serious damage.

The study of Porter *et al.*[145] appropriately demonstrates the effect of changing the nature of the trigylceride involved in the formulation on drug absorption. These workers studied three lipid-based danazol formulations; namely a long-chain triglyceride solution (LCT-solution), a SMEDDS based on long (C18) chain lipids

(LC-SMEDDS) and a SMEEDS formulation containing medium (C8-C10) chain lipids (MC-SMEDDS). These formulations were administered to fasted beagle dogs and their absorption, compared with that obtained with a micronized danazol formulation administered postprandially and in the fasted state. Although both the LCT-solution and LC-SMEDDS formulations were found to significantly enhance the oral bioavailability of danazol, when compared with fasted administration of the micronized formulation, the MC-SMEDDS produced little improvement in danazol bioavailability. This result was partly attributed to the fact that upon digestion of the medium-chain formulation, significant drug precipitation was observed.

Khoo *et al.*[94] also considered the effect of formulating halofantrine as a pre-microemulsion concentrate in a formulation based on either a medium- or long-chain triglyceride. Both formulations, which were administered as soft-gelatin capsules, contained the same amount of medium or long chain trigylceride and were stabilized by the same surfactant/cosurfactant mixture, consisting of Cremophor EL and ethanol. Although the plasma levels of the drug were not significantly different between the two formulations, the amount of drug absorbed lymphatically varied in that 28.3% of the dose administered in the long-chain trigylceride formulation was transported lymphatically, as opposed to only 5.0% of the dose administered in the medium-chain formulation.

Kim *et al.*[98] attempted to improve the solubility and bioavailability of biphenyl dimethyl dicarboxylate, a drug used in treating liver diseases, by formulating it as a premicroemulsion concentrate. In order to optimize drug loading in the formulation, these workers screened drug solubility in a range of surfactants and oils, and on the basis of these results selected: Tween 80 and Neobee M-5. However, care must be taken when using this approach to optimize the formulation with respect to drug loading, as it has shown that solubility of drug in the bulk components is not a reliable indicator of solubility, in the final microemulsion formulation.[120,122] The danger of predicting drug solubility in the final formulation, on the basis of bulk solubility, can be seen in the study of Kim *et al.*[98] where the solubility of the drug in a formulation consisting of a 2:1 weight ratio of Tween 80 to Neobee M-5 was 7 times that of the formulation containing a Tween 80:Neobee M-5 weight ratio of 1:4, despite the solubility of the drug in Neobee M-5 being 10 times that seen in Tween 80. The final formulation, which consisted of 35 wt% triacetin and 65 wt% Tween 80 and Neobee M-5 at a weight ratio of 2:1, greatly enhanced the oral bioavailability of BDD, possibly due to the increased solubility of the drug and its immediate dispersion in the gastrointestinal tract.

Itoh *et al.*[79] optimized the formulation of the poorly water-soluble drug N-4472, N-[2-(3,5-di-tert-butyl-4-hydroxyphenethyl)-4,6-difluorophenyl]-N-[4-(Nbenzylpiperidyl)] urea, by complexing it with L-ascorbic acid and incorporating the complex into a SMEEDS comprising Gelucire 44/14, HCO-60 and sodium dodecyl sulfate. Upon dilution with water, the SMEEDS formulation produce a fine

dispersion of 18 nm droplets which were stable over the pH range of 2.0 to 7.0. The oral bioavailability of the drug was between 2–4 times that which was obtained with an aqueous solution of the complex.

## 4.2. *Buccal*

To date, very little work has been performed on investigating the use of microemulsions as vehicles for buccal delivery. In 1988, Ceschel *et al.*[31] showed that the penetration of the essential oil, Salvia sclarea L. through porcine buccal mucosa *in vitro* was increased when formulated as a microemulsion, as opposed to the pure essential oil. Scherlund *et al.*[58] investigated the potential of lidocaine and prilocaine thermosetting microemulsions and mixed micellar solutions as drug delivery systems for anesthesia of the periodonlal pocket. The formulations contained between 2–10 wt% of a eutectic mixture of lidocaine or prilocaine (melting point 18°C), while the block copolymer surfactants, Pluronic F127 and F68, were present at between 13 and 17 wt% for F127, and between 2 and 6 wt% for F68. F127 was chosen, as it is known to gel at body temperature and it is important that the formulation is easy to apply, remain at the application site, have a fast onset time, be non-irritant, and stable under normal storage conditions. The pH of the formulations was varied between 5 and 10. Most of the combinations were found to result in clear solutions, presumably oil-in-water microemulsions or mixed micellar solutions, depending on the pH of the system. At low pH, lidocaine and prilocaine are positively charged, and they could be expected to behave largely as water-soluble cationic surfactants, hence possibly forming mixed micelles. On the other hand, at high pH, the drug substances are poorly soluble and could be expected to act largely as hydrophobic solutes and form the core of the microemulsion droplets.

## 4.3. *Parenteral*

In recent years, considerable emphasis has been given to the development of injectable microemulsions (o/w) for the intravenous delivery of drug, in order to increase the solubility of the drug[39,138,139] to reduce drug toxicity,[25,26,126] to reduce hypersensitivity,[72] and to improve drug solubility and reduce pain upon injection.[109] A very recent development is the formulation of microemulsions as long circulating vehicles, and more recently, as drug tageting agents. In addition, water-in-oil microemulsions have been investigated as depot vehicles for the intramuscular delivery of drugs.[22,64]

The first published study which established the potential of microemulsions for use in intravenous delivery was probably that of von Corswant *et al.* in Ref. 39. These researchers prepared a pharmaceutically acceptable, bicontinuous microemulsion from a medium-chain triglyceride oil, poly(ethylene

glycol) 400 and ethanol cosolvents and stabilized by soybean phosphatidylcholine and poly(ethylene glycol)(660)-12-hydroxystearate. Prior to administration, the microemulsion required dilution with a suitable aqueous phase. Upon dilution, the microemulsion formed an oil-in-water microemulsion with droplets of size between 60 and 200 nm, smaller than the size of the droplets in a commercial intravenous emulsion, namely Intralipid. From their animal studies, the authors concluded that the microemulsion they developed was suitable for adminstion by intravenous infusion to conscious rats. Unfortunately, although the researchers did determine drug solubility in the bicontinuous microemulsions, they did not report this.

Park and Kim[138] also investigated the formulation of poorly water-soluble flurbiprofen at ~8 times its aqueous solubility into an oil-in-water microemulsion suitable for intravenous administration. The microemulsions were prepared using varying weight ratios of oil (ethyl oleate) to surfactant (Tween 20), and contained a range of isotonic solutions as the polar (aqueous) phase. Unfortunately, insufficient information was supplied regarding the precise compositions of the microemulsions, in particular, how much oil and surfactant were present, so as to draw conclusions about the formulation; (perhaps surprisingly) the ratio of oil to surfactant used did not seem to have any effect on the amount of drug solubilized and that the presence of too much drug had a destabilizing effect on the microemulsion. Disappointingly, the pharmacokinetic parameters of flurbiprofen, after intravenous administration of flurbiprofen-loaded microemulsion to rats, were also not significantly different from those of flurbiprofen in phosphate buffered saline solution. In a later publication, Park *et al.*[138] overcame the problem of stability seen in their earlier study by replacing the surfactant Tween 20 with lecithin and distearoylphosphatidyl- ethanolamine-*N*-poly(ethyleneglycol) 2000 (DSPE-PEG) and using ethanol as a cosolvent. Due to the presence of the long chain polyoxyethylene groups on the exterior surface of the microemulsion droplets, it was perhaps unsurprising that the biodistribution of flurbiprofen administered in this microemulsion was quite different. In particular, reticuloendothelial uptake of flurbiprofen decreased, suggesting that it may ultimately be possible to target drugs incorporated in this microemulsion to different sites of the body.

As part of a series of papers, Brime *et al.*[25,26] and Moreno *et al.*[126] prepared a novel amphotericin B lecithin-based oil-in-water microemulsion, in an attempt to produce a formulation with less toxic effects than the currently available commercial formulation, Fungizone. The microemulsion which contained as oil isopropyl mystriate and a mixture of either Tween 80 or Brij 96 with lecithin as surfactant. In some instances, formulation was lyophilized in an attempt to increase its stability. The overall results of the toxicity studies were encouraging as the amphotericin B-containing microemulsions exhibited a low toxicity, suggesting a potential therapeutic application.

Zhang *et al.*[191] prepared a lecithin-based SMEDDS formulation of the drug norcantharidin. Upon dilution, the release rate of norcantharidin contained in the SMEEDS formulation was found to be dependent on the size of the disperse phase and the type of lecithin used. Interestingly, although norcantharidin was poorly soluble in the ethyl oleate and only slightly soluble in water, microemulsions containing ethyl oleate oil exhibited a significant increase in solubilization over the corresponding aqueous solution.

Clonixic acid is currently marketed in salt form because of its poor water-solubility. However, the commercial dosage form causes severe pain after intramuscular or intravenous injection. To improve the apparent aqueous solubility of clonixic acid and to reduce the pain it causes on injection, Lee *et al.* (2000) incorporated 3 mg/mL clonixic acid into oil-in-water microemulsions (size 120 nm) prepared from pre-microemulsion concentrate of castor oil, and a mixture of Tween 20 and Tween 85 surfactants (present in a weight ratio of 5:12:18). Although the microemulsion formulation significantly reduced the number of rats licking their paws as well as the total licking time, suggesting less pain induction by the microemulsion formulation; the pharmacokinetic parameters of clonixic acid after intravenous administration were not significantly different from those of the commercial formulation, lysine clonixinate. The results of the study suggested that a microemulsion formulation is an alternative vehicle for clonixic acid.

Paclilaxel (Taxol) injection is known to cause hypersensitivity reactions. Consequently, He *et al.*[72] explored whether it was possible to prepare a non-sensitizing paclitaxel microemulsion using egg phosphatidylcholine, Piyronic F68 ancl Cremophor EL as surfactants, and ethanol as cosurfactant. Note that there was no mention of the presence of a specific oil. The study showed that for an equivalent dose, the paclitaxel microemulsion did not cause any hypersensitivity reaction, whereas Taxol did. In addition, the bioavailability of the paclitaxel in the new microemulsion was significantly higher and the elimination rate slower than that achieved with Taxol. The authors suggested that the drug molecules, trapped in the oil droplets, diffused into the systemic circulation slowly. Furthermore, the small particle size of the droplets (10–50 nm) meant that the microemulsion droplets could escape from uptake and phagocytosis of RES. Infact it was previously suggested that it should be possible to modify the surface of the microemulsion droplets, with polyoxyethylene chains, to significantly improve circulation time.[57,118,190]

Kanga *et al.*[86] have recently explored the possibility of optimizing the release of paclitaxel from a SEEDS formulation using the polymer, PLGA. The SEEDS formulation, which was a mixture of drug, tetraglycol, Cremophor ELP, and Labrafil 1944 also contained PLGA of varying molecular weight. The droplet size of the microemulsions was in the range of 45–270 nm, with the systems without PLGA exhibiting the smaller size. The release rate of paclitaxel decreased in the order of

PLGA, PLGA 8 K, PLGA 33 K, and PLGA 90 Kg/mol, suggesting that the molecular weight of PLGA in microemulsion could control the release rate of paclitaxel from microemulsion.

### 4.3.1. *Long circulating microemulsions*

Long circulating microemulsions have been suggested as an alternative formulation to long circulating vesicles on the basis of their small size, thus avoiding uptake by the RES, their stability and their ability to solubilize lipophilic compounds more effectively than vesicles, and their ease of preparation.

Wang *et al.*,[83] and Junping *et al.*,[84] have determined the potential of intravenous delivery systems of emulsion/microemulsion systems based on vitamin E, cholesterol and $PEG_{2000}$-lipid. In their first study, Wang *et al.*,[183] prepared emulsions containing 1 part drug, 3 parts vitamin E, 3 parts cholesterol and 3 parts $PEG_{2000}$-DSPE with the final formulation containing 5 mg of drug in 10 mL of saline solution. Although the emulsion was reported to form spontaneously on the addition of the required amount of saline, the formulation was homogenized to produce a more uniform particle size distribution of $123.0 \pm 1.2$ nm; no information was given as to the size of the droplets prior to homogenization. The zeta potential and drug loading efficiency of the sub-micron emulsion were $-12.67 + 1.35$ mv and $96.3 + 0.3$. Although the size and loading efficiency of the formulation remained uncharged when stored at 7 to 8°C for a year, ~6.5% decomposition of the drug was observed. The plasma area under the curve (AUC) of the drug in the sub-micron emulsion

microemulsion exhibited a low acute toxicity and a high potential antitumor effect

microemulsions as cutaneous drug delivery vehicles in 2002. In the present review,

work prior to 2002 will not be dealt with in any detail. In addition, due to the large

amount of research in the area, the review is not exhaustive.

## Proteins and peptides

Recently, the transdermal route has received attention as a promising means to

the microemulsion formulation. In this study, the microemulsion performed less

ous solution of the drug. This observation is perhaps not surprising as various

enhancers were involved in the microemulsion droplets and were not available to improve drug penetration. Also, as it is likely that the drug was predominately in the continuous phase of the microemulsion, it is not surprising that the formulation behaved in a similar manner to an aqueous solution.

containing PEG-8 caprylic/capric glycerides (surfactant), polyglyceryl-6 dioleate (cosurfactant), isopropyl myristate and water was determined by Djordjevic.[49] The phase behavior of the microemulsions was determined in the absence of drug. In the

and propylene glycol were used as the hydrophilic phase. These microemulsions were able to solubilize up to 20 wt% of the eutectic mixture.

In an attempt to enhance the transdermal delivery of the poorly water soluble drug, triptolide, and to reduce the toxicity problems associated with its usage, a water-in-oil microemulsion was compared with that of solid lipid nanoparticles.[124] The microemulsion which was formulated using 40 wt% isopropyl myristate, 50 wt% Tween-80:1,2-propylene glycol (5:1, v/v) and water and contained 0.025 wt% of triptolide, gave a steady-state flux (for over 12 hours) and a permeability coefficient of triptolide of $6.4 \pm 0.7$ mg/cm$^2$ per h and $0.0256 \pm 0.002$ cm/h; a value which was approximately double that of the solid liquid nanoparticles and 7 times higher than that of triptolide solution of the same concentration. In another study, Chen *et al.*[33] also studied the incorporation of the drug, into a similar microemulsion using oleic acid as oil. Oleic acid was added because it is a known penetration enhancer, although there was no evidence of it acting as such in the present formulation. The addition, however, of 1 wt% menthol to the formulation slightly increased penetration from $1.58 \pm 0.04$ to $2.08 \pm 0.06$ μg/cm$^2$ per h ($p < 0.05$). Encouragingly, no obvious skin irritation was observed for the formulation studied, suggesting that microemulsions are promising vehicles for the transdermal delivery of triptolide.

Ross *et al.*[153] examined the transdermal penetration, across full thickness hairless mouse skin, of the insect repellant, *N,N*-diethyl-m-toluamide (DEET), contained in either a 1:1 v/v ethanol:water solution (containing 20 wt% DEET) or one of two commercially available microemulsion formulations (3M Ultrathon Insect Repellant (containing 31.6 wt% DEET; 3M, St. Paul, MN), and Sawyer Controlled Release DEET Formula (19.0%; Sawyer Products, Safety Harbor, FL). Both formulations were of interest because they were marketed as retarding the absorption of DEET due to being microemulsions. All of the DEET preparations exhibited considerable penetration, e.g., the ethanolic DEET formulation had a time to detection of approximately 30 min with steady stale at 85 min. The penetration obtained with the Sawyer was no different from that obtained from the ethanolic solution. The other microemulsion formulation (3M) demonstrated a different profile; despite being a higher concentration of DEET (30 wt% versus 20 wt%) and a comparable time to detection (40 min), the time to reach steady state was delayed, although there was still substantial absorption at steady state.

Sintov and Shapiro[168] prepared a high surfactant lidocaine microemulsion, containing as surfactant a mixture of glyceryl oleate and either PEG-40 stearate or PEG-40 hydrogenated castor oil, isopropyl myristate as oil, tetraglycol as cosurfactant, water, and up to 10 wt% of drug, although 2.5 wt% was generally used. The microstructure of the microemulsion went from oil-in-water, through bicontinuous to water-in-oil. The penetration of the drug from the various formulations showed that the surfactant mixture containing PEG-40 stearate was best, while the

water and surfactant/cosurfactant concentration was also important. Significantly, the lag time for penetration was reduced, suggesting that these microemulsions loaded with drug would provide rapid local analgesia.

Priano *et al.*[147] investigated the delivery from a water-in-oil microemulsion, of apomorphine present as ion-pair complex with octanoate to increase its lipophilicity and to diminish its dissociation. As the drug was present at a high concentration, the dispersed phase acted as a reservoir, making it possible to maintain an almost constant concentration in the continuous phase and therefore achieving pseudo-zero-order release kinetics. The composition of the microemulsion was complex, containing 18.2 wt% water, 42.1 wt% of oily phase of isopropyl miristate-decanol 1:1.5 v/v, 3.9 wt% R-apomorphine hydrochloride, 7.3 wt% Epikuron 200, 7.1 wt% benzyl alcohol, 4.6 wt% octanoic acid 3.5 wt% sodium octanoate, 5.7 wt% sodium taurocholate, 7.6 wt% 1,2-propanediol. The microemulsion was thickened by the addition of 5.9 wt% Aerosil 2000. The microemulsion was able to provide *in vitro*, through hairless mouse skin, a flux of 88 g/h per $cm^2$ for 24 hrs, with a kinetic release of pseudo-zero-order, and was chosen for *in vivo* study; all the components were biocompatible and safe. The flux gave a first approximation of the feasibility of the transdermal administration in man.

The pain and discomfort caused by the injection of local anesthetics has stimulated research into developing topical anesthetics. However, the currently available formulations, such as Ametop®gel, (4 wt% amethocaine base preparation) have a number of disadvantages, in particular a long delay of typically 40–60 min between application and anesthetic effect and the requirement for a plastic occlusive dressing. Arevalo *et al.*[13] have recently developed a decane-in-water microemulsion stabilized by lauromacrogol 300 and containing 4 wt% of amethocaine in an attempt to achieve faster drug permeation, thus reducing the time to reach optimum anesthetic effect. The amethocaine microemulsion proved to be a promising fast-acting analgesic in experimental preclinical studies.

## Mixtures of hydrophilic and hydrophobic drugs

Although microemulsions have long been suggested as suitable formulations for the co-adminstration of drugs of very varying physico-chemical properties, it is only very recently that anyone has reported doing so. Lee *et al.*[110] have developed a novel microemulsion enhancer formulation for the co-administration of hydrophilic (lidocaine HCl, diltiazem HCl) and lipophilic (lidocaine free base, estradiol) drugs. The microemulsions composed of isopropyl myristate and water, and were stabilized by the nonionic surfactant, Tween 80. Transdermal enhancers such as *n*-methyl pyrrolidone (NMP) and oleyl alcohol were incorporated into all systems without apparent disruption of the system. Unfortunately, the authors did not give the precise,

composition of the microemulsions tested; it was only mentioned that they contained a 1:1 v:v mixture of water and ethanol, isopropylmyristate as oil and Tween 80 as surfactant, and were either oil-in-water or water-in-oil. Interestingly, regardless of the physico-chemical nature of the drug, the oil-in-water microemulsions provided significantly better flux for all drugs studied ($p < 0.025$). Enhancement of drug permeability from the oil-in-water systems was 17-fold for lidocaine base, 30-fold for lidocaine HCl, 58-fold for estradiol, and 520-fold for diltiazem HCl. Significantly, the simultaneous delivery of estradiol with diltiazem hydrochloride did not affect the transport of either drug ($p > 0.5$).

### Immunization

Traditionally, vaccines have been administered by injection using needles, although the concept of topical immunization through *intact* skin has attracted much attention. Cui *et al.*[42] recently hypothesized that a fluorocarbon-based microemulsion system could be one possible way to deliver plasmid DNA across the skin. Cui *et al.*[42] screened a range of fluorosurfactants for their ability to form ethanol-in-perfluorooctyl bromide microemulsions. Note that the authors provided no evidence of a microemulsion being formed. The stability of plasmid DNA in the formulations was also examined. From the surfactant screen, the commercially available Zonyl® FSN-100, an ethoxylated nonionic fluorosurfactant, was selected for further study. Significant enhancements in luciferase expression and antibody and T-helper type-1 based immune responses, relative to those of "naked" pDNA in saline or ethanol, were observed after topical application of plasmid DNA in ethanol-in-perfluorooctyl bromide microemulsion system. From these studies, it can be concluded that fluorocarbon-based microemulsions are suitable for DNA vaccine delivery, although the mechanism(s) of the immune response induction is not known. It is possible that the transport of the molecules across the skin is via the hair-follicles, because DNA is too large and highly charged to cross intact stratum corneum.

## 4.5. *Ophthalmic*

Conventional ophthalmic dosage forms tend to be either simple solutions of water-soluble drugs or suspension or ointment formulations of water-insoluble drugs. Unfortunately, as these delivery vehicles generally result in poor levels of drug absorption across the cornea, most of the applied drug does not reach its intended site of action. However, because of the relative safety and convenience of topical application in ophthalmology, as well as the relatively low risk (compared with other routes of administration) of systemic side-effects, topical administration

of ophthalmic agents is the preferred route of delivery. Microemulsions and sub-microemulsions should offer a possible solution to the problem of poor delivery to the cornea, by sustaining the release of the drug, as well as by providing a higher penetration of drug into the deeper layers of the eye. In addition, they offer the potential of increasing the solubility of the drug in the ophthalmic delivery vehicle.[162]

Gallarate *et al.*[61] were probably the first to examine the potential of microemulsions as vehicles for ophthalmic delivery. Since then, a number of groups have successfully demonstrated the ability of microemulsions (sub-microemulsions) to prolong the ocular delivery of drug. In their study, Gallarte *et al.*[61] were able to further prolong the release of timolol by forming an ion pair with octanoic acid. Garty and Lusky[63] demonstrated that the delivery of pilocarpine from an oil-in-water microemulsion was delayed to such an extent that the instillations of the microemulsion formulation twice daily were equivalent to four times daily the applications of conventional eye drops. A similar result was reported by Muchtar *et al.*[130] who determined *in vitro* that the corneal penetration of indomethacin formulated in a sub-microemulsion was more than three times that obtained using commercially available drops. A number of researcher have investigated the potential of positively charged microemulsions to retain the delivery vehicle in the eye, thereby sustaining delivery.[23,52]

To date, a range of drugs have been formulated in a microemulsion in an attempt to sustain release including adaprolol maleate,[11,125] timolol,[61] levobunolol,[62] chloramphenicol[162] tepoxalin,[54] piroxicam,[100] delta-8-tetrahydrocannabinol,[129] pilocarpine,[21,52,63,71,133] indomethacin,[130] antibodies[20] and dietary iso-flavonoids and flavonoids.[83] In general, these studies showed that it was possible to delay the effect of drug incorporated in a microemulsion, thereby improving bioavailability. The proposed mechanism of the delayed action is that microemulsion droplets are not eliminated by the lachrymal drainage, thereby acting as drug reservoirs. The first studies conducted on man with microemulsions containing adaprolol maleate and pilocarpine, confirmed the results of the earlier studies performed mainly using rabbits.[18,178] Vandamme[178] has recently reviewed the use of microemulsions as ocular delivery system, and thus only studies since then will be considered in the present review.

Fialho and da Silva-Cunha[58] recently investigated the long term application of a microemulsion system in rabbits intended for the topical ocular administration of dexamethasone. The formulation contained 5 wt% isopropyl myristate as oil, 15 wt% Cremophor EL as surfactant, and a polar phase of water and 15 wt% propylene glycol, with dexamethasone present at a concentration of 0.1 wt%. Significantly, ocular irritation tests in rabbits suggested that the microemulsion did not provide significant alteration to eyelids, conjunctiva, cornea and iris over a Fe

3-month period. In addition, the formulation exhibited greater penetration of dexamethasone in the anterior segment of the eye and longer release of the drug when compared with a conventional preparation. The area under the curve obtained for the microemulsion system was more than two-fold that of the conventional preparation ($p < 0.05$).

Gulsen and Chauhan[68] have recently developed a disposable soft contact lens of a drug-containing microemulsion dispersed in a poly 2-hydroxyethyl methacrylate (HEMA) hydrogel, suitable for ophthalmic delivery, in an attempt to reduce drug loss and side-effects. Upon insertion into the eye, the lens will slowly release the drug into the pre lens (the film between the air and the lens) and the post-lens (the film between the cornea and the lens) tear films, thus providing a sustained delivery of drug. Assuming the size and drug loading of the microemulsions is low, the lenses should be transparent. It was found using these microemulsion-containing lenses, with and without a stabilizing silica shell, that drug could be released for a period of >8 days. By altering droplet size and loading, it is possible to tailor release.

## 4.6. *Vaginal*

In their 2001 review, D'Cruz and Uckun proposed that microemulsion gel formulations had great potential as intravaginal/rectal drug delivery vehicles for lipophilic drugs, such as microbicides, steroids, and hormones, because of their high drug solubilization capacity, increased absorption, and improved clinical potency, as long as a non toxic formulation could be prepared. In their review, D'Cruz and Uckun reported the formulation of two microemulsion-based gels using commonally available pharmaceutical excipients. Repeated intravaginal applications of formulations to rabbits and mice were found to be safe and did not cause local, systemic, or reproductive toxicity. D'Cruz and Uckun investigated the potential of the microemulsion-based gels as delivery vehicles of two lipophilic drugs, WHI-05 and WHI-07, which exhibit potent anti-HIV and contraceptive activity. As AIDS is spread largely through sexual intercourse, the development of a dual action vaginal spermicidal microbicide to curb mucosal viral transmission, as well as to provide fertility control would have a tremendous impact world wide. D'Cruz and Uckun[46–48] investigated the formulation of 2 wt% of the lipophilic drugs in a microemulsion-based gel, composed of Phospholipon 90G and Captex 300 as the oil phase, with Pluronic F68 and Cremophor EL as surfactants, and seaspan carragennan and Xantral as gelling agents. The microemulsions were gelled to obtain the necessary viscosity for the gel-microemulsion formulation. Under the conditions of their intended use, intravaginal application of the gel-microemulsions containing 2 wt% of drug in a rabbit model resulted in marked contraceptive activity, as well as exhibiting a lack of toxicity. Therefore, as a result of its dual anti-HIV and

spermicidal activities, the drug-containing gels shows unique clinical potential as a vaginal prophylactic contraceptive for women who are at a high risk of acquiring HIV by heterosexual transmission.

## 4.7. *Nasal*

Nasal route has been demonstrated as being a possible alternative to the intravenous route for the systemic delivery of drugs. In addition to rapid absorption and avoidance of hepatic first-pass metabolism, the nasal route allows the preferential delivery of drug to the brain via the olfactory region, and is therefore a promising approach for the rapid-onset delivery of CMS medications. The solution-like feature of microemulsions could provide advantages over emulsions in terms of the sprayability, dose uniformity and formulation physical stability.

Li *et al.*[114] developed a diazepam-containing ethyl laurate-in-water microemulsion, stabilized by Tween 80 and containing propylene glycol and ethanol as cosolvents for the rapid-onset intranasal delivery of diazepam. A single isotropic region, which was considered to be a bicontinuous microemulsion, was seen at high surfactant concentrations but at various Tween 80: propylene glycol: ethanol ratios. Increasing Tween 80 concentration increased the microemulsion area, microemulsion viscosity, and the amount of water and oil solubilized. In contrast, increasing ethanol concentration produced the opposite effect. A microemulsion consisting of 15 wt% ethyl laurate, 15 wt% water and 70 wt% Tween 80:propylene glycol:ethanol at a 1:1:1 weight ratio contained 41 mg/mL of the poorly-water soluble diazepam. The nasal absorption of diazepam from the formulation was fairly rapid with a maximum drug plasma concentration being obtained within 2 to 3 min, while bioavailability at 2 hrs post-administration was ~50% of that obtained with intravenous injection.

Zhang *et al.*[192] attempted to prepare an oil-in-water microemulsion, containing a high concentration of nimodipine, suitable for brain uptake via the intranasal route of delivery. Three microemulsion systems stabilized by either Cremophor RH 40 or Labrasol, and containing a variety of oils, namely isopropyl myristate, Labrafil M 1944CS and Maisine 35-1, were developed and characterized. The nasal absorption of the drug from the three microemulsions was studied in rats. The formulation composed of 8 wt% Labrafil M 1944CS, 30 wt% Cremophor RH 40/ethanol (3:1 weight ratio) and water solubilized up to 6.4 mg/mL of drug and exhibited no ciliotoxicity. After intranasal administration, the peak plasma concentration was obtained of 1 hr, while the absolute bioavailability was ~32%. Significantly, uptake of the drug in the olfactory bulb after nasal administration was three times that which was obtained from intravenous injection. In addition, the ratios of the AUC in brain tissues and cerebrospinal fluid to that in plasma obtained after nasal administration

were significantly higher than those seen after administration. In conclusion, the microemulsion system appears to be a promising approach for the intranasal delivery of nimodipine.

Richter and Keipert[51] investigated the *in vitro* permeability of the highly lipophilic material, androstenedione, across excised bovine nasal mucosa, porcine cornea and an artificial cellulose membrane. In order to control release, the two microemulsion formulations studied contained either hydroxypropyl-$\beta$-cyclodextrin or propylene glycol. Both microemulsions were prepared from 5 wt% isopropyl myristate, 20 wt% Cremophor EL and water. The permeation of the drug through the three tissues was influenced by the microemulsion. For example, the apparent permeability coefficients ($P_{app}$) of the drug from the microemulsions across nasal mucosa did not differ from the $P_{app}$ of the drug contained in solution. In the case of the other two membranes, release from both of the microemulsion formulations exhibited extended time lags, so no $P_{app}$ could be calculated. It seems that the composition of the microemulsion had a greater impact on the $P_{app}$ of cornea than on the $P_{app}$ of the other tissues. The structure of the different membranes is probably responsible for the observed differences in permeation.

## 4.8. *Pulmonary*

Emulsions and (to a far lesser extent) microemulsions have been investigated as vehicles for pulmonary delivery. By far, the most widely studied systems are those containing fluorocarbon oil and are stabilized by a (predominately) fluorinated surfactant. Fluorocarbon oils are of pharmaceutical interest because of their biological inertness and their high (and unique) ability to dissolve gas, which means they can support the exchange of the respiratory gases in the lungs. In addition, a fluorocarbon oil, namely perfluorooctylbromide, is in Phase II:III clinical trials in the United States, for the treatment of acute respiratory distress by liquid ventilation. It should be noted that en-large hydrocarbon surfactants are ineffective solubilizers in fluorocarbon-based systems. Instead, fluorocarbon surfactants are required. To date, fluorocarbon-based (micro)emulsions have been investigated for use as oil-in-water systems for *in vivo* oxygen delivery (blood substitutes), targeted systems for diagnosis and therapy, and water-in-fluorocarbon systems for pulmonary drug delivery.[40,102] Water-in-perfluorooctylbromide microemulsions have been shown to deliver homogeneous and reproducible doses of a tracer (caffeine) using metered-dose inhalers (pMDI) pressurized with hydrofluoroalkanes (HFAs).[27]

Lecithin-based reverse microemulsions have also been investigated as a means of pulmonary drug delivery.[170,171] In these studies, dimethylethyleneglycol (DMEG) and hexane were used as models for the propellants, dimethyl ether (DME) and

propane respectively. A combination of equilibrium analysis and component diffusion rate determination (by pulsed-field gradient [PFG]-NMR) and iodine solubilization experiments were used to confirm the formation of a microemulsion. Water soluble solutes, including selected peptides and fluorescently labeled poly-$\alpha,\beta$-[N-(2-hydroxyethyl) D,L-aspartamide] were dissolved in the microemulsions in a lecithin- and water-dependent manner. Experiments with DME/lecithin demonstrated microemulsion characteristics similar to those in the model propellant and produced a droplet size and a fine particle fraction suitable for pulmonary drug delivery.

Patel *et al.*[140] have prepared water-in-hydrofluorocarbon (specifically 134a) microemulsions using a combination of fluorinated polyoxyethylene ether surfactants and a short chain hydrocarbon alcohol such as ethanol. In the absence of a hydrocarbon alcohol, only cosolvent systems, but not microemulsions, were formed. Due to the high molecular weight of the fluorocarbon surfactant, large concentrations of fluorocarbon surfactant are required to solubilize relatively small amounts of water compared with comparable hydrocarbon-based surfactants. This has obvious implications for the pharmaceutical application of such systems.

To date, very little on the potential of oil-in-water microemulsions for pulmonary drug delivery has been investigated, yet they are attractive vehicles because of their ability to solubilize high amounts of drug.[157]

### 4.8.1. *Antibacterials*

Al-Adham *et al.*[6] demonstrated that microemulsion formulations have a significant antimicrobial action against planktonic populations of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* (i.e. greater than a 6 log cycle loss in viability over a period as short as 60 s). Transmission electron microscopy studies indicated that this activity may in part be due to significant losses in outer membrane structural integrity. Nevertheless, these results have implications for the potential use of microemulsions as antimicrobial agents against this normally intransigent microorganism.

More recently, the same group[6] have determined the antibiofilm activity of an oil-in-water microemulsion, prepared from 15 wt% Tween 80, 6 wt% pentanol and 3 wt% ethyl oleate, by incubating the microemulsion with an established biofilm culture of *Ps. aeruginosa* PA01 for a period of 4 hrs. The planktonic MIC of sodium pyrithione and the planktonic and biofilm MICs of cetrimide were used as positive controls and a biofilm was exposed to a volume of normal sterile saline as a treatment (negative) control. The results showed that exposure to the microemulsion resulted in a three log-cycle reduction in biofilm viability, as compared to a one long-cycle reduction in viability observed with the positive

control treatments, suggesting that microemulsions are highly effective antibiofilm agents.

## 5. Conclusion

As can be seen, microemulsions are attractive drug delivery vehicles that offer much scope for improving drug delivery. Although microemulsions have been seriously studied as a delivery vehicle in the last >20 years, there are few microemulsion products currently on the market. Comparing microemulsions with vesicular drug delivery systems, it is pertinent to note that it took >25 years before vesicles were commercially exploited as drug delivery vehicles, and this was with the immense research effort expended in their study. Microemulsions have by contrast been much less widely studied. It is only a matter of time before more microemulsion-based formulations appear on the market.

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# 8

# Lipoproteins as Pharmaceutical Carriers

*Suwen Liu, Shining Wang and D. Robert Lu*

## 1. Introduction

Large protein structures (in nanometer range) may be utilized as pharmaceutical carriers of drugs and DNA for targeted and other specialized delivery in biological systems. Lipoproteins are such structures which function as natural biological carriers and transport various types of lipids in blood circulation. There are many studies suggesting that lipoproteins can serve as efficient carriers for anticancer drugs, gene or other type of compounds.[1–4] Previous results showed that hydrophobic cytotoxic drugs could be incorporated into lipoproteins, without changing the integrity of native lipoprotein structure. Lipoproteins as drug carriers offer several advantages.[5–6] Firstly, they are endogenous components and do not trigger immunological response. They have a relatively long half-life in the circulation. Secondly, they have small particle size in the nanometer range, allowing the diffusion from vascular to extravascular compartments. Thirdly, lipoproteins can potentially serve as the carriers for targeted drug delivery through specific cellular receptors. For example, low density lipoprotein (LDL)-drug complexes may target cancer cells which, in many cases, have higher LDL-receptor expression than normal cells. Fourthly, the lipid core of lipoprotein provides a suitable compartment for carrying hydrophobic drugs.

As a result of these advantages, lipoproteins have received wide attentions in recent years in the development of drug-targeting strategies to use them as specialized delivery vehicles. This review intends to provide an overview of the development and the specialized utilization of lipoproteins for drug delivery purpose. After

briefly introducing the structure and the basic biological functions of lipoproteins, we will focus on four classes of lipoproteins, namely, chylomicron, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), as the carriers for various drug compounds. Cholesterol-rich emulsions (LDE) and artificial lipoproteins as drug carriers will also be discussed.

## 2. The Structure of Lipoproteins

Lipoproteins, as implied by their names, are biological protein-lipid complexes. Lipoproteins serve the functions of carrying hydrophobic substances in blood circulation and transporting them to various biological sites through the protein-receptor interactions.[6,7] The size of lipoproteins is in the nanometer range and they have a spherical shape with complex physicochemical properties. Figure 1 illustrates the general structure of lipoprotein. The hydrophobic core contains water-insoluble substances and is surrounded by a polar shell. The polar shell consists of phospholipids, unesterified cholesterol and different types of apolipoproteins, which bind to various cellular receptors for specific biological functions. Therefore, based on their physicochemical properties, lipoproteins are nanoemulsions with targeting functions provided by the apolipoproteins. Owing to the unique structure of lipoproteins, they can serve a two-mode function of solubilizing hydrophobic substances, including triglycerides and cholesteryl esters, within the nanoemulsion core and allow themselves to float in blood circulation.

Lipoproteins can be classified into five major classes, based on their densities from gradient ultracentrifugation experiments. The lipoprotein classification includes chylomicron, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). These classes of lipoproteins have different sizes, different protein to lipid ratios and different types of apolipoproteins. In general, chylomicrons act on transporting dietary triacylglycerols and cholesterol to the adipose tissue and liver, following the absorption of dietary hydrophobic substances from the intestines. Very

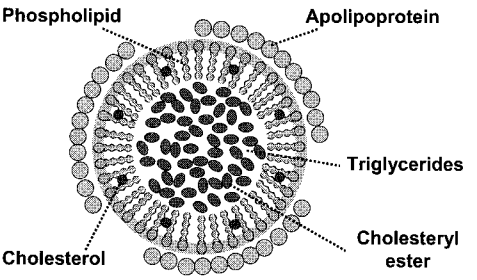


**Fig. 1.** General structure of lipoproteins.

**Table 1** Physicochemical properties of lipoproteins.

| Lipoprotein | Transport Route | Size (nm) | Protein (%) | Total lipids (%) |
|---|---|---|---|---|
| Chylomicron | Intestines to Liver | 75–1200 | 1.5–2.5 | 97–99 |
| VLDL | Liver to tissues | 30–80 | 5–10 | 90–95 |
| IDL | Liver to tissues | 25–35 | 15–20 | 80–85 |
| LDL | Liver to tissues | 18–25 | 20–25 | 75–80 |
| HDL | Tissues to liver | 5–12 | 40–55 | 45–60 |

low density lipoprotein, intermediate density lipoprotein and low density lipoprotein work at different stages to transport triacylglycerols and cholesterol from the liver to various tissues. High density lipoprotein brings endogenous cholesterol from the tissues back to the liver. The general physicochemical properties of lipoproteins can be seen in Table 1.

## 3. Chylomicron as Pharmaceutical Carrier

Chylomicrons are assembled in the intestine from the absorbed dietary lipids and transported by lymphatic system. Although most of the drugs administered orally are absorbed directly into the portal blood to reach the systemic circulation, an alternative absorption route through the intestinal lymphatics may be available for hydrophobic drugs. It is estimated that a high hydrophobicity (log o/w partition co-efficient > 5) of drug molecules is required for intestinal lymphatic transport.[8] Chylomicrons can thus potentially serve as an important natural carrier for hydrophobic drugs to transport through lymphatic system.[9] It is known that targeted drug delivery through the lymphatics is important for anti-viral drug molecules for the protection of B- and T-lymphocytes, which maintain relatively higher concentrations through the lymphatics than the systemic circulation. Chylomicrons have a much larger size than other lipoproteins, and thus can carry more drug molecules from the absorption site. With the presence of food, chylomicrons are the predominant lipoprotein produced by the small intestine to carry dietary lipids efficiently because of its large size.

Various types of bioactive molecules have been incorporated into reconstituted chylomicron structure for delivery purposes. In gene delivery, Hara *et al.*[10,11] developed reconstituted chylomicron which incorporated a hydrophobic DNA complex and used it as an *in vivo* gene transfer vector. They found that the DNA-incorporated chylomicrons induced a high gene expression in mouse liver after the reconstituted chylomicron was administered through portal vain injection. Furthermore, it was also reported that artificial, protein-free lipid emulsions could be utilized to model the metabolism of lymph chylomicron in rats, not only in the initial partial

hydrolysis by lipoprotein lipase, but also in the delivery of a remnant-like particle to the liver.[12] As a targeted therapeutic approach to hepatitis B, anti-viral iododeoxyuridine was incorporated into recombinant chylomicrons, resulting in the drug molecules being selectively targeted to the liver parenchymal cells.[13] It has been suggested that chylomicron can serve as a special carrier for liver cell targeting.[14] Due to the targetability, this approach could be further developed as an effective therapy for hepatitis B patients.

## 4. VLDL as Pharmaceutical Carrier

VLDL particles have a size range of 30–80 nm. They are assembled in the endoplasmic reticulum (ER) and matured in Golgi apparatus of hepatocytes before secretion.[15] After entering into the plasma, VLDL particles are catabolized by a series of biochemical actions, including apolipoprotein exchange of apoC-I, apoC-II, apoC-III, and apoE; lipolysis by triglyceride lipase; and cell-surface receptor-mediated uptake. As lipolysis proceeds, VLDL particles become smaller and are eventually converted to IDL. Some of the IDL particles are rapidly taken up by hepatocytes via a receptor-mediated mechanism while others undergo further hydrolysis before being converted to LDL. The catabolism route of VLDL suggests the possibility of using VLDL as a drug carrier for targeted delivery. ApoE is a protein ligand present on the surface of VLDL and it is well known that the receptor of apoE is overexpressed on some types of cancer cells. Therefore, VLDL can potentially serve as an antineoplastic drug carrier.

As a drug carrier, VLDL is an interesting candidate because it contains a relatively small amount of proteins (about 5–10% protein) and a large amount of triglycerides (about 50–65% within the emulsion core) which can be used to solubilize hydrophobic substances sufficiently. By mimicking the compositions and structure of VLDL, Shawer *et al.* developed a VLDL-resembling phospholipid nanoemulsion system that carried a new anti-tumor boron compound for targeted delivery to cancer cells.[16] The nanoemulsion demonstrated sufficient capability to solubilize the hydrophobic compound. The structure of the phospholipid nanoemulsion was verified based on the changes in the molecular surface area and the molecular volume of each component of the nanoemulsion when the particle size is changed (from different size fractions). If certain molecules are located at the core of nanoemulsion, their numbers per overall volume should not be changed when the particle size is increased. If certain molecules are located at the surface of nanoemulsion, their numbers per overall volume should decrease when particle size is increased. This is because the overall surface area decreases when particle size is increased. Similar to the natural lipoproteins, it was demonstrated that phospholipid was predominately

located at the surface and the hydrophobic substances, triolein and cholesteryl oleate, were mainly located in the core of the phospholipid nanoemulsion.

Recently, a similar nanoemulsion formulation was used to encapsulated quantum dots (QD) as a new bioimaging carrier.[17] Quantum dots (QDs) are semiconductor nanocrystals that are emerging as unique fluorescence probes in biomedicine.[18–21] When manufactured, most of the quantum dots have organic ligand coating on their surface and are extremely hydrophobic. The research goal was to encapsulate QDs in phospholipid nanoemulsion and to examine the physical stability, size distribution and their interactions with cancer cells. It was found that CdSe QDs can be efficiently encapsulated in the phospholipid nanoemulsion. The QD-encapsulated phospholipid nanoemulsion are stable and interact well with cultured cells to deliver the QDs inside the cells for fluorescence imaging.[17] In other studies, it has been demonstrated that cytotoxic drugs such as 5-fluorouracil (5-FU), 5-iododeoxyuridine (IudR), doxorubicin (Dox), and vindesine can be effectively incorporated into VLDL, and the resultant complexes showed effective cytotoxicity to human carcinoma cells.[22]

## 5. LDL as Pharmaceutical Carrier

LDL (18–25 nm) is not directly synthesized in human body. Instead, most of them are formed through the VLDL pathway. LDL is the major circulatory lipoprotein for the transport of cholesterol and cholesteryl esters, and it can be internalized by cells via LDL receptor-mediated endocytosis. The internalization process of LDL has been well characterized and the understanding of the mechanism can potentially help the designing of the drug targeting strategy through the LDL receptor (Fig. 2). The binding of dephosphorylated adaptor protein to the plasma membrane

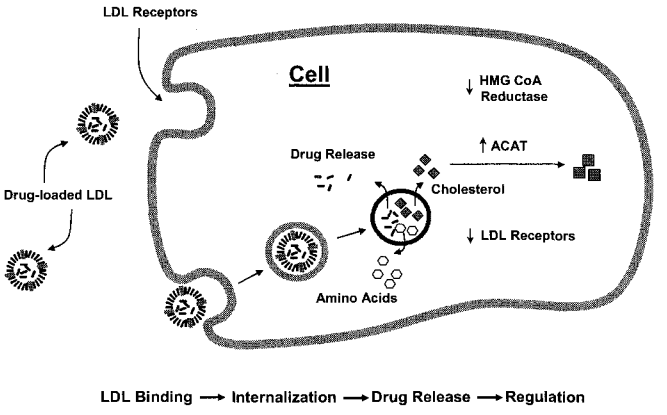


**Fig. 2.** LDL receptor pathway and targeted drug delivery.

initiates the formation of coated pits which are covered by the protein clathrin. The receptors from the surrounding regions of the plasma membrane shift towards the binding site for internalization. Apolipoproteins including apo B-100 and apo E are recognized and bound by the LDL receptor on the cell surface to form a complex which is internalized into the coated pits. After internalization of the LDL, the coated pits are pinched off and within a very short time, they shed off their clathrin coating. The internalized LDL particle is transferred to endocytotic vesicles or endosomes. Due to the acidic pH within the endosomes, LDL dissociates from its receptor. This is followed by the fusion of the endosomes with lysosomes which contain hydrolases. The protein component of LDL is broken into free amino acids, while the cholesteryl ester component is cleaved by lysosomal lipase. The free cholesterol is released and incorporated into the cell membrane. Excess cholesterol is re-esterified by the action of acyl-CoA:cholesterol acyltransferase (ACAT).

Among various lipoproteins, LDL has been widely studied as a drug carrier for targeted and other specialized deliveries, because many types of cancer cells show elevated expression of LDL receptors than the corresponding normal cells.[23–26] Comparing with chylomicron, VLDL, and IDL, LDL also has a longer serum half-life of 2–4 days,[27] making it a desirable drug carrier. Low density lipoprotein was found to be suitable as carriers for cytotoxic drugs to target cancer cells. LDL-drug complexes can be formed through various processes without changing the lipoprotein integrity.[28–31]

## 5.1. *LDL as anticancer drug carriers*

Doxorubicin (Dox) is widely used in treating different tumors. Its main side effects are cadiotoxicity and multidrug resistance, especially during prolonged treatment in the patients. LDL has been studied as a target carrier for Dox in nude mice, bearing human hepatoma HepG2 cells.[32] Both *in vitro* and *in vivo* studies indicated that when Dox was incorporated into LDL, the multidrug resistance could be circumvented and the cardiotoxicity could be reduced as well.[33] Kader and Pater[22] used VLDL, LDL and HDL as carriers to deliver four cytotoxic drugs, 5-fluorouracil (5-FU), 5-iododeoxyuridine (IUdR), doxorubicin (Dox) and vindesine. They found that significant drug loading was achieved in all three classes of lipoproteins, consistent with the sizes and hydrophobicity of the drug. Experiments were carried out to examine the changes in drug cytotoxicity against HeLa cervical and MCF-7 breast carcinoma cells, after the incorporation into lipoprotein. The results demonstrated that VLDL-drug complex did not affect their $IC_{50}$ on both HeLa and MCF-7 cell lines, when compared with free drugs. However, the $IC_{50}$ values of LDL- and HDL-drug complexes were significantly lower compared with free drugs. Their studies further indicated that drugs were incorporated into lipoproteins without disrupting

their integrity; drugs remained in their stable forms inside lipoproteins; and human LDL and HDL could be particularly useful in the delivery of antineoplastic drugs.

### 5.2. *LDL as carriers for other types of bioactive compounds*

Although LDL has been widely studied as a carrier to deliver anticancer compounds, it may also be useful to deliver other types of bioactive compounds. LDL may serve as a carrier for site-specific delivery of drugs to atherosclerotic lesions.[34] When dexamethasone palmitate (DP), a steroidal anti-inflammatory drug, was incorporated in LDL, an inhibitory effect of this complex on foam cell formations was demonstrated. The study indicated that LDL could potentially carry DP to atherosclerotic lesions.[34] Fluorophore-labeled LDL was also used for optical imaging in tumors diagnosis. For example, carbocyanine dyes can be used as near infrared (NIR) optical imaging probes with long wavelength absorption, high extinction coefficients and high fluorescence quantum yield. *In vitro* confocal microscopic study and *ex vivo* low-temperature fluorescent scanning demonstrated that carbocynine-labled LDL probes, DiI-LDL, could be selectively delivered to B16/HepG2 tumor cells and the corresponding animal tumors via the LDL receptor pathway.[35] It was also proposed that DiI is located and oriented in the phospholipid monolayer when it binds to LDL.

### 5.3. *LDL for gene delivery*

LDL has also been investigated as gene delivery carriers. Comparing with viral gene-delivery vectors and some other types of non-viral gene delivery vectors, the LDL system shows certain advantages in transfection efficiency and safety considerations.[5] Several LDL based gene delivery systems have been reported. Kim's group developed a terplex system which comprises LDL, lipidized poly(L-lysine) and plasmid DNA. The complex had a diameter of about 100 nm. The studies showed high efficiency to deliver plasmid DNA to smooth muscle cells and fibroblast cells.[36,37] In addition, a novel LDL-DNA complex was formulated by Khan *et al.* [38] LDL was cationized using carbodiimide and the modified lipoprotein complex significantly increased the DNA binding capacity with improved stability. The novel delivery system also demonstrated the ability to target cells through LDL receptor.[38]

## 6. HDL as Pharmaceutical Carriers

Among various lipoproteins, HDL has the smallest size with a diameter of 5–12 nm. It shares common structural characteristics with other lipoproteins. However, its

polar shell contributes more than 80% of the total mass. Newly synthesized HDL hardly contains any cholesteryl ester molecules. Cholesteryl esters are gradually added to the particles by lecithin via enzymatic reaction: cholesterol acyltransferase (LCAT), which is a 59-kD glycoprotein associated with HDL. The interaction of HDL with cells appears similar to that of LDL.[39] Although the function of HDL in the human body is not well-defined, it generally transports excess cholesterol and cholesteryl esters from various tissue cells back to the liver. Comparing with other types of lipoproteins, small size and fast internalization by tumor cells are the major advantages of utilizing HDL for drug delivery and targeting.

HDL has mainly been utilized for the delivery of water insoluble anticancer drugs through the targeting function.[40–41] When the anticancer drug, Taxol, was incorporated into HDL, stable complexes were formed and they were examined for cancer-cell targeting.[41] Reconstituted HDL was explored as a drug carrier system for a lipophilic prodrug, IDU-$Ol_2$.[42] The studies indicated that the lipophilic prodrug could be efficiently incorporated into reconstituted HDL particles. This approach may also be useful to encapsulate other lipophilic derivatives of water-soluble drugs. The utilization of HDL for drug targeting may lead to a more effective therapy for infectious diseases, such as hepatitis B, since the HDL-drug complexes were demonstrated to be selectively taken by parenchymal liver cells.[42] Comparing with free drugs in cytotoxicity assays, the IC values of HDL-drug complexes were significantly decreased, about 2.5 to 23-fold lower.[22] Interestingly, it was observed that HDL-drug complex specifically increased the cytotoxicity to carcinoma cells. Earlier studies showed that HDL could increase the sensitivity of HeLa cells to the cytotoxic effects of Dox.[43] Similar to LDL-drug complex, the lipoprotein receptor pathway appears to be involved in the interactions between HDL-drug complex and cancer cells.

## 7. Cholesterol-rich Emulsions (LDE) as Pharmaceutical Carriers

LDE is a lipid based formulation, an emulsion with a lipid structure resembling LDL particle and it is made without protein incorporation. Essentially, it is composed of a cholesteryl ester core surrounded by a monolayer of phospholipids. Comparing with native LDL, LDE is removed from the blood circulation more rapidly.[44] It appears possible that LDE can acquire apoE and other apolipoproteins from native lipoproteins in plasma. ApoE can be recognized by LDL receptors, thus allowing the binding of LDE to the receptors. However, it is known that LDE binds to receptors through apoE, but not through apoB100. The interaction between apoE and the receptor appears stronger than that of apoB100.[45]

LDE is considered as a potential carrier for anticancer drugs to deliver chemotherapeutic agents to neoplastic cells. Although there is no protein in the LDE formulations, previous studies showed that the LDL receptor could still play an important role in the cellular uptake of these lipid complexes.[46–56] LDE binds to low-density lipoprotein receptors which are upregulated in cancer cells, leading to a higher concentration in neoplastic tissues.[24,57] LDE-carmustine complex was studied with a neoplastic cell line and its biodistribution was studied in mice. An exploratory clinical study was also conducted. The result showed that the uptake of LDE-carmustine complex by tumor was several fold greater than the uptake by the corresponding normal tissue. The association of carmustine with LDE preserves the tumor-cytotoxicity of carmustine with reduced side effects.[58] Preliminary clinical study[59] was also carried out using LDE-carmustine complex to treat patients with advanced cancers. The results demonstrated that the systemic toxicity of the drug was significantly reduced.

Rodrigues *et al.* investigated the formulation of LDE containing antineoplastic compound paclitaxel.[55] The experiments revealed a 75% incorporation efficiency and the stable complex of the drug molecules incorporated in LDE emulsion. Its $LD_{50}$ was ten-fold greater than that of a commercial formulation of paclitaxel. It was suggested by the authors that the cellular uptake and the cytotoxic activity of LDE-paclitaxel complex might be mediated by the LDL receptors due to the cholesterol moiety in the LDE formulation.[55]

In addition to LDE, artificial lipoproteins have been constructed. Several research groups have developed various types of artificial lipoproteins.[44,60–62] Most of them constructed the artificial lipoproteins by incorporating natural apoB protein into lipid microemulsion for the purpose of examining the lipoprotein metabolism. Artificial lipoproteins containing poly-lysine has also been investigated as the DNA carrier for cellular transfection, with the potential to reduce the cytotoxicity and to improve the transfection efficiency.[63,64]

## 8. Concluding Remark

Lipoproteins are natural nanostructures in biological systems. They have unique physicochemical properties which may be utilized as pharmaceutical carriers for drug compounds and other bioactive substances. Owing to the structural diversity of lipoproteins, including chylomicron, VLDL, LDL and HDL, various specialized delivery systems may be developed to fully utilize their delivery potentials. New nanostructures, such as LDE and artificial lipoproteins, can also be constructed to mimic the structure of natural lipoproteins. As these new nanostructures are built from scratch, they may be more efficient in encapsulating drug and other bioactive molecules, and more effective for specialize drug delivery.

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# 9

# Solid Lipid Nanoparticles as Drug Carriers

*Karsten Mäder*

## 1. Introduction: History and Concept of SLN

Nanosized drug delivery systems have been developed to overcome one or several of the following problems: (i) low and highly variable drug concentrations after peroral administration due to poor absorption, rapid metabolism and elimination (ii) poor drug solubility which excludes i.v. injection of an aqueous drug solution (iii) drug distribution to other tissues combined with high toxicity (e.g. cancer drugs). Several systems, including micelles, liposomes, polymer nanoparticles, nanoemulsions and nanocapsules have been developed. During the last few years, solid lipid nanodispersions (SLN) have attracted increased attention. It is the aim of this chapter to discuss the general features of these systems with respect to manufacturing and performance.

In the past, solid lipids have been mainly used for rectal and dermal applications. In the beginning of the 80s, Speiser and coworkers developed solid lipid microparticles (by spray drying)[1] and "Nanopellets for peroral administration".[2] These Nanopellets were produced by dispersion of melted lipids with high speed mixers or ultrasound. The manufacturing process was unable to reduce all particles to the submicron size. A considerable amount of microparticles was present in the samples. This might not be a serious problem for peroral administration, but it excludes an intravenous injection. "Lipospheres", described by Domb, are

close related systems.[3–5] They are also produced by means of high shear mixing or ultrasound and also often contain considerable amounts of microparticles.

The quality of the SLN has been significant improved by the use of high pressure homogenization (HPH) in the early 90s.[6–8] Higher shear forces and a better distribution of the energy force more effective particle disruption, compared with high shear mixing and ultrasound. Dispersions obtained by this HPH are called Solid Lipid Nanoparticles (SLN$^{TM}$). Most SLN dispersions produced by high pressure homogenization (HPH) are characterized by an average particle size below 500 nm and a low microparticle content. Other production procedures are based on the use of organic solvents HPH/solvent evaporation[9] or on dilution of microemulsions.[10,11] The ease and efficacy of manufacturing lead to an increased interest in SLN.

Furthermore, it has been claimed that SLN combine the advantages yet without inheriting the disadvantages of other colloidal carriers.[12,13] Proposed advantages include:

- Possibility of controlled drug release and drug targeting
- Increased drug stability
- High drug pay load
- Feasibility to incorporate lipophilic and hydrophilic drugs
- No biotoxicity of the carrier
- Avoidance of organic solvents
- No problems with respect to large scale production and sterilization.

However, during the last years, some of these claims have been questioned and it became evident that SLN are rather complex systems which possess not only advantages but also serious limitations.

## 2. Solid Lipid Nanoparticles (SLN) Ingredients and Production

### 2.1. *General ingredients*

General ingredients include solid lipid(s), emulsifier(s) and water. The term lipid is used generally in a very broad sense and includes triglycerides (e.g. tristearine, hard fat), partial glycerides (e.g. Imwitor), pegylated lipids, fatty acids (stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetylpalmitate). All classes of emulsifiers (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion. The most frequently used compounds include different kinds of poloxamer, polysorbates, lecithin and bile acids. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently.

Unfortunately, poor attention has been given by most investigators to the physicochemical properties of the lipid. Fatty acids, partial glycerides and other polar lipids are able to interact with water to much a greater extent, compared with a long chain triglyceride (e.g. they might form liquid crystalline phases). Polar lipids will have much more interaction with stabilizers (e.g. formation of mixed micelles), while more lipophilic lipids will show phase segregation. The author strongly suggests to follow the proposal by Small and to classify lipids according to their interactions with water.[14]

## 2.2. *SLN preparation*

### 2.2.1. *High shear homogenization and ultrasound*

High shear homogenization and ultrasound are dispersion techniques which were initially used for the production of solid lipid nanodispersions.[1–3] Both methods are widespread and easy to handle. However, dispersion quality is often poor due to the presence of microparticles. Furthermore, metal contamination has to be considered if ultrasound is used.

Ahlin *et al.* used a rotor-stator homogenizer to produce SLN from different lipids, including trimyristin, tripalmitin, tristearin, partial glycerides (Witepsol®W35, Witepsol®H35) and glycerol tribehenate (Compritol®888) by melt-emulsification.[15] They investigated the influence of different process parameters, including emulsification time, stirring rate and cooling conditions on the particle size and the zeta potential. Poloxamer 188 was used as steric stabilizer (0, 5%w/w). For Witepsol®W35 dispersions, the following parameters were found to produce the best SLN quality: stirring 8 min at 20 000 rpm, the optimum cooling conditions 10 min at 5000 rpm at room temperature. In contrary, the best conditions for Dynasan®116 dispersions were 10 min emulsification at 25 000 rpm and 5 min of cooling at 5000 rpm in cool water (T = 16°C). An increased stirring rate did not significantly decrease the particle size, but improved the polydispersity index slightly. No general rule can be derived from differences in the established optimum emulsification and cooling conditions. In most cases, average particle sizes in the range of 100–200 nm were obtained in this study.

## 2.3. *High pressure homogenization (HPH)*

HPH has emerged as a very reliable and probably the most powerful technique for the preparation of SLN. HPH has been used for many years for the production of nanoemulsions for parenteral nutrition. In most cases, scaling up represents zero or limited problems. High pressure homogenizers push a liquid with high pressure (100–2000 bar) through a narrow gap (in the range of few microns). The

fluid accelerates on a very short distance to very high velocities. The high shear stress disrupts the particles down to the submicron range. Typical lipid contents are in the range of 5 to 10%. Even higher lipid concentrations (up to 40%) have been homogenized to lipid nanodispersions.[16]

Two general approaches of the homogenization step, the hot and the cold homogenization techniques, can be used for the production of SLN.[17,18] In both cases, a preparatory step involves the drug incorporation into the bulk lipid by dissolving the drug in the lipid melt.

## 2.4. *Hot homogenization*

The hot homogenization is carried out at temperatures above the melting point of lipid. Therefore, it is in fact the homogenization of an emulsion. A preemulsion of the drug loaded lipid melt and the aqueous emulsifier phase (same temperature) is obtained by high-shear mixing device (Ultraturrax). The quality of the preemulsion is very important for the final product quality. In general, higher temperatures result in lower particle sizes due to the decrease of the viscosity of the inner phase.[19] However, high temperatures may also increase the degradation rate of the drug and the carrier. The homogenization step can be repeated several times. It should be kept in mind however, that HPH increases the temperature of the sample (approximately 10°C for 500 bar[20]). In most cases, 3 to 5 homogenization cycles at 500 to 1500 bar are sufficient. Increasing the homogenization pressure or the number of cycles often results in an increase of the particle size due to particle coalescence, which occurs as a result of the high kinetic energy of the particles.[21]

It is important to note that the primary product of the hot homogenization is a nanoemulsion due to the liquid state of the lipid. Solid particles are expected to be formed by the following cooling of the sample to room temperature, or to temperatures below. Due to the small particle size and the presence of emulsifiers, lipid crystallization may be highly retarded and the sample may remain as a supercooled melt for several months.[22]

## 2.5. *Cold homogenization*

Cold homogenization has been developed to overcome the following three problems of the hot homogenization technique:

(1) Temperature induced drug degradation
(2) Drug distribution into the aqueous phase during homogenization
(3) Complexity of the crystallization step of the nanoemulsion, leading to several modifications and/or supercooled melts

The first preparatory step for cold homogenization is the same as in the hot homogenization procedure and includes the solubilization of the drug in the melt of the

bulk lipid. However, the following steps differ. The drug containing melt is rapidly cooled. The high cooling rate favors a homogenous distribution of the drug within the lipid matrix. The solid, drug containing lipid is milled to microparticles. Typical particle sizes obtained by means of ball or mortar milling are in the range of 50 to 100 microns. Low temperatures increase the fragility of the lipid, and therefore favor particle disruption. The solid lipid microparticles are suspended in a chilled emulsifier solution. The preemulsion is subjected to HPH at or below room temperature. An effective temperature control and regulation is needed in order to ensure the unmolten state of the lipid due to the increase in temperature during homogenization.[20] In general, compared with hot homogenization, larger particle sizes and a broader size distribution are observed in cold homogenized samples.[23] A modified version of this technique has been recently published by the group of Müller-Goymann. They dispersed a solid 1:1 lecithin-hardfat mixture (described as solid reversed micelles) in Tween containing water using high pressure homogenization.[24]

### 2.5.1. *SLN prepared by solvent emulsification/evaporation*

The solvent emulsification/evaporation processes adapts techniques which have been previously used for the production of polymeric micro- and nanoparticles. The solid lipid is dissolved in a water-immiscible organic solvent (e.g. cyclohexane, or chloroform) that is emulsified in an aqueous phase. Upon evaporation of the solvent, a nanoparticle dispersion is formed by precipitation of the lipid in the aqueous medium. Westesen prepared nanoparticles of tripalmitate by dissolving the triglyceride in chloroform.[25] This solution was emulsified into an aqueous phase by high pressure homogenization. The organic solvent was removed from the emulsion by evaporation under reduced pressure. The mean particle size ranges from approximately 30 to 100 nm depending on the lecithin/co-surfactant blend. Particles with very small diameters (30 nm) were obtained by using bile salts as co-surfactants. Comparable small particle size distributions were not achievable by melt emulsification of similar composition. The mean particle size depends on the concentration of the lipid in the organic phase. Very small particles could only be obtained with low fat loads (5 w%) related to the organic solvent. With increasing lipid content, the efficacy of the homogenization declines due to the higher viscosity of the dispersed phase.

### 2.5.2. *SLN preparations by solvent injection*

The solvent injection method has been developed by Fessi to produce polymer nanoparticles.[26] Nanoparticles were only produced with solvents which distribute very rapidly into the aqueous phase (e.g. ethanol, acetone, DMSO), while larger

particle sizes were obtained with more lipophilic solvents. According to Fessi, the particle size is critically determined by the velocity of the distribution processes and only water miscible solvents can be used. The solvent injection method can also be used for the production of solid lipid nanoparticles.[27, 28] However, the method is limited to lipids which dissolve in the polar organic solvent. Advantages of the method are the avoidance of elevated temperatures and high shear stress. However, the lipid concentration in the primary suspension will be less compared with High-Pressure-Homogenization. Furthermore, the use of organic solvents clearly represents a drawback of the method.

### 2.5.3. *SLN preparations by dilution of microemulsions or liquid crystalline phases*

SLN preparation techniques which are based on the dilution of microemulsions have been developed by Gasco and coworkers. Unfortunately, there is no common agreement within the scientific community about the definition of a microemulsion. One part of the scientific community understands under microemulsions high fluctuating systems which can be regarded as a critical solution, and therefore do not contain an inner and outer phase. This model has been confirmed by self-diffusion NMR studies of Lindman.[29] In contrast, Gasco and other scientists understand microemulsions as two systems composed of an inner and outer phase (e.g. O/W-microemulsions). They are made by stirring an optical transparent mixture at 65–70°C, typically composed of a low melting lipid fatty acid (e.g. stearic acid), emulsifier (e.g. polysorbate 20, polysorbate 60, soy phosphatidylcholin, taurodeoxycholic acid sodium salt), co-emulsifiers (e.g. Butanol, Na-monooctylphosphate), and water. The hot microemulsion is dispersed in cold water (2–3°C) under stirring. Typical volume ratios of the hot microemulsion to the cold water are in the range of 1:25 to 1:50. The dilution process is critically determined by the composition of the microemulsion. According to the literature, the droplet structure is already contained in the microemulsion, and therefore, no energy is required to achieve submicron particle sizes.[30,31] The temperature gradient and the pH-value determine the product quality in addition to the composition of the microemulsion. High temperature gradients facilitate rapid lipid crystallization and prevent aggregation.[32,33] Due to the dilution step, lipid contents which are achievable are considerably lower, compared with the HPH based formulations. Another disadvantage includes the use of organic solvents.

Recent work describes a similar approach to produce SLN. A hot liquid crystalline phase (instead of a microemulsion) is diluted in cold water to yield a solid lipid nanodispersion.[34] This approach avoids the use of high pressure homogenization and organic solvents, and therefore represents an interesting opportunity.

## 2.6. *Further processing*

### 2.6.1. *Sterilization*

Sterility is required for parenteral formulations. Dry or wet heat, filtration, $\gamma$-irradiation, chemical sterilization and aseptic production are general, opportunities to achieve sterility. The sterilization should not change the properties of the sample with respect to physical and chemical stability and the drug release kinetics.

Sterilization by heat is a reliable procedure which is most commonly used. It was also applied for Liposomes.[35,36] Steam sterilization will cause the formation of an oil in water emulsion, due to the melting of the lipid particles. The formation of SLN requires recrystallization of the lipids. Concerns are related to temperature induced changes of the physical and chemical stability. The correct choice of the emulsifier is of significant importance for the physical stability of the sample at high temperatures. Increased temperatures will affect the mobility and the hydrophilicity of all emulsifiers, but to a different extent. Schwarz found that Lecithin is preferable to Poloxamer for steam sterilization, as only a minor increase in the particle size and the number of microparticles was observed after steam sterilization.[37,38] An increase in particle size for Poloxamer 188 stabilized Compritol-SLN was observed after steam sterilization. It was found that a decrease of the sterilization temperature from 121°C to 110°C can reduce sterilization induced particle aggregation to a large extent. This destabilization can be attributed to the decreased steric destabilization of the Poloxamer. It is well known for PEG-based emulsifiers that increased temperatures lead to dehydration of the ethylenoxide chains, pointing to a decrease of the thickness of the protecting layer. It has been demonstrated by 1H-NMR spectroscopy on Poloxamer stabilized lipid nanoparticles, that even a moderate temperature increase from RT to 37°C decreases the mobility of the ethylenoxide chains on the particle surface.[39] Results of Freitas *et al.* indicate that the lowering of the lipid content (to 2%), and the surface modification of the glass vials and nitrogen purging might prevent the particle growth to a large extent and avoid gelation.[40] Further studies of Cavalli *et al.*[41] and Heiati [42] demonstrate the possibility of steam sterilization of drug loaded SLN.

Filtration sterilization of dispersed systems requires very high pressure and is not applicable to particles >0, 2 $\mu$m. As most SLN particles are close to this size, filtration is of no practical use, due to the clocking of the filters. Few studies investigated the possibility of $\gamma$-sterilization. It must be kept in mind that free radicals are formed during $\gamma$-sterilization in all samples, due to the high energy of the $\gamma$-rays. These radicals may recombine with no modification of the sample or undergo secondary reactions which might lead to chemical modifications of the sample. The degree of sample degradation depends on the general chemical reactivity and the molecular mobility and the presence of oxygen. It is therefore not surprising

that chemical changes of the lipid bilayer components of liposomes were observed after $\gamma$-irradiation.[43] Schwarz investigated the impact of different sterilization techniques [steam sterilization at 121°C (15 min) and 110°C (15 min); $\gamma$-sterilization] on SLN characteristics.[37,38] In comparison to lecithin stabilized systems, Poloxamer stabilized SLN were less stable than steam sterilization. However, this difference was not detected for $\gamma$-sterilized samples. Compared with steam sterilization at 121°C, the increase in particle size after $\gamma$-irradiation was lower, but comparable to that at 110°C.

Unfortunately, most investigators did not search for steam sterilization or irradiation induced chemical degradation. It should be kept in mind that degradation does not always cause increased particle sizes. In contrast, the formation of species like lysophosphatides or free fatty acids could even preserve small particle sizes, but might cause toxicological problems. Further studies with more focus on chemical degradation products are clearly necessary to permit valid statements of the possibilities of SLN sterilization.

### 2.6.2. *Drying by lyophilization, nitrogen purging and spray drying*

SLN are thermodynamic unstable systems, and therefore, particle growth has to be minimized. Furthermore, SLN ingredients and incorporated drugs are often unstable, hydrolyzing or oxidizing. The transformation of the aqueous SLN-suspension in a dry, redispersible powder is therefore often a necessary step to ensure storage stability of the samples. Lyophilization is widely used and is a promising way to increase chemical and physical SLN stability over extended periods of time. Lyophilization also offers principle possibilities for SLN incorporation into pellets, tablets or capsules.

Two additional transformations are necessary which might be the source of additional stability problems. The first transformation, from aqueous dispersion to powder, involves the freezing of the sample and the evaporation of water under vacuum. Freezing of the sample might cause stability problems due to the freezing out effect which results in the changes of the osmolarity and the pH. The second transformation, resolubilization, involves situations at least in its initial stages which favor particle aggregation (i.e. low water and high particle content, high osmotic pressure).

The protective effect of the surfactant can be compromised by lyophilization.[44] It has been found that the lipid content of the SLN dispersion should not exceed 5%, so as to prevent an increase in the particle size. Direct contact of lipid particles are decreased in diluted samples. Furthermore, diluted SLN dispersions will also have higher sublimation velocities and a higher specific surface area.[45] The addition of cryoprotectors (e.g. Sorbitol, Mannose, Trehalose, Glucose, and

Polyvinylpyrrolidon) will be necessary to decrease SLN aggregation and to obtain a better redispersion of the dry product. Schwarz *et al.* investigated the lyophilization of SLN in detail.[46] Best results were obtained with the cryoprotectors, Glucose, Mannose, Maltose and Trehalose, in the concentration range between 10% and 15%. The observations come into line with the results of the studies on liposome lyophilization, which indicated that Trehalose was the most sufficient substance to prevent liposome fusion and the leakage of the incorporated drug.[47] Encouraging results obtained with unloaded SLN cannot predict the quality of drug loaded lyophilizates. Even low concentrations of 1% Tetracain or Etomidat caused a significant increase in particle size, excluding an intravenous administration.[46]

Westesen investigated the lyophilization of tripalmitate-SLN using glucose, sucrose, maltose and trehalose as cryoprotective agents.[48] Handshaking of redispersed samples was an insufficient method, but bath sonification produced better results. Average particle sizes of all lyophilized samples with cryoprotective agents were 1.5 to 2.4 times higher than the original dispersions. One year storage caused increased particle sizes of 4 to 6.5 times compared with the original dispersion. In contrast to the lyophilizates, the aqueous dispersions of tyloxapol/phospholid stabilized tripalmitate SLN exhibited remarkable storage stability. The instability of the SLN lyophilizates can be explained by the sintering of the particles. TEM pictures of tripalmitate SLN show an anisometrical, platelet-like shape of the particles. Lyophilization changes the properties of the surfactant layer due to the removal of water, and increases the particle concentration which favors particle aggregation. Increased particle sizes after lyophilization (2.1 to 4.9 times) were also reported by Cavalli.[41] Heiati compared the influence of four cryoprotectors (i.e. trehalose, glucose, lactose and mannitol) on the particle size of azidothymidine palmitate loaded SLN lyophilizates.[42] In agreement to other reports, Trehalose was found to be the most effective cryoprotectant. The freezing procedure will affect the crystal structure and the properties of the lyophilizate. Literature data suggest that the freezing process needs to be optimized to a particular sample size. Schwarz recommended rapid freezing in liquid nitrogen.[46] In contrast, other researchers observed the best results after a slow freezing process.[49] Again, best results were obtained with samples of low lipid content and with the cryoprotector trehalose. Slow freezing in a deep freeze (−70°C) was superior to rapid cooling in liquid nitrogen. Furthermore, introduction of an additional thermal treatment of the frozen SLN dispersion (2 hr at −22°C; followed by 2 hr temperature decrease to −40°C) was found to improve the quality of the lyophilizate. Lately, lyophilization has been used to stabilize retinoic acid loaded SLN.[50]

An interesting alternative to lyophilization has been recently suggested by Gasco's group. Drying with a nitrogen stream at low temperatures of 3 to 10°C has been found to be superior.[51] Compared with lyophilization, the advantages of

this process are the avoidance of freezing and the energy efficiency resulting from the higher vapor pressure of water.

Spray drying has been scarcely for SLN drying, although it is cheaper compared with lyophilization. Freitas obtained a redispersable powder with this method, which meets the general requirements of i.v.-injections, with regard to the particle size and the selection of the ingredients.[52] Spray drying might potentially cause particle aggregation due to high temperatures, shear forces and partial melting of the particles. Freitas recommends the use of lipids with high melting points >70°C to avoid sticking and aggregation problems. Furthermore, the addition of carbohydrates and low lipid contents favor the preservation of the colloidal particle size in spray drying.

## 3. SLN Structure and Characterization

The characterization of SLN is a necessity and a great challenge. Lipid characterization itself is not trivial as the statement by Laggner shows[53]: "Lipids and fats, as soft condensed material in general, are very complex systems, which not only in their static structures but also with respect to their kinetics of supramolecular formation, Hysteresis phenomena or supercooling can gravely complicate the task of defining the underlying structures and boundaries in a phase diagram". This is especially true for lipids in the colloidal size range. Therefore, possible artifacts caused by sample preparation (removal of emulsifier from particle surface by dilution, induction of crystallization processes, changes of lipid modifications) should be kept in mind. For example, the contact of the SLN dispersion with new surfaces (e.g. a syringe needle) might induce lipid crystallization or modification, and sometimes result in the spontaneous transformation of the low viscous SLN-dispersion into a viscous gel. The most important parameters of SLN include particle size and shape, the kind of lipid modification and the degree of crystallization, and the surface charge.

Photon correlation spectroscopy (PCS) and Laser Diffraction (LD) are the most powerful techniques for routine measurements of particle size. It should be kept in mind that both methods are not "measuring" particle sizes. Rather, they detect light scattering effects which are used to calculate particle sizes. For example, uncertainties may result from nonspherical particle shapes. Platelet structures commonly occur during lipid crystallization[54] and are very often described in the SLN literature.[55–59] The influence of the particle shape on the measured size is discussed by Sjöström.[55] Further difficulties arise both in PCS and LD measurements for samples which contain several populations of different size. Therefore, additional techniques might be useful. For example, light microscopy is recommended although it is not sensitive to the nanometer size range. It gives a fast indication about the

presence and the character of microparticles. Electron Microscopy provides, in contrast to PCS and LD, direct information on the particle shape.[57,58] Atomic force microscopy (AFM) has attracted increasing attention. A cautionary note applies to the use of AFM in the field of nanoparticles, as an immobilization of the SLN by solvent removal is required to assess their shape by the AFM tip. This procedure is likely to cause substantial changes of the molecular structure of the particles. Zur Mühlen demonstrated the ability of AFM to image the morphological structure of SLN.[60] The sizes of the visualized particles are of the same magnitude, compared with the results of PCS measurements. The AFM investigations revealed the disk-like structure of the particles. Dingler investigated cetylpalmitate SLN (stabilized by polyglycerol methylglucose distearate, Tego Care 450) by electron microscopy and AFM and found an almost spherical form of the particles.[61] The usefulness of cross flow Field-Flow-Fractionation (FFF) for the characterization of colloidal lipid nanodispersions has been recently demonstrated.[58] Lipid nanodispersions with constant lipid content, but different ratios of liquid and solid lipids did show similar particle sizes in dynamic light scattering. However, retention times in FFF were remarkably dissimilar due to the different particle shapes (i.e. spheres vs. platelets). Anisotropic particles such as platelets will be constrained by the cross flow much more heavily compared with the spheres of similar size. The very high anisometry of the SLN particles has been confirmed by electron microscopy, where very thin particles of 15 nm thickness and the length of several hundred nanometers became visible.

The measurement of the zeta potential allows predictions about the storage stability of colloidal dispersions.[62] In general, particle aggregation is less likely to occur for charged particles (i.e. high zeta potential) due to electric repulsion. However, this rule cannot strictly apply to systems which contain steric stabilizers, because the adsorption of steric stabilizer will decrease the zeta potential due to the shift in the shear plane of the particle.

Particle size analysis is just one aspect of SLN quality. The same attention has to be paid on the characterization of lipid crystallinity and modification, because these parameters are strongly correlated with drug incorporation and release rates. Thermodynamic stability and lipid packing density increase, and drug incorporation rates decrease in the following order:

$$\text{supercooled melt} < \alpha\text{-modification} < ß'\text{-modification} < ß\text{-modification}$$

In general, it has been found that melting and crystallization processes of nanoscaled material can differ considerable from that of the bulk material.[63] The thermodynamic properties of material having small nanometer dimensions can be considerably different, compared with the material in bulk form (e.g. the reduction

of melting point). This occurs because of the tremendous influence of the surface energy.

This statement is also valid for SLN, where lipid crystallization and modification changes might be highly retarded,[64] due to the small size of the particles and the presence of emulsifiers. Moreover, crystallization might not occur at all and has been shown that samples which were previously described as SLN (solid lipid particles) were in fact supercooled melts (liquid lipid droplets).[65] The impact of the emulsifier on SLN lipid crystallization has been shown by Bunjes.[66] The same group demonstrated also a size dependent melting of SLN.[67]

Differential Scanning Calorimetry (DSC) and X-ray scattering are most commonly applied to asses the status of the lipid. DSC uses the fact that different lipid modifications possess different melting points and melting enthalpies. By means of X-ray scattering, it is possible to assess the length of the long and short spacings of the lipid lattice. It is highly recommended to measure the SLN dispersion themselves, because solvent removal will lead to modification changes. Sensitivity problems and long measurement times of convential X-ray sources might be overcome by synchrotron irradiation.[64] In addition, this method permits to conduct time resolved experiments and allows the detection of intermediate states of colloidal systems which will be non detectable by convential X-ray methods.[53] Recent work shows that SLN might form superstructures by parallel alignment of SLN platelets. These reversible particle self-assemblies were observed by Illing *et al.* in tripalmitin dispersions when the lipid concentration exceeds 40 mg/g. Higher lipid concentrations did enhance particle self-assembly. The tendency to form self-assemblies has been found to depend on the particle shape, the lipid and the surfactant concentration.[68] Infrared and Raman Spectroscopy are useful tools to investigate structural properties of lipids and they might give complentary information to X-ray and DSC.[54] Raman measurements on SLN show that the arrangement of lipid chains of SLN dispersions changes with storage.[69]

Rheometry might be particularly useful for the characterization of the viscoelastic properties of SLN dispersions. The rheological properties are important with respect to the dermatological use of SLN, but they also provide useful information about the structural features of SLN dispersions and their storage dependency. Studies of Lippacher show that the SLN dispersion posses higher elastic properties than emulsions of comparable lipid content.[70–72] Furthermore, a sharp increase of the elastic module is observed at a certain lipid content. This point indicates the transformation from a low viscous lipid dispersion to an elastic system, with a continuous network of lipid nanocrystals. Illing and Unruh did compare the rheological properties of trimyristic, tripalmitic and tristearic SLN suspensions. The results indicate that the viscosity of triglyceride suspensions increases with the lipid chain length and an increased anisotropy of the particles.[73] Souto *et al.* used

rheology to study the influence of SLN addition on the rheological properties of hydrogels.[74]

The co-existence of additional colloidal structures (micelles, liposomes, mixed micelles, nanodispersed liquid crystalline phases, supercooled melts, drug-nanoparticles) has to be taken into account for all SLN dispersions. Unfortunately, many investigators neglect this aspect, although the total amount of surface active compounds is often comparable to the total amount of the lipid. The characterization and quantification are serious challenges due to the similarities in size. In addition, the sample preparation will modify the equilibrium of the complex colloidal system. Dilution of the original SLN dispersion with water might cause the removal of surfactant molecules from the particle surface and induce further changes such as crystallization or the changes of the lipid modifications. It is therefore highly desirable to use methods which are sensitive to the simultaneous detection of different colloidal species, which do not require preparatory steps such as Raman, NMR and ESR spectroscopy.

NMR active nuclei of interest are $^{1}H$, $^{13}C$, $^{19}F$ and $^{35}P$. Due to the different chemical shifts, it is possible to attribute the NMR signals to particular molecules or their segments. For example, lipid methyl protons give signals at 0.9 ppm, while protons of the polyethylenglycole chains give signals at 3.7 ppm. Simple $^{1}H$-spectroscopy permits an easy and rapid detection of supercooled melts, due to the low linewidths of the lipid protons[69,75–77]. This method is based on the different proton relaxation times in the liquid and semisolid/solid state. Protons in the liquid state give sharp signals with high signal amplitudes, while semisolid/solid protons give very broad or invisible NMR signals under these circumstances. NMR has been used to characterize calixarene SLN[78] and hybrid lipid particles (NLC), which are composed of liquid and solid lipids.[59] Protons from solid lipids are not detected by standard NMR, but they can be visualized by solid state NMR. A drawback of solid state NMR is the rapid spinning of the sample that might cause artifacts. A recent paper describes the use of this method to monitor the distribution of Q10 in lipid matrices.[79] Unfortunately, the authors did use "drying of the sample to constant weight" as a preparatory step, which will cause significant changes of the sample characteristics.

ESR requires the addition of paramagnetic spin probes to investigate SLN dispersions. A large variety of spin probes is commercially available. The corresponding ESR spectra give information about the microviscosity and micropolarity. ESR permits the direct, repeatable and non-invasive characterization of the distribution of the spin probe between the aqueous and the lipid phase.[80] Experimental results demonstrate that storage induced crystallization of SLN leads to an expulsion of the probe out of the lipid into the aqueous phase.[81] Furthermore, using an ascorbic acid reduction assay, it is possible to monitor the time scale of the exchange between

the aqueous and the lipid phase.[59] The transfer rates of molecules between SLN and liposomes or cells have been determined by ESR.[82]

## 4. The "Frozen Emulsion Model" and Alternative SLN Models

Lipid nanoemulsions are composed of a liquid oily core and a surfactant layer (lecithin). They are widely used for the parenteral delivery of poorly soluble drugs.[83–85] The original idea of SLN was to achieve a controlled release of incorporated drugs by increasing the viscosity of the lipid matrix. Therefore it is not surprising that in original model, SLN is being described as "frozen emulsions" (see Fig. 1, left and middle).[86,87] However, lipids are known to crystallize very frequently in anisotropic platelet shapes[54] and anisotropic. Sjöström *et al.* described in 1995 that the particle shape of Cholesterylacetate SLN did strongly depend on the emulsifier.[55] Platelet shaped particles have been detected for lecithin stabilized particles, while PEG-20-sorbitanmonolaurate stabilized particles preserved their spherical shape. Anisotropic particles have been found in numerous other SLN dispersions.[56–59] Based on the experimental results, a platelet shaped SLN model can be proposed as an alternative (see Fig. 1, right).

In the year 2000, Westesen questioned the frozen emulsion droplet model with the following statement[88]:

"Careful physicochemical characterization has demonstrated that these lipid-based nanosuspensions (solid lipid nanoparticles) are not just emulsions with solidified droplets.

During the development process of these systems, interesting phenomena have been observed, such as gel formation on solidification and upon storage, unexpected dynamics of polymorphic transitions, extensive annealing of nanocrystals over significant periods of time, stepwise melting of particle fractions in the

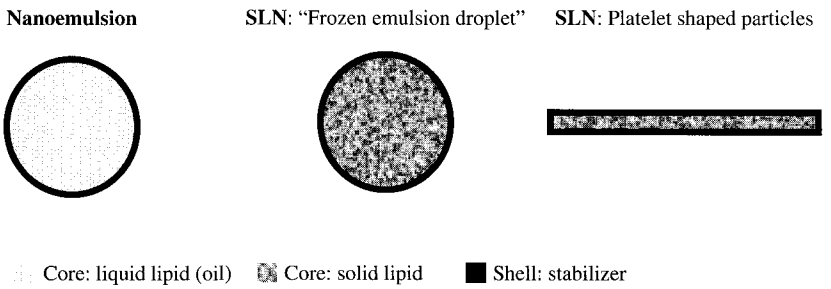


**Fig. 1.** General structure of a nanoemulsion (left), and proposed models for SLN: Frozen emulsion droplet model (middle) and platelet shaped SLN model (right).

lower-nanometer-size range, drug expulsion from the carrier particles on crystallization and upon storage, and extensive supercooling."

Her comment highlights the complex behavior and changes of SLN dispersions. In addition, the presence of competing colloidal structures (e.g. micelles, liposomes, mixed micelles, nanodispersed liquid crystalline phases, supercooled melts and drug-nanoparticles) should be considered. Additional colloids might have an impact on very different aspects, including the correct measurement of particle size, drug incorporation and toxicity. A recent study shows that the cell toxicity of the SLN dispersion was reduced by dialysis due to the removal of water soluble components.[89]

## 5. Nanostructured Lipid Carriers (NLC)

Nanostructured lipid carriers (NLC) have been recently proposed as a new SLN generation with improved characteristics.[90] The general idea behind the system is to improve the poor drug loading capacity of SLN by "mixing solid lipids with spatially incompatible lipids leading to special structures of the lipid matrix",[91] while still preserving controlled release features of the particles. Three different types of NLC have been proposed (NLC I: The imperfect structured type, NLC II: The structureless type and NLC III: The multiple type). Unfortunately, these structural proposals have not been supported by experimental data. They assume a spherical shape and they are not compatible with lipid platelet structures.

For example, NLC III structures should contain small oily droplets in a solid lipid sphere (Fig. 2, left). Detailed analytical examination of NLC systems by Jores *et al.* demonstrate that "nanospoon" structures are formed, in which the liquid oil adheres on the solid surface of a lipid platelet (Fig. 2, right).

Jores *et al.* did conclude that "Neither SLN nor NLC lipid nanoparticles showed any advantage with respect to incorporation rate or retarded accessibility to the drug, compared with conventional nanoemulsions. The experimental data concludes that NLCs are not spherical solid lipid particles with embedded liquid

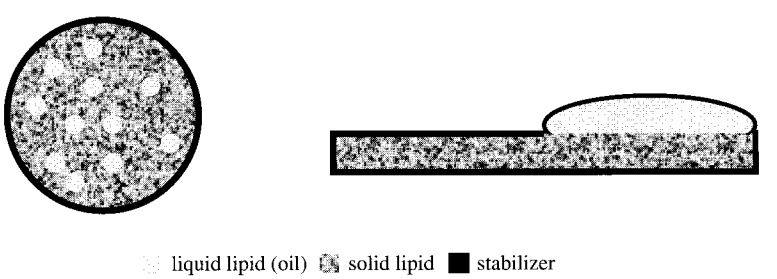


**Fig. 2.** Proposed NLC III structure (modified after[91]) and experimental determined "nanospoon" structure described by Jores *et al.* (side view of particle).[58,59]

droplets, but rather, they are solid platelets with oil present between the solid platelet and the surfactant layer". Very similar structures have been found on Q10 loaded SLN by Bunjes *et al.*[92]

## 6. Drug Localization and Release

Proposed advantages of SLN, compared with nanoemulsions, include increased protection capacity against drug degradation and controlled release possibilities due to the solid lipid matrix. The general low capacity of crystalline structures to accommodate foreign molecules is a strong argument against the proposed rewards. It is therefore necessary to distinguish between drug association and drug incorporation. Drug association means that the drug is associated with the lipid, but it might be localized in the surfactant layer or between the solid lipid and the surfactant layer (similar to the oil in Fig. 2, right). Drug incorporation would mean the distribution of the drug within the lipid matrix. Another limiting aspect comes from the fact that the platelet structure of SLN, which is found in many systems, leads to a tremendous increase in surface area and the shortening of the diffusion lengths. Furthermore, additional colloid structures present in the sample are alternatives for drug localization the SLN for drug incorporation as it was pointed out by Westesen[88]: "The estimation of drug distribution is difficult for dispersions consisting of more than one type of colloidal particle. Depending on the type of stabilizer and on the concentration ratio of stabilizer to matrix material significant numbers of particles such as liposomes and/or (mixed) micelles may coexist with the expected type of particles".

The detailed investigation of drug localization is very difficult and only a few studies exist. Parelectric spectroscopy has been used to investigate the localization of glucocorticoids. The results indicate that the drug molecules are attached to the particle surface, but not incorporated into the lipid matrix. With Betamethasonvalerate, the loading capacity of the particle surface was clearly below the usual concentration of 0.1%.[93] Lukowski used Energy Dispersive X-ray Analysis and found that the drug Triamcinolone, Dexamethasone and Chloramphenicol are partially stored at the surface of the individual nanoparticles.[94]

The importance of the emulsifier is reflected in a study from Danish scientists.[95] They produced gamma-cyhalothrin (GCH) loaded lipid micro- and nanoparticles. GCH had only limited solubility in the solid lipid and was expulsed during storage. The appearance of GCH crystals was strongly dependent from the solubility of the GCH in the emulsifier solutions. Emulsifier with high GCH solubility provoked rapid crystal growth. This observation is in accordance with a mechanism of crystal growth according to Ostwald ripening. Slovenian scientist found that ascorbylpalmitate was more resistant against oxidation in non-hydrogenated soybean

lecithin liposomes, compared with SLN.[96] It shows that liposomes might have a higher protection capacity compared with SLN.

Fluorescence and ESR studies have been used by Jores *et al.* to monitor the microenvironment and the mobility of model drugs. The results indicate that even highly lipophilic compounds are pushed into a polar environment during lipid crystallization. Therefore, the incorporation capacity of SLN is very poor for most molecules.[69] A nitroxide reduction assay gave results in accordance with the results of the distribution. Compared with nanoemulsions, nitroxides were more accessible in SLN and NLC to ascorbic acid, localized in the aqueous environment. Therefore, nanoemulsions were more protective than SLN and NLC systems.

Drug release from SLN and NLC could be either controlled by the diffusion of the drug or the erosion of the matrix. The original idea was to achieve a controlled release of SLN due to the slowing down of drug diffusion to the particle surface. This idea is, however, questionable due to drug expulsion during lipid crystallization. In addition, very short diffusion lengths in nanoscaled delivery systems lead to short diffusion times, even in highly viscous or solid matrices. In most cases, the delivery of the drug will be controlled by the slow dissolution rate in the aqueous environment. Drug release rate will be highly dependent on the presence of further solubilizing colloids (e.g. micelles), which are able to work as a shuttle for the drug and the presence or absence of a suitable acceptor compartment. Many investigators studied only the release in buffer media. A controlled release pattern under such conditions is not surprising, as it is caused by low solubilization kinetics due to the poor solubility of the drug. *In vivo*, acceptor compartments will be present (e.g. lipoproteins, membranes) and will speed up release processes significantly. Whenever possible, drug loaded SLN should be compared with nanosuspensions to separate the general features of the drug and the influence of the lipid matrix.

Results by Kristl *et al.* indicate that lipophilic nitroxides diffuse between SLN and liposomes. The diffusion kinetics was strongly dependent on the nitroxide structure. In contrast, uptake of nitroxides in cells was similar between lipophilic nitroxides, suggesting endocytosis as the main mechanism.[82] The detailed mechanisms of drug release *in vivo* are poorly understood. *In vitro* data by Olbrich demonstrate that SLN are degraded by lipases.[97,98] Degradation by lipase depends on the lipid and strongly on the surfactant. Steric stabilization (e.g. by poloxamer) of SLN and NLC are less accessible because lipase needs an interface for activation. It is also known that highly crystalline lipids are poorly degraded by lipase.

## 7. Administration Routes and *In Vivo* Data

SLN and NLC can be administrated at different routes, including peroral, dermal, intravenously and pulmonal. Peroral administration of SLN could enhance the drug

absorption and modify the absorption kinetics. Despite the fact that in most of the SLN, the drug will be associated but not incorporated in the lipid, SLN might have advantages due to enhanced lymphatic uptake, enhanced bioadhesion or increased drug solubilization by SLN lipolysis products such as fatty acids and monoglycerides. A serious challenge represents the preservation of the colloidal particle in the stomach, where low pH values and high ionic strengths favor agglomeration and particle growth. Zimmermann and Müller studied the stability of different SLN formulations in artificial gastric juice.[99] The main findings of this study are that (i) some SLN dispersions preserve their particle size under acidic conditions, and (ii) there is no general lipid and surfactant which are superior to others. The particular interactions between lipid and stabilizer are determining the robustness of the formulation. Therefore, the suitable combination of ingredients has to be determined on a case by case basis.

Several animal studies show increased absorption of poorly soluble drugs. The efficacy of orally administrated Triptolide free drug and Triptolide loaded SLN have compared in the carrageenan-induced rat paw edema by Mei *et al.*[100] Their results suggest that SLN can enhance the anti inflammatory activity of triptolide and decrease triptolide-induced hepatotoxicity. The usefulness of SLN to increase the absorption of the poorly soluble drug all-trans retinoic acid has been shown by Hu *et al.* on rats.[101] Gascos group investigated the uptake and distribution of Tobramycin loaded SLN in rats.[102,103] They observed an increased uptake into the lymph, which causes prolonged drug residence times in the body of the animals. Furthermore, AUC and clearance rates did depend on the drug load. The same group described also enhanced absorption of Idarubicin-loaded solid lipid nanoparticles (IDA-SLN), in comparison to the drug solution. Furthermore, the authors described that SLN were able to pass the blood-brain barrier and concluded that duodenal administration of IDA-SLN modifies the pharmacokinetics and tissue distribution of idarubicin.[104]

Parenteral administration of SLN is of great interest too. To avoid the rapid uptake of the SLN by the RES system, stealth SLN particles have been developed by the adoption of the stealth concept from liposomes and polymer nanoparticles. Reports indicate that Doxorubicin loaded stealth SLN circulate for long period of time in the blood and change the tissue distribution.[105] Therefore, SLN could be alternatives to marketed stealth-liposomes, which can decrease the heart toxicity of this drug due to changed biodistribution. Long circulation times have also been observed for Poloxamer stabilized SLN with Paclitaxel.[106]

The dermal application is of particular interest and it might become the main application of SLN.[107] SLN pose occlusive properties which are related to the solid structure of the lipid.[108] Human *in vivo* results of the group of Müller demonstrate that SLN can improve skin hydration and viscoelasticity.[109] SLN have also

UV protection capacity due to their reflection of UV light.[110] Furthermore, data by Schäfer-Korting suggest SLN can be used to decrease drug side effects due to SLN mediated drug targeting to particular skin layers.[111]

Further reports describe additional applications of SLN as well as gene delivery,[112] delivery to the eye,[113] pulmonary delivery,[114] and drug targeting of anticancer drugs.[115] Studies of the different groups also propose the use of SLN for brain targeting to deliver MRI contrast agents[116] or antitumour drugs.[117,118]

## 8. Summary and Outlook

SLN and NLC are now investigated by many scientists worldwide. In contradiction to early proposals, they certainly do not combine all the advantages of the other colloidal drug carriers and avoid the disadvantages of them. SLN are complex colloidal dispersions, not just "frozen emulsions". SLN dispersions are very susceptible to the sample history and storage conditions. Disadvantages of SLN include gel formation on solidification and upon storage, unexpected dynamics of polymorphic transitions, extensive annealing of nanocrystals over significant periods of time, stepwise melting of particle fractions in the lower-nanometer-size range, drug expulsion from the carrier particles on crystallization and upon storage, and extensive supercooling. The anisotropic shape of many SLN dispersions increases the surface area significantly, decreases the diffusion lengths to the surface and changes the rheological behavior dramatically (e.g. gel formation). Furthermore, the presence of alternative colloidal structures (micelles, liposomes) has to be considered to contribute to drug localization. In most cases, the drug will be associated with the lipid and not incorporated. Studies demonstrate that SLN and NLC might have no advantages compared with submicron emulsions, in regard to protection from the aqueous environment.

On the other side, animal data suggest that SLN can change the pharmacokinetics and the toxicity of drugs. In many cases, drug incorporation might not be required and drug association with the lipid can be sufficient for lymphatic uptake. Clearly, more detailed studies are necessary to get a deeper understanding of the *in vivo* fate of these carriers. Whenever possible, SLN and NLC systems should be compared directly with alternative colloidal carriers (e.g. liposomes, nanoemulsions, nanosuspensions) to evaluate their true potential.

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# 10

# Lipidic Core Nanocapsules as New Drug Delivery Systems

*Patrick Saulnier and Jean-Pierre Benoît*

A new generation of controlled size Lipidic core NanoCapsules (LNC) is presented with respect to their simple formulation, interfacial characteristics, pharmacokinetic and biodistribution properties. We describe their ability to load and release hydrophobic drugs.

## 1. Introduction

The ultimate goal of therapeutics is to deliver any drug at the right time in a safe and reproducible manner to a specific target at the required level. A great deal of effort is currently made to develop novel drug delivery systems that are able to fulfil these specifications. Among them, nanoscale drug carriers appear to be promising candidates. Colloidal carriers are particularly useful because they can provide protection of a drug from degradation in biological fluids and promote its penetration into cells. However, because the body is so well equipped to reject any intruding object, for the materials to stand any chance of success within this hostile yet sensitive environment, they must be chosen very carefully. In particular, attention has to be turned to the composition of the surface of colloidal drug carriers.[1] Indeed, their clearance rate from the circulatory system is determined by their uptake by the mononuclear phagocytic system (MPS), which in turn depends on their physico chemical surface characteristics. In order to enhance circulation time, steric protection of various nanoparticulate drug carriers can be achieved by the presence of hydrophilic and flexible polymers to their surface. In the search

for injectable, biocompatible and long-circulating systems, many colloidal systems have been evaluated.

Different kinds of vectors can be used. For example, molecular vectors where the drug is complexed or associated to a transport molecule are currently used. Many vectors are also constituted by viruses or hybrid viruses, following the modification of their genomes in order to avoid the possibility of replication. In this way, they are used as gene delivery systems. However, we will focus on non viral vectors in this chapter. They are always formulated using soft physico chemical methods, by taking advantage of macromolecular self-assembly properties at the colloidal state in order to produce well-controlled particles. The number of required biological and physico chemical properties of these systems is high in order to formulate operant vectors. One of the most important specifications of these systems is the biocompatibility and biodegradability of each component that needs to be chosen carefully from a restricted list of molecules. Secondly, they need to be well constructed in terms of size and interfacial properties, in order to constitute stealthy systems that will not be phagocyted by the MPS and consequently will have the longest residence time in blood.

We should not forget that such vectors exist biologically. Low density lipoproteins (LDL) are interesting systems possessing many of the required specifications. Unfortunately, their extraction, purification or reconstitution is still a challenge with strong physico chemical problems to solve. No convenient common solvent of proteins and lipids exists in order to reconstitute a similar supra-molecular framework. Consequently, we have to keep in mind a formulation of nanoparticles with biomimetic properties to those related to LDL as close as possible.

We would now like to describe a novel class of nanoparticles (Lipidic core NanoCapsules:LNC) formulated without organic solvents with biocompatible and biodegradable molecules.[2] We will see that after modification of the composition, we can control their size without difficulty in the 10–200 nm range, with a monomodal and narrow size distribution.

Initially, we suggest describing the LNC formulation following some particular auto-organizational properties of Poly Ethylene Glycol (PEG)-like surfactants, induced by several emulsion-phase inversions in which they are incorporated. We will particularly emphasize the different physical methods that determine the characterization of the final structure of LNC, as well as their stability in suspensions. Then, we will describe strong correlations between their stealthy properties in blood and structural characteristics, mainly size and interfacial properties. In specific, we have evaluated the activation of the complement system in an original *in vitro* model. These nanocapsules are devoted to the encapsulation of drugs that need to be dispersed in their oily core. As a proof that the concept works, we will describe the ability of LNC to encapsulate and release simple lipophilic molecules, ibuprofene and amiodarone, in the last paragraph.

## 2. Lipidic Nanocapsule Formulation and Structure

### 2.1. *Process*

The first step of the process consists of the formulation of a stable emulsion characterized by its oily phase (O), aqueous phase (W) and finally its surfactants mixture (S).

Due to the complexity of the mixture, the brand names will be used throughout the following text. It is important to note that no organic solvent or medium-chain alcohols are used in the formulation. All these molecules are known to be biocompatible and biodegradable. This indicates that the lack of residual toxicity can guarantee the safe use of LNC for human administration. Solutol® is mainly comprised of 12-hydroxystearate of PEG 660 that corresponds to a hydrophilic surfactant (HLB = 11). The lecithin used is a mixture of hydrophobic phospholipids. The main compounds of each phase are reported in Table 1.

The beginning of the formulation (see Fig. 1) corresponds to a magnetic stirring of all the components for which the proportions will be defined later, with a gradual rise in temperature from room temperature to 80°C at a rate of 4°C/min, leading to an W/O emulsion characterized by low conductivity. The system is

**Table 1** Compounds used in the LNC formulation.

| | |
|---|---|
| S | • Solutol® HS-15: 12-hydroxysterarate of PEG 660 and PEG 660 (low content)<br>• Lipoïd®: lecithin |
| O | • Labrafac®: triglycerides (C8-C10) |
| W | • Purified water<br>• NaCl |

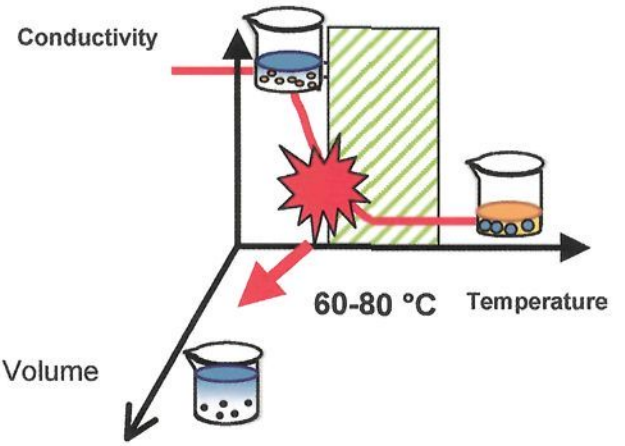


**Fig. 1.** Emulsion-phase inversion induced by temperature changes and the principle of LNC formulation.

cooled from 80 to 55°C (4°C/min), leading to an O/W emulsion characterized by its high conductivity. Between these two kinds of emulsion, a transition zone called the Phase Inversion Zone (PIZ) is defined where the system is known to be in bicontinuous states.[2,3]

In order to provide appropriate and optimal interfacial properties to the water-oil interfaces, the formulation typically requires three temperature cycles across the PIZ. The system is stopped at a temperature corresponding to the beginning of the PIZ, just before performing a final, fast-cooling dilution process in cold water (2°C). This second step of the formulation leads to LNC in suspension in an aqueous phase.

The interfacial rheology method developed in several papers demonstrates that the interfacial association of all the implicated molecules of the process is different from other commoner systems.[4] Cohesion energy at the interface, as well as the interaction of the interfacial molecules with the adjacent phases, reaches a minimum for the concentrations used. We think that this particularity can explain why the system can be broken down in an ideal way during final dilution. The surfactants involved in the stabilization of the bicontinuous systems can easily leave the microemulsion in order to constitute the colloidal structures (LNC).

It might be noted that temperatures corresponding to the PIZ are much too high to decline this method to the simple encapsulation of thermo-sensitive molecules. Fortunately, we have shown that the electrolyte concentration (NaCl) strongly influences the location of PIZ on the temperature scale. When we increase the electrolyte concentration, we decrease the PIZ temperature to reach acceptable levels.

## 2.2. *Influence of the medium composition*

Obviously, the presence or not of LNC strongly depends on the composition of the system reported in Fig. 2(a) as a pseudo-ternary diagram.[5] Each point corresponds to strictly similar formulation processes and the entire diagram describes the appropriate feasibility zone.

It should be noticed that the optimal formulation corresponds to w/w concentration of around 20% for the oil phase, 60% for the water phase and 20% for Solutol®. In the zone corresponding to the LNC formulation, a statistical model is applied in order to approximate the influence of the composition on the size distribution measured by the dynamic light scattering method.

Polynomial interpolations between well-controlled points are performed. The corresponding results are reported in Fig. 2(b) where different iso-size curves are presented. The same procedure was applied to the size variation coefficients. These two curve beams are powerful tools, allowing an optimized formulation to be found, once a given and reproducible size distribution is elaborated just by tuning the composition.

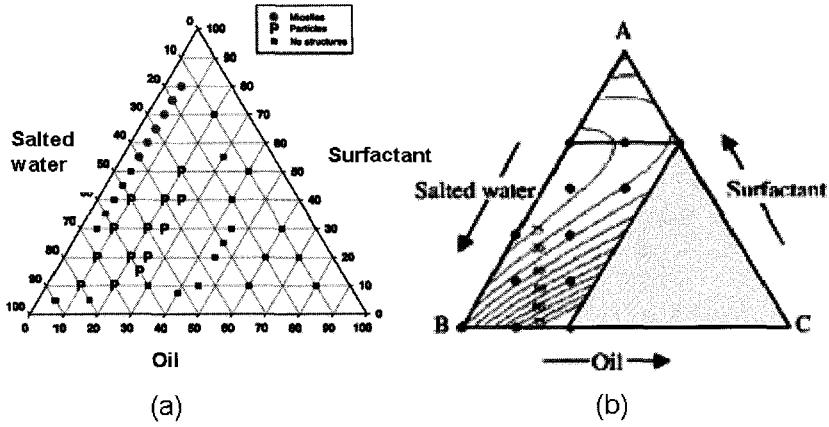


**Fig. 2.** Feasibility diagram of LNC. a: zone of favorable formulation; b: iso-size curves in the favorable zone.

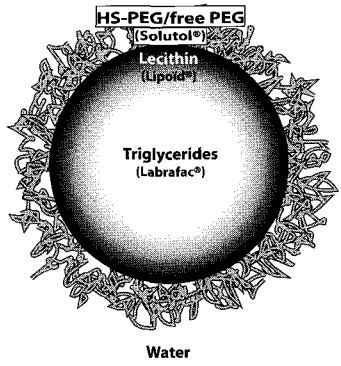


**Fig. 3.** Schematic representation of LNC.

It is important to note that LNC have demonstrated very good freeze-drying and stability characteristics in storage conditions for several months, as determined by DSC measurements,[6] confirming the structure presented in Fig. 3.

LNCs are constituted of a lipidic core surrounded by a surfactant shell, where lecithin is located in the inner part of the shell and the Solutol® in the outer part.

## 2.3. *Structure and purification of the LNC by dialysis*

Considering that in the biological environment of the blood stream, the particles interact strongly with various interfaces, one possible model for studying the interfacial behavior of these particles is their spreading at the air-water interface. Classically, the Langmuir balance was used to describe interfaces composed by simple

mixtures. The basic technique was the measurement of the surface pressure ($\pi$)-area (A) isotherm, by determining the decrease in surface tension as a function of the area available for each molecule on the aqueous sub phase. This included the study of the monolayer formation, the compressibility of the interface, the mutual interactions of molecules in the monolayer, but also interactions with the sub-phase molecules across interfacial rheological measurements.[7] Following this, these suspension spreading results were compared with zeta potential measurements. These studies[8,9] clearly indicate that the mother suspension, just after dilution in cold water, is composed of

- Stable nanocapsules as described before; these objects diffuse strongly in the aqueous phase after spreading at the air/water interface.
- Unstable nanocapsules with similar size, but with a lower amount of phospholipids (Lipoid®) in the inner part of their shell. These capsules are not sufficiently robust to support the interfacial energies during spreading. Consequently, the components or fragments of the initial particles can be detected at the air-water interface.
- Free PEG (minor component of the Solutol®) released from the outer part of the shell.

It is obvious that the excess of PEG, as well as an important fraction of the unstable particles could be limited by dialysis. We will see in the next chapter an original investigation of these dialysis effects.

## 2.4. *Imagery techniques*

AFM images [Fig. 4(a)] were obtained after spreading the initial suspension of 50 nm (±10 nm) LNC on a fresh mica plate, and then allowing a complete evaporation of the water at room temperature. A contact mode was applied with a contact force of 10 nN, as well as a non contact mode without modification of the related images. The particle shape looked like a cylinder, 2 nm high and 275 nm wide, corresponding to a total volume similar to a 60 nm sphere. We demonstrate the deformation of LNC after water evaporation, but without fusion of the particles, something that often occurs with liposomes.

Classical TEM images were taken of the covered copper grids, following staining with a 2% phosphotungstic acid aqueous solution. It is noted on Fig. 4(b) that the lateral diameters are relatively polydispersed in a 20–70 nm range.

Fig. 4(c) corresponds to a cryo-TEM image (kindly provided by Olivier Lambert, IECB-UBS UMR CNRS 5471) where individualized LNC are detectable. It is important to note that this image was performed after a dialysis, followed by an appropriate dilution of the mother suspension.

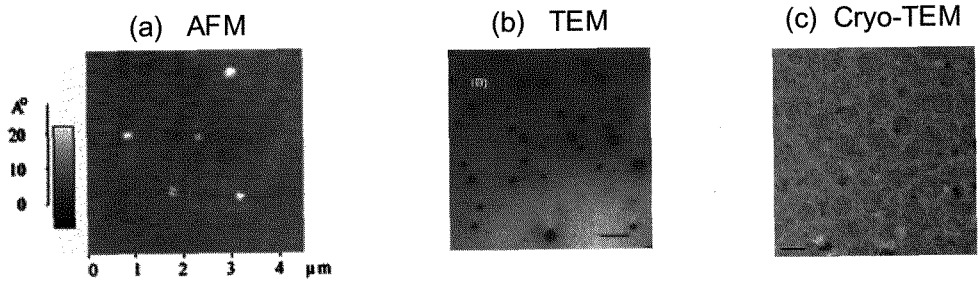


**Fig. 4.** Visualization of LNC by (a) AFM, (b) TEM and (c) cryo-TEM.

# 3. Electrical and Biological Properties

## 3.1. *Electro kinetic comportment*

The stable Lipid NanoCapsules (LNC) contain pegylated 12-hydroxy stearate, as well as free PEG in the outer part of the shell, which can be an important biological specification that we will describe latter. The distribution of PEG chains at the surface was determined by their electrokinetic properties. Thus, electrophoretic mobility was measured as a function of ionic strength and pH, for particles differing in sizes, dialysis effects, and the presence or not of lecithin in their shell. The study enabled us to find the isoelectric point (IEP) as well as the charge density (ZN) in relation to the dipolar distribution in the polyelectrolyte accessible layer (thickness $1/\lambda$), by using soft particle electrophoresis analysis[10] (see Fig. 5).

This study showed that LNC presented electrophoretic properties conferred by PEG groups at the surface constituting dipoles that are able to interact with counter ions ($H^+$, $Na^+$) or water dipoles.

The levels of IEP, ZN and $1/\lambda$ changed after dialysis, due to the removal of molecules that were poorly linked (mainly free PEG) at the outer part of the surface, allowing accessibility to the inner adjacent part of the shell.

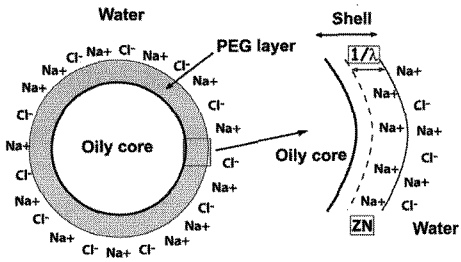


**Fig. 5.** Accessible layer to counter ions characterized by its thickness ($1/\lambda$) and its dipolar charge density (ZN).

100 nm LNC presented the best-organized and the accessible part of the shell, compared with other sizes of LNC, before and after dialysis. Lecithin was found to be present in the inner part of the polyelectrolyte layer and was found to play a role in the disorganization of the outer part. Dialyzing LNC formulated with lecithin led to stable and well structured nanocapsules, ready for an *in vivo* use as a drug delivery system.[11]

### 3.2. *Evaluation of complement system activation*

Generally, after intravenous administration, nanoparticles (NP) are rapidly removed from the blood stream because they are recognized by cells of the MPS such as Küpffer cells in the liver, or spleen and bone-marrow macrophages. However, a brush of PEG chains grafted on the surface is known to decrease the recognition of nanoparticles by the immune system after intravenous administration.[12] One has demonstrated that a strong correlation prevails between the complement activation and the stealthy properties of LNC.

Therefore, these properties were evaluated by measuring the degree of complement activation[11] [CH50 technique and crossed immunoelectrophoresis (C3 cleavage)] and the level of macrophage uptake, in relation to the organization of PEG chains, according to the electrokinetic properties of the LNC surface. These experiments were performed on 20, 50 and 100 nm LNC before and after dialysis. The CH50 technique is presented in Fig. 6.

Nanoparticles are dispersed in human serum with sensitized erythrocytes. After incubation, lysis is evaluated by a classical spectrophotometric method. The measured absorbance is related to the consumption of complement proteins by particles.

The main conclusions are that whatever the *in vitro* test, all LNC were not recognized by the non specific components of the immune system. It was probably due to the strong density of PEG chains at their surface. Furthermore, dialysis maintains a sufficiently high density of PEG and had no incidence on the complement consumption.

## 4. Pharmacokinetic Studies and Biodistribution

At first, the biodistribution of radiolabeled nanocapsules was studied by scintigraphy and $\gamma$ counting, after intravenous administration in rat whereby the $^{99m}$Tc-oxine was incorporated in the lipid core and $^{125}$I labelled the shell of the nanocapsules.[13] Dynamic scintigraphic acquisition was carried out 3 hrs after administration and $\gamma$ activity in blood and tissues was followed for more than 24 hrs (see Fig. 7).

An early half-disappearance time of about 47 $\pm$ 6 min was found for $^{125}$I and 41 $\pm$ 11 min for $^{99m}$Tc. These ranges of residence times were interesting for specific

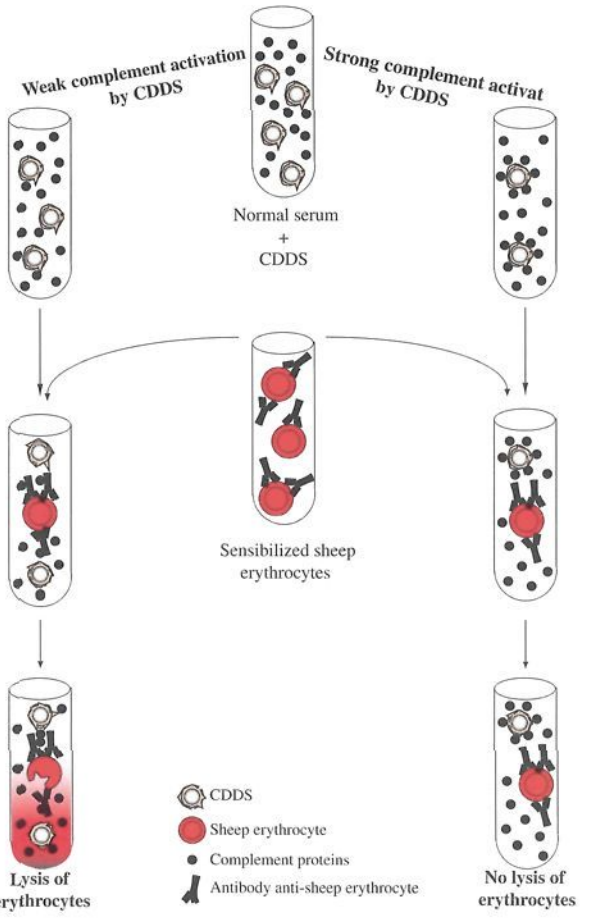


**Fig. 6.** CH50 method for the evaluation of complement system activation.

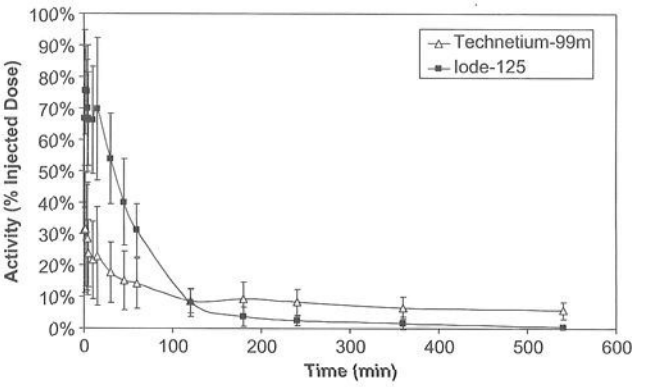


**Fig. 7.** Evolution of radioactivity blood repartition after the intravenous administration of LNC expressed as a percentage of the injected dose.

site delivery. Meanwhile, it appears that the length of the PEG chain (in this case, 15 ethylene oxyde groups per molecule) should be increased to extend the vascular residence time.

Recently, it has been shown that adding different DSPE-PEG to the system enhances the $t^{1/2}$ values to several hours, depending on the concentration and the PEG length.[14] $t^{1/2}$ (half-life), MRT (Mean Residence Time) in blood and AUC (Area Under Curve) were evaluated by using [$^3$H]-cholesteryl hexadecyl ether mixed with the lipid and the surfactant at the beginning of the formulation.

The main conclusion was that the LNC formulated in this study compared advantageously with other nanoparticulate systems, particularly for their residence time in blood. Nanocapsule uptake by the different organs of rat was evaluated 24 hrs after intravenous administration. It was shown that LNC deposited mainly in the liver and the spleen, but also in the heart, and the results were comparable to a liposome reference.

## 5. Drug Encapsulation and Release

### 5.1. *Ibuprofene*

LNC were characterized for their suitability as an ibuprofene delivery device for pain treatments.[15] After *in vitro* investigations, ibuprofene- loaded LNC were evaluated after intravenous and oral administration in rats. For each system, the carrier was evaluated through its potential antinociceptive efficiency.

We present in Fig. 8, the release of ibuprofene in a phosphate buffer after its incorporation in LNC during formulation. For each case, LNC provide high ibuprofene loadings (95%). The main feature is an initial burst followed by a

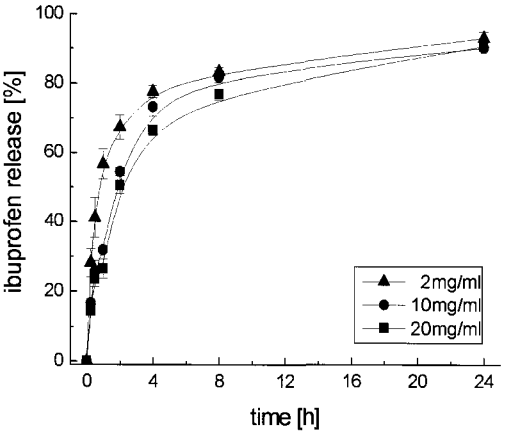


**Fig. 8.** Ibuprofene release from three batches of drug-loaded LNC in phosphate buffer (pH = 7.4).

sustained release, where the time for 50% drug released values are 0.84, 1.78 and 2.29 hrs for 2, 10 and 20 mg/ml loaded amounts respectively.

Furthermore, after oral administration, these nanocarriers offered a better bioavailability as well as prolonged antinociceptive effects than other nanoparticulate systems.

### 5.2. *Amiodarone*

Amiodarone is widely used because of its anti-anginal and anti-arrythmic properties. Unfortunately, this molecule can provoke severe adverse effects due to its accumulation in other tissues, after classical intravenous or intraperitoneal administration. In that manner, the use of LNC was evaluated in *in vitro* conditions in order to incorporate and release amiodarone from their lipidic core.[16] It was found that sustained drug release was achieved over a range of significant period between 25 hrs and 263 hrs depending on the pH of the release medium.

## 6. Conclusions

A new kind of colloidal drug carrier, the LNC, was formulated without organic solvent or toxic surfactants via a rapid and easy protocol. These nanoparticulate systems were designed in order to have biomimetic properties and can be considered as pseudo-lipoproteins. A lipidic core is surrounded by a surfactant shell, stabilized by phospholipids in the inner part of the shell and by stearate of PEG in the external part of the shell. The structural characteristics of these carriers allow for the incorporation in their core of different lipophilic drugs, initially dispersed in the oily phase at the beginning of the formulation. The narrow size distribution can be selected anywhere in a 10–200 nm range. One of their most important features is the presence of PEG groups on the surface ideally presented, providing very low recognition. These surface properties are crucial in order to hide the LNC from the MPS system. In addition, the presence of hydroxyl groups should allow the functionalization of the LNC surface to attach ligands of interest and to improve the specificity of drug targeting.

These PEG groups could participate to the inhibition of the efflux pumps involved in multidrug resistance. In this context, preliminary *in vitro* studies are very promising. Different strategies of incorporation or attachment of a specific ligand of the BBB and also of a glioblastoma tumor are studied. The linkage of a grafted monoclonal antibody is currently evaluated. It could provide an interesting way of accumulating LNC in the brain after intravenous administration.

The release of simple lipophilic drugs taken as models were analyzed and have shown sustained release over several days. Furthermore, since we are able to elaborate stable lipidic complexes (hydrophilic molecules associated to a cationic lipid

for example), these vectors could provide an interesting alternative to liposomes for the delivery of hydrophilic compounds like DNA or proteins.

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# 11

# Lipid-Coated Submicron-Sized Particles as Drug Carriers

*Evan C. Unger, Reena Zutshi, Terry O. Matsunaga and Rajan Ramaswami*

Lipid-coated submicron-sized particles afford a new platform for drug delivery and therapy. In this chapter, we will discuss the characteristics and some of the potential clinical applications of submicron-sized particles.

## 1. Technology

In general, bubbles present a hydrophilic exterior and hydrophobic interior stabilized by detergents. Detergents are characterized by their polar head group and a hydrophobic domain consisting of long chain fatty acids, alcohols, ethers, etc. In bubbles, (Fig. 1) detergents aggregate by orienting the hydrophilic polar groups on the outside in contact with aqueous environment and stacking their hydrophobic sections of alkyl chains on the inside, away from the water.[1] This results in an energy minimized spherical structure that can incorporate gas and/or other hydrophobic materials inside. Phospholipids are specialized surfactants with characteristics similar to that of detergents and can stabilize micron- and submicron-sized gas bubbles, especially when perfluoropropane is used.

Perfluorocarbon (PFC) gases have low solubility in aqueous media, relatively high molecular weight and can be used to prepare stable microbubbles or submicron-sized bubbles (SMBs) that are less than 1 micron in diameter. Fully

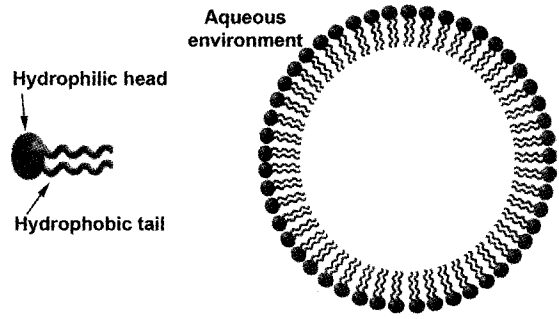


**Fig. 1.** The structure of bubbles.

halogenated PFCs are inert and generally not metabolized in the body. The table below lists some of the different PFCs and other gases useful in making lipid-coated microbubbles and PFC emulsions.

| Compound | Molecular Weight | Boiling Point | Solubility in Water |
|---|---|---|---|
| Nitrogen | 28 | −195.8 | Sparingly |
| Sulfur hexafluoride (SF6) | 147.07 | Sublimes | Sparingly |
| Perfluoropropane (C3F8) | 188.2 | −36.7 | Insoluble |
| Perfluorobutane (C4F10) | 238.04 | −2 | Insoluble |
| Perfluoropentane (C5F12) | 288.05 | 29.5 | Insoluble |
| Perfluorohexane (C6F14) | 338.06 | 57.11 | Insoluble |

Lipid-coated submicron-sized particles may be prepared by agitating an aqueous mixture of lipids with a selected gas (as in the approved product Definity® microbubbles ultrasound contrast agent, marketed by Bristol-Meyers Squibb), by lyophilizing the material and storing with a head space of the pre-selected gas, spray-drying or by creating an emulsion of the gas in the bubbles, e.g. when a PFC material is used to formulate the particles below the boiling point of the gas, while the PFC is in its liquid state.

The properties of the lipid-coated bubble will vary in part, depending upon the gas or material that is encapsulated in the bubble. Lipid-coated microbubbles of air or nitrogen will be relatively short lived. Following intravascular injection, bubbles composed of air or nitrogen may be stable enough to pass from the right heart through the pulmonary circulation and into the left heart, but will likely be unstable to undergo multiple passes through the circulation. When bubbles are prepared from air or nitrogen, the gas is relatively water-soluble and diffuses rapidly

across the lipid membrane into the blood. PFC gases have much lower solubility in the blood and therefore make more stable bubbles. The solubility of the PFC in part reflects the molecular weight of the compound with the higher molecular weight materials generally having lower solubility, and the boiling point of the materials increases with increasing molecular weight. PFCs with 4 or less carbon atoms will be gases at room temperature. Dodecafluoropentane has a boiling point of about 28.5°C. Liquid perfluoropentane filled phospholipid-coated submicron droplets (SMDs) may volatilize *in vivo* to form gas bubbles after intravenous injection. Perfluorohexane will be a liquid at physiological temperature, but because of its vapor pressure, a small fraction of the material may be in gaseous state at physiological temperature.

A wide variety of different lipids can potentially be employed to make the lipid-coated microbubbles. Experiments were performed using agitation to prepare the microbubbles. Microbubbles were more stable when prepared when the lipids were at gel state and when the same chain length of lipids was used in the formulation. The product Definity was developed using a blend of lipids: dipalmitoylphosphatidylcholine (DPPC), dipalmitoyl-phosphatidic acid (DPPA) and dipalmitoylphosphatidylethanolamine-PEG5000 (DPPE-PEG5000, polyethyleneglycol, MW = 5000). The product is primarily composed of the neutral lipid DPPC. The anionic lipid in the formulation may aid in the electrostatic repulsion of the bubbles and the PEG may form a steric barrier to further prevent aggregation or fusion of the bubbles.

The microbubbles have various potential medical applications:

(a) They can be used as the active drug product.
(b) They can be coadministered with other biologically active drug substances.
(c) Biologically active drug materials can be incorporated into the hydrophobic domain of the microbubbles.
(d) Biologically active gases can also be entrapped inside the bubbles and used for delivery.

In (a), the bubbles themselves can be used as contrast agents with diagnostic ultrasound, or as therapeutic agents with therapeutic ultrasound. An unusual feature of lipid-coated microbubbles compared with other delivery systems is that these agents can be activated by energy, particularly using ultrasound for localized therapy. The ultrasound energy can be targeted precisely to small regions in the body. Localized therapy and drug delivery can be accomplished using ultrasound to activate the bubbles to disperse a clot, increase local capillary permeability or to release drugs from the bubbles [as in (c) & (d) above]. Figure 2 below shows some of the ways that bubbles may be used to deliver therapeutic agents. The following sections will review some of the specific biomedical applications.

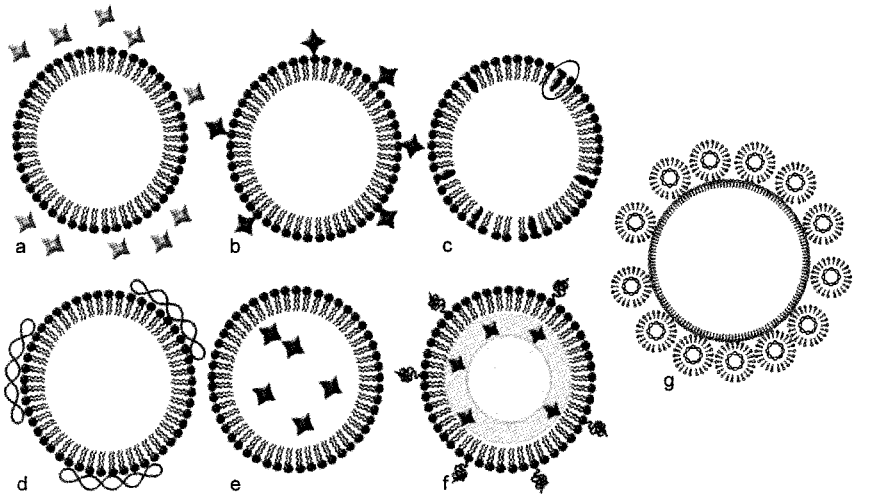


**Fig. 2.** Microbubbles can be used to transport materials in a variety of methods. In (a), the drug is injected in conjunction with the bubbles and driven into the target tissue by the acoustic activation of the bubbles. To create drug carrying bubbles, the agent may be: (b) attached to the outside of the lipid, (c) embedded in the lipid layer, (d) associated to the membrane by electrostatic interactions, (e) encapsulated directly in the bubble, or (f) dissolved in an oil or other compatible liquids and then encapsulated within the bubble. Also, smaller bubbles or spheres (e.g. delivering gene products) may encapsulate the agent and then associate with larger bubbles as in (g).

## 2. Ultrasound Contrast Agents

In biological fluids and tissues, microbubbles are efficient reflectors of sound. Ultrasound is the most common biomedical imaging modality and ultrasound contrast agents are used to increase the reflectivity or backscatter of blood and tissues. In ultrasound imaging the reflectivity of the bubbles is proportional to $r^6$ where $r$ = the radius of the microbubble.[2,3] This implies that the larger the bubble, the more efficient it is as a reflector of sound. For intravascular applications, however, the bubbles must be smaller than the diameter of a red blood cell to safely pass through the capillaries without causing vascular blockade. The relationships between reflectivity and effectiveness as an ultrasound contrast agent are far more complex than the prediction based upon mere size or diameter of the microbubbles.

Biomedical ultrasound is commonly performed over a range of ultrasound frequency from 1.5 MHz to 20 MHz. The resolution of ultrasound increases as the frequency increases (shorter wavelengths of ultrasound), but penetration in tissues also decreases linearly with frequency. The highest frequencies of ultrasound (e.g. 20 MHz) are mainly used for imaging with catheter-based ultrasound

(e.g. for looking within vessels), or for imaging very superficial tissues such as the skin. The lower frequencies (e.g. 3 to 7 MHz) will penetrate abdominal tissues and other structures for general purpose imaging.

Bubbles have resonant properties and reflect sound most efficiently at their resonant frequencies. For example, the resonant frequency of a 1 micron bubble is approximately 9.5 MHz and the resonant frequency of a 5 micron bubble is approximately 1.3 MHz.[4] When insonated at their resonant frequencies, microbubbles will emit harmonic signals at higher frequencies. For example, when microbubbles are insonated with a fundamental insonation frequency = $B_o$, the bubbles will reflect signals at the $B_o$ frequency as well as signaling powerfully at 2x $B_o$.

The images below (Fig. 3) show fundamental and harmonic images of the heart pre- and post-contrast, using the lipid-coated microbubble contrast agent, Definity®. As shown in the images, the harmonic image, obtained from the signal twice the insonation frequency, has higher contrast and greater suppression of background signal. Harmonic imaging (sampling the 2x $B_o$ signal) as well as other techniques enable ultrasound to suppress signal from the background and to enhance the signal from blood.

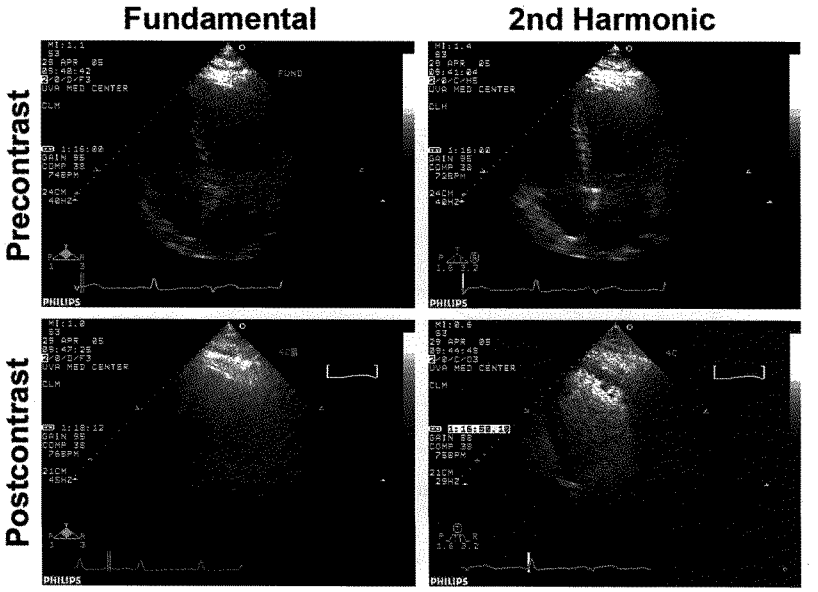


**Fig. 3.** Comparison of fundamental and second harmonic imaging. The second precontrast harmonic image is clearer. The contrast was unclear post-contrast, when the sonographer switched back to fundamental imaging. The infusion rate was then increased, resulting in excessive shadowing, thereby further obscuring the details. Courtesy of Kevin Wei, MD, University of Virginia.[5]

Another factor contributing to the effectiveness of the ultrasound contrast agent is the elasticity of the shell surrounding the microbubbles. The more elastic the shell, the more efficient the bubble may be as a reflector of sound.[6] A less elastic shell will not only decrease the efficiency with which sound is reflected, but will also raise the frequency at which the bubble resonates. Lipid coatings surrounding microbubbles are thin and are most likely monolayers or bilayers of lipid, and as such, are relatively elastic compared with other materials that may be used to coat bubbles such as cross-linked synthetic polymers. The lipid materials used in coating the microbubbles, enable production of highly elastic and efficient reflectors of ultrasound. In preparing bubbles for drug delivery, as materials are added to the bubbles, the bubbles may become less elastic and require higher amounts of ultrasound energy for activation.

In certain imaging regimes, bubbles can be ruptured from the ultrasound energy. As shown in Fig. 4 below, microbubbles lower the threshold of ultrasound energy for cavitation to occur.[7,8] Cavitation creates an acoustic signal that can be detected. Cavitation can occur as a stable inertial cavitation of a bubble, where the bubble expands and collapses in concert with the phase of the waves of ultrasound. At higher energy, cavitation can lead to localized energy deposition analogous to a local explosion on the microscopic scale. Cavitation can be used for therapeutic purposes as described below, or it can be used to create a strong ultrasonic signal

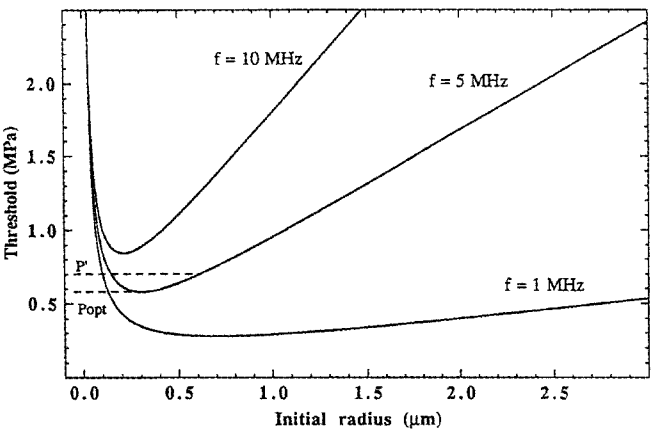


**Fig. 4.** Plot of the cavitation threshold in water as a function of initial nucleus radius for three frequencies of insonification: 1, 5, and 10 MHz. Nuclei consist of air bubbles initially at 300 K that undergo growth in a single cycle of ultrasound and collapse adiabatically to a temperature of 5000 K. Surface tension, viscosity, and inertia of the host fluid are included in this analytical model (Holland and Apfel, 1989). For 5 MHz, the optimal nucleus radius is 0.3 $\mu$m with a corresponding cavitation threshold, $P_{opt}$, of 0.58 MPa peak negative pressure. Note that at a pressure $P'$ greater than $P_{opt}$, a broader size range of nuclei cavitate. (Reproduced by permission of Apfel and Holland, *Ultrasound Med Biol.*)

for diagnosis and detection of diseases. Calculations indicate that with cavitation imaging, it is possible to detect a single microbubble.[9]

Bubbles as ultrasound contrast agents open the field of diagnostic ultrasound to molecular imaging. In terms of sensitivity to concentration of material, ultrasound imaging using bubbles rivals the most sensitive imaging techniques such as nuclear medicine. In addition to cavitation-based imaging which may detect a single bubble, when bubbles are targeted to a certain structure and accumulate to present an interface of several or more bubbles, they may form a so-called specular reflector or highly efficient interface for the reflection of ultrasound. Our group has created targeting ligands for incorporation into the microbubbles and performed imaging of models of disease with these contrast agents.[10,11] The images below show a thrombus in the left atrial appendage in a dog, pre- and postcontrast. The thrombus is not visible on the ultrasound images precontrast, but is readily detected when it is postcontrast.

Figure 6 is a depiction of the bioconjugate that was synthesized to develop the thrombus targeted ultrasound contrast agent. The lipid in the bioconjugate serves as a hydrophobic anchor to bind the bioconjugate to the surface of the bubble. A small number of bubbles bound to the surface of a target such as a thrombus, appear to be sufficient for contrast enhancement and detection on ultrasound.

A number of different molecular targets have been imaged with ultrasound using targeted bubbles. Some of the different diseases that have been imaged include vulnerable plaque, inflammation, angiogenesis and ischemia. Figure 7 shows P-selectin targeted imaging in a model of ischemia with myocardial contrast echocardiography (MCE).

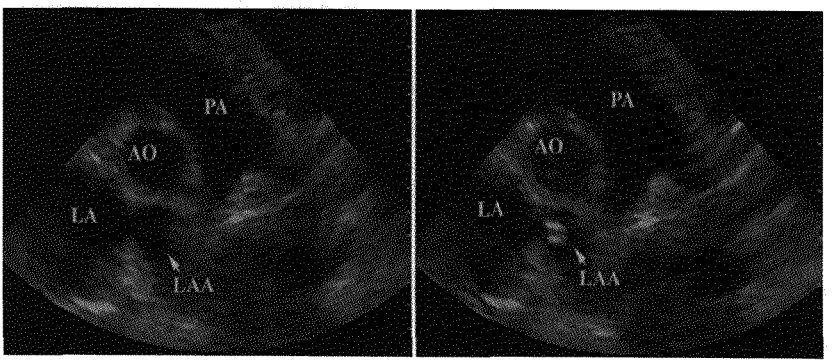


**Fig. 5.** Ultrasound images of left atrial appendage (LAA) clot enhancement in the canine model using an intravenous infusion of targeted microbubbles at a dose of 0.01 $cm^3$/kg: precontrast image (left); postcontrast infusion image (right), highlighting clear enhancement of the clot in the left atrial appendage. AO, aorta; LA, left atrium; PA, pulmonary artery. [Reproduced with permission of Unger *et al.*, in *Ultrasound Contrast Agents, 2nd Edition*, Goldberg, Raichlen & Forsberg (eds.).]

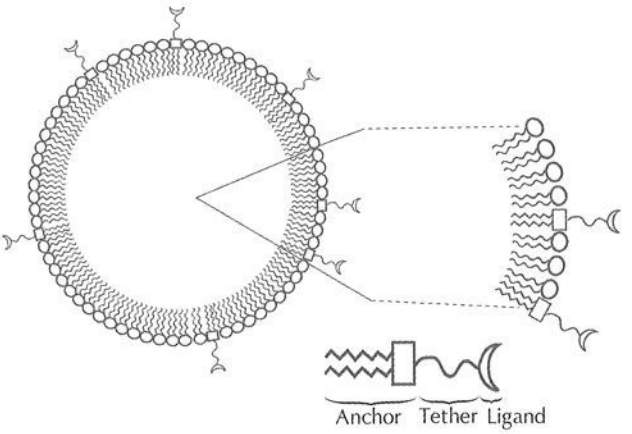


**Fig. 6.** Microbubble with bioconjugates attached with enlarged view showing the anchor, tether and ligand. Reproduced by permission Unger *et al., EJR.*[12]

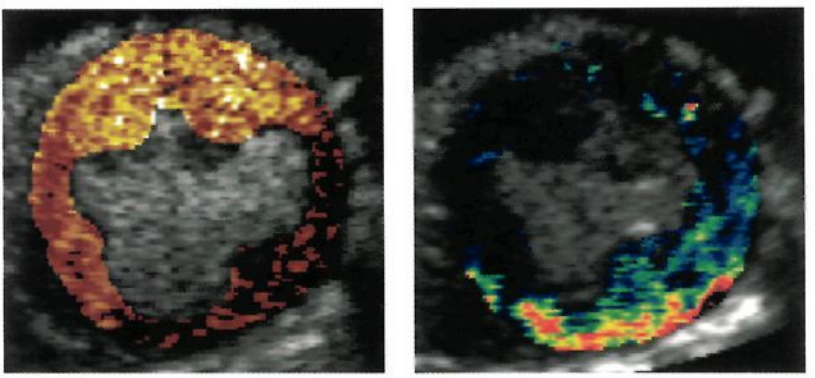


**Fig. 7.** The left panel shows an area of hypoperfusion imaging with MCE during myocardial ischemia of the left circumflex artery. The right panel shows enhancement 60 min after reflow from P-selectin-targeted imaging in the risk area. Courtesy of Jonathan R. Lindner, MD, University of Virginia.[13]

## 3. Sonothrombolysis

Bubble-assisted sonothrombolysis is the term we use to describe the ultrasound-mediated cavitation of bubbles to aid in the lysis of venous and arterial thrombi. MRX-815 is the designation for ImaRx Therapeutic Inc.'s (ImaRx) manufactured phospholipid-coated submicron-sized bubble product that will be used in clinical trials. MRX-815 is the next generation bubble, developed based on the Definity bubble. The bubbles in MRX-815 exhibit a size profile where 70% of the particle

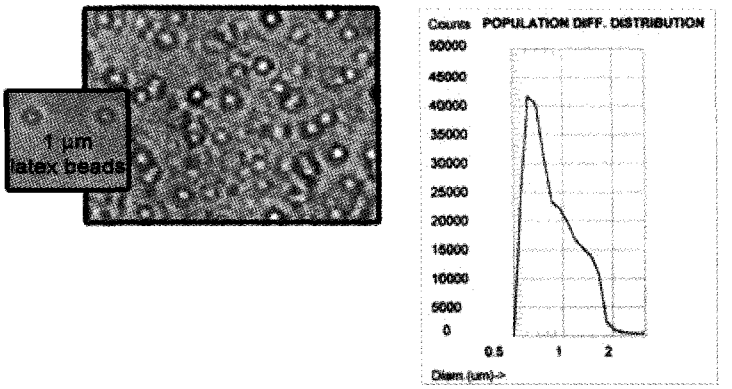


**Fig. 8.** Sizing studies of submicron bubbles.

distribution is less than one micron and the mean size is less than 1 micron in diameter.[14,15]

The images below depict a photomicrograph of MRX-815 bubbles alongside a photomicrograph of one-micron size latex microbeads. The bubbles are one micron in diameter and smaller. We found in our lab that the smallest bubbles are not well shown on the light microscopy due to limitations of the imaging technique. The sizing profile shows that there are bubbles up to approximately two microns in diameter, but more than 70% of the bubbles are smaller than one micron in size.[16,17]

Investigators have demonstrated that ultrasound can be used to generate cavitation in an aqueous medium.[18] Cavitation research has led to studies involving ultrasound-mediated clot lysis at a variety of frequencies.[19–23] Furthermore, microbubbles and submicron-sized bubbles provide a nucleus at which cavitation can occur, thereby lowering the ultrasound energy requirements.[24]

While intravenous administration with local application of ultrasound appears to be effective for sonothrombolysis in both pre-clinical and clinical models, applications using an infusion catheter are also being investigated.[25] It is believed that submicron-sized bubbles and ultrasound-mediated cavitation are able to affect the thrombus architecture by increasing permeability through the thrombus matrix, thereby improving accessibility and the penetration of thrombolytic enzymes to more efficiently lyse clots. Studies by Francis *et al.*,[26,27] demonstrated that ultrasound alone increased the spacing between fibrin strands in clots, presumably improving the penetration of lytic enzymes, such as t-PA, into the clot.

By way of explanation, when bubbles are insonified, these bubbles can oscillate in response to the acoustic pressure wave. If driven with a sufficient acoustic pressure, the rapid expansion and contraction of the bubble will result in local

velocities at the bubble surface on the order of hundreds of meters per second. If the expansion of the bubble is large enough, the bubble will become unstable, resulting in the destruction of the bubble into smaller fragments.[28] The rapid oscillation of the bubble in response to an acoustic pulse is referred to as "cavitation". Bubbles undergoing this violent expansion and contraction produce liquid jets, local shock waves, and free radicals. Although the exact mechanism is still being studied, the effect of cavitating bubbles has been demonstrated to have several effects on the surrounding tissues, including the poration of cell membranes resulting in enhanced membrane permeability (sonoporation) or the disruption of local thrombus. Thus, the combination of ultrasound with microbubbles has potential applications in blood clot dispersion and local drug delivery to treat cardiovascular disease, cancer, and diseases of the central nervous system. The figure below shows individual images from ultra-high speed videomicroscopy of a single bubble. The bubble is shown in the resting state on the far left hand side of the figure. The bubble expands after the application of the ultrasound pulse, then collapses and fragments. The daughter bubbles expand and collapse again, leaving behind small nano-sized fragments.[29] Localized activation of bubbles with ultrasound can be used for a number of different medical applications including SonoLysis.

Whereas in diagnostic ultrasound contrast imaging where there is an $r^6$ dependence between size and ultrasound reflection for therapy, it is advantageous to have much smaller bubbles. As shown in Fig. 10, when bubbles are cavitated by ultrasound, they may undergo a relatively greater increase in the expansion ratio $r_i/r_o$, where $r_i$ is the maximum size for the radius of the bubble after insonation, and $r_o$ = the initial resting radius.[31] The relative expansion with insonation is greatest for the smallest diameter submicron-sized bubbles. This conceivably results in a more effective cavitational force, and hence more efficient lysis of thrombi.

Another effect of ultrasound on microbubbles which has the potential to be utilized therapeutically is the use of acoustic radiation force to selectively concentrate microbubbles at a target site.[32,33,34] Microbubbles driven with ultrasound, experience radiation force in the direction of ultrasound wave propagation.[35] Pulses of

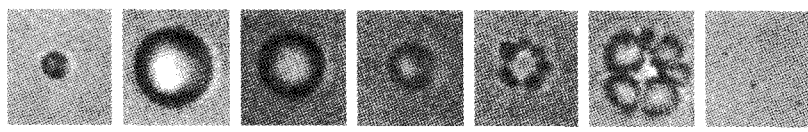

**Fig. 9.** In the images above, a single 3 $\mu$m bubble is shown (far left) in the resting state. Insonation with a single pulse of ultrasound energy causes the bubble to expand, collapse, and fragment, yielding nanometer-sized fragments. As the bubbles expand and collapse, they generate a local shockwave that can be used therapeutically. Reproduced with permission from Chomas *et al., Appl Phys Lett, 2000.*[30]

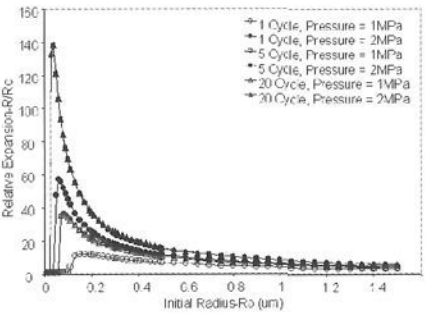


**Fig. 10.** The relationship between nanobubbles' size at resting state and expansion ratio under insonation. Reproduced with permission of D. Patel *et al.*, IEEE Ultrasonics, Ferroelectrics, and Frequency Control. In press.

many cycles can deflect resonant microbubbles over distances in the order of millimeters. Thus, it may be possible to bring microbubbles circulating in the blood pool into contact with targeting sites on a blood vessel wall, in a region selected by the positioning of the ultrasound beam. This effect has been demonstrated to increase the retention of microbubbles at a target site over an order of magnitude.[36]

In addition to favorable acoustic characteristics, submicron-sized bubbles have other potential advantages for therapy, compared with larger-sized microbubbles. The smaller bubbles may penetrate a clot more easily and may have better biodistribution characteristics for targeting.

The pictorial representation below (Fig. 11) is the hypothetical mechanism of action for MRX-815 bubbles flowing through the vasculature in association with

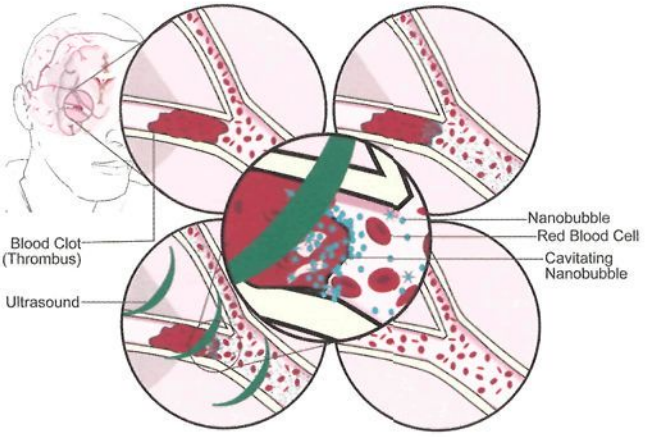


**Fig. 11.** It is hypothesized that when submicron-sized bubbles are injected systemically, some will aggregate on the thrombus, and due to their small size, work into the clot. When the bubbles cavitate, the kinetic energy disperses the clot, both from its periphery, and due to the fact that bubbles are able to penetrate the clot from within.

a thrombus. Ultrasound could cause cavitation of the bubbles, transferring their dispersive energy to the clot and dispersing the clot safely and painlessly. Particle sizing studies of the effluent from *in vitro* studies of SMB-assisted sonothrombolysis have shown that the particles are submicron in size.[37]

The figures below show the experimental set-up used in our lab for a flow through phantom for testing sonothrombolysis, and then treatment of a clot in the phantom. The clot was exposed to 1 MHz ultrasound and tissue plasminogen activator (t-PA), followed by an infusion of MRX-815 microbubbles. As shown in the figures, after 40 min of treatment there is near complete resolution of the clot.

The graph below shows the results from a series of clots exposed to t-PA, t-PA + ultrasound and t-PA + ultrasound + MRX-815 bubbles in our lab. Note that the greatest reduction of thrombi was in the group exposed to bubbles.

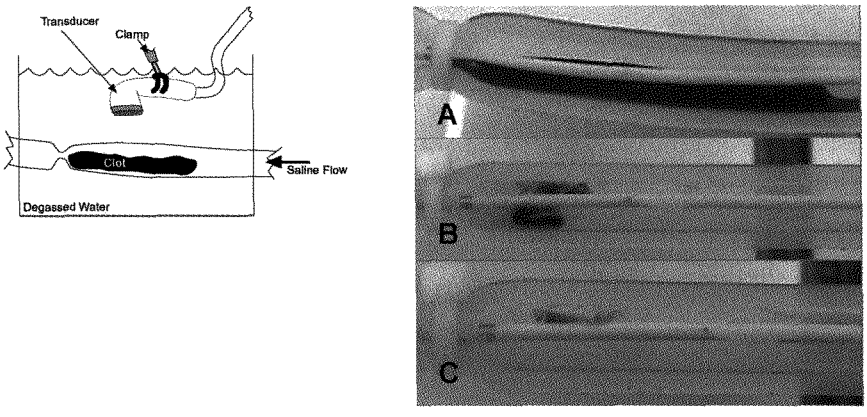


**Fig. 12.** Above a schematic of the experimental set-up: (A) the clot pre-treatment, (B) after 32 min of treatment, (C) after 40 min of treatment. The clot was 96% dissolved.

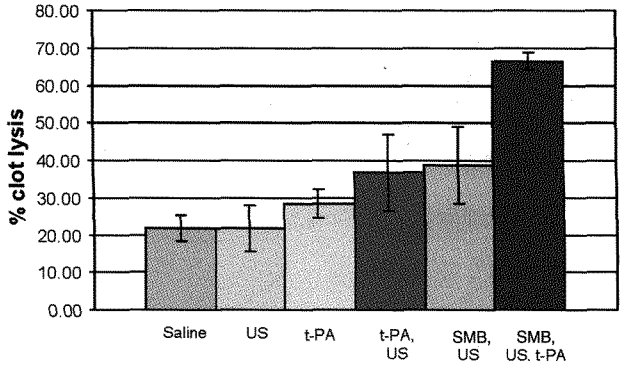


**Fig. 13.** SMB = Bubbles.

## 4. Clinical Studies

Vascular thrombosis is a major cause of death in industrialized countries, responsible for myocardial infarction, stroke and peripheral arterial occlusions.[38] In addition, deep vein thrombosis (DVT), which afflicts one in twenty Americans during their lifetime,[39,40] may also be an application for sonothrombolysis.

ImaRx completed a Phase I/II clinical trial in thrombosed dialysis grafts for the purpose of preliminary feasibility and safety for sonothrombolysis treatment of clotted grafts. Initial studies in thrombosed dialysis grafts provided a venue to evaluate the principle of sonothrombolysis in vascular thrombosis. As such, clinical trial efforts will move forward to address the treatment of stroke, peripheral arterial occlusions (PAO) and deep vein thrombosis (DVT).

Below are examples shown from clinical trials for sonothrombolysis in dialysis grafts and DVT. The examples are not an indication that all sonothrombolysis treatments will have similar outcomes.

Images from a venogram in a patient with DVT showed that the patient was administered bubbles via infusion catheter into the popliteal vein over a period of 1 hr, while ultrasound was applied across the skin. No thrombolytic drug such as t-PA was administered. Clinically, this particular patient had marked reduction in pain post-treatment with sonothrombolysis.

Stroke is the third most common cause of death, after heart disease and cancer in North America. It incurs far more expenses than any other diseases due to its long term disability.[41] In the US, stroke accounts for over $50 billion each year to the health care system.[42] The only approved pharmacologic therapy to help restore blood flow in stroke patients is t-PA (Activase®). Less than 5% of patients are treated with t-PA due to concerns over bleeding and the risk relative to the benefit.[43] Encouraging results have been obtained, however, in human studies with ultrasound and t-PA, and most recently, with ultrasound + t-PA + microbubbles.

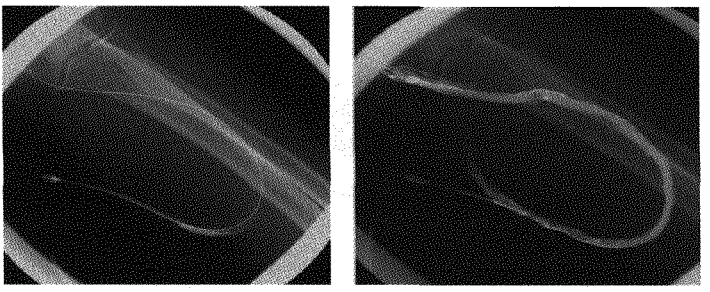

**Fig. 14.** The angiogram on the left is of a clotted dialysis graft. Very little contrast enters the graft as it is filled with clot. The image on the right, post-bubble treatment, shows complete opacification of the graft due to successful dissolution of thrombosis by sonothrombolysis.

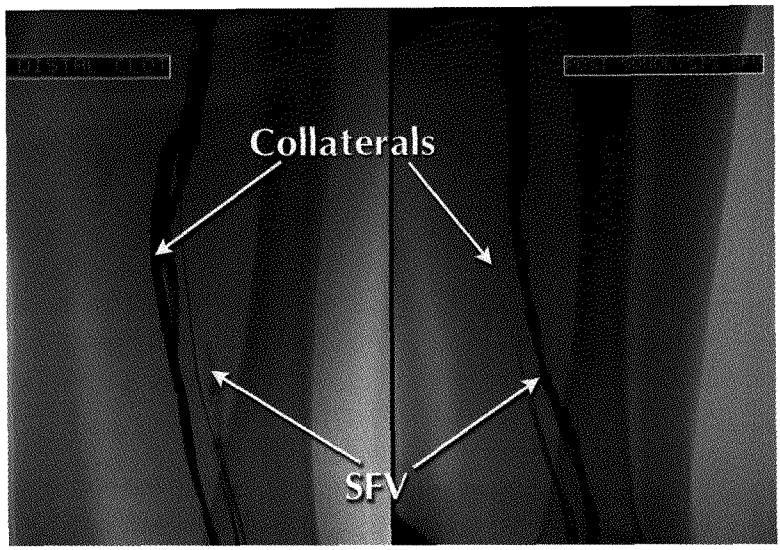


**Fig. 15.** On the pre-treatment image (left), there is complete occlusion of the superficial femoral vein (SFV). Collateral veins are seen carrying the blood flow that would normally be carried by the SFV. Post-treatment, there is good flow in the SFV and much less flow is seen in the collateral vessels due to the increased flow in the SFV.

Dr. Andrei Alexandrov from the University of Texas in Houston led a study of ultrasound + t-PA in acute ischemic stroke.[44,45] In this study, 126 patients were randomized prospectively to receive either a 1 hr infusion of t-PA at a dose of 0.9 mg/kg alone, or t-PA plus 2 hrs of continuous trans-cranial Doppler (TCD) ultrasound applied through the temporal window where the skull is thinnest and most easily penetrated by ultrasound. Of the 63 patients treated with t-PA alone, there was a 13% recanalization rate of the intra-cranial circulation at 2 hrs.[46,47] In the same number of patients receiving t-PA + ultrasound, there was a highly significant increase in recanalization to 38% at two hours, indicating that ultrasound-mediated therapy aided in thrombus dispersion.

Dr. Carlos Molina, from Barcelona, Spain, conducted a similar study but with microbubbles.[48] The addition of microbubbles enhances the cavitational nuclei with a decrease in power requirements. Dr. Molina's study demonstrated that the recanalization rate increased impressively to 55%.[49] In this study, Dr. Molina administered three doses of Levovist®, a microbubble agent comprised of air-filled galactose microparticles. Dr. Molina's pioneering work has demonstrated the utility of using bubbles in conjunction with ultrasound to improve the clinical outcome of acute stroke. ImaRx is currently moving MRX-815 into stroke treatment trials.

SMB-assisted sonothrombolysis therapy could move beyond the current clinical regimens by eliminating the thrombolytic agent. Pre-clinical trials in both canine and porcine models have been encouraging.[50,51,52] Human studies will be conducted to determine if lipid-coated bubbles will improve recanalization rates in patients treated with this new ultrasound-mediated paradigm.

## 5. Blood Brain Barrier

Poor transport into the CNS is an obstacle to effectively treat diseases including brain tumors, Alzheimer's and other neuro-degenerative diseases. There are two principal barriers to drug transport into the CNS: (a) the blood brain barrier (BBB) and (b) the ABC transporters, ABCC1 and ABCB1.

Unlike the rest of the body, the capillary foot processes of the cerebral endothelial cells are tight, preventing peptides and macromolecules from leaking through to the brain.[53] Although the BBB may be permeable to selected ions and small molecules, ABCB1, also known as the P-glycoprotein, acts to remove the molecules by a drug-efflux system before they enter the brain. Several different strategies have been developed to overcome these limitations.[54] One approach to drug delivery to the brain is by the transient opening of the BBB.

Hypertonic solutions containing mannitol, which act by shrinking the endothelial cells when co-administered with drugs, have been shown to result in enhanced cerebral drug uptake.[55,56] However, to cause minimum side effects, it is essential for the therapy to be regional and localized. Recently, Hynynen *et al.*[57] have shown that the BBB can be transiently opened using ultrasound and microbubbles (Illustrated in Fig. 16). When bubbles were administered intravenously and focused ultrasound was applied across the intact skull, the BBB could be reversibly opened, permitting passage of hydrophilic low molecular weight molecules such as gadolinium-DTPA, and macromolecules such as fluorescently labeled albumin (Fig. 17) into the CNS.[58] The permeability resolved over a period of hours without damage to the neurons.

Similar studies have been performed in a porcine model showing that non-focused ultrasound with microbubbles can be used to open the BBB.[59] Figure 18 shows increased dye deposition in the cerebral tissue.

Introduction of microbubbles as the cavitation nucleus prior to the application of ultrasound, lowered the energy needed to open the BBB, thereby lowering the bioeffects of ultrasound.[60] Using this technique, large biomolecules such as horseradish peroxidase (a 40 kDa protein) have been shown to pass through the BBB with minimal damage to the brain tissues.[61]

It can be envisaged that drugs (small or macromolecules) bound to the microbubbles would function as a more efficient drug delivery vehicle, since these

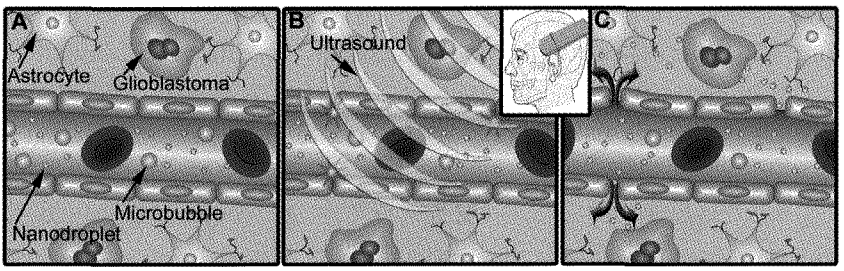


**Fig. 16.** Cartoon representation of hypothesized ultrasound mediated drug delivery to the brain. (A) Cerebral capillaries with tight endothelial junctions prevent passage of molecules (including microbubbles and nanoparticles) into the brain. (B) Ultrasound is applied to the skull through the temporal window where the skull is thinnest (inset), cavitating the microbubbles and opening up the endothelial junctions. (C) Therapeutic agents may now pass through the opened junctions.

| Location | Pressure amplitude values |
|---|---|
| 1 | 4.7 MPa |
| 2 | 2.3 MPa |
| 3 | 3.3 MPa |
| 4 | 1.0 MPa |

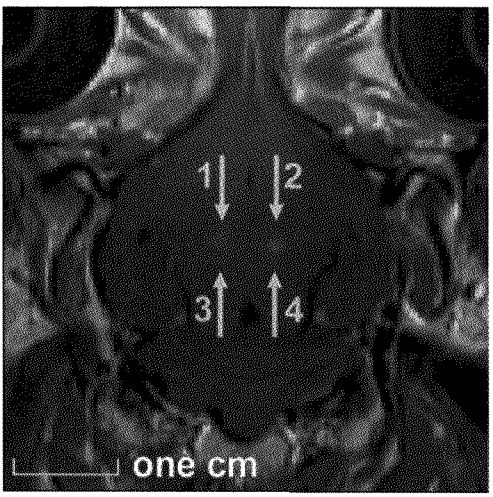


**Fig. 17.** T1-weighted MR images of rabbit brain after treatment shows contrast enhancement at 4 locations (arrows), coronal image across focal plane. Reproduced with permission from Hynynen *et al., Radiology*.

would provide the cavitation nuclei and the drug payload in one entity, circumventing the co-administration of drug and microbubble. In such instances, the drug could be (a) bound to the lipid membrane (hydrophobic drugs), (b) bound to the charged lipids on the surface (gene delivery), or (c) buried in the interior in an oily layer of a droplet (hydrophobic drugs) (Fig. 19). Furthermore, (d) these drug loaded bubbles or droplets may have the potential to be targeted to a specific site in the brain by surface ligands.

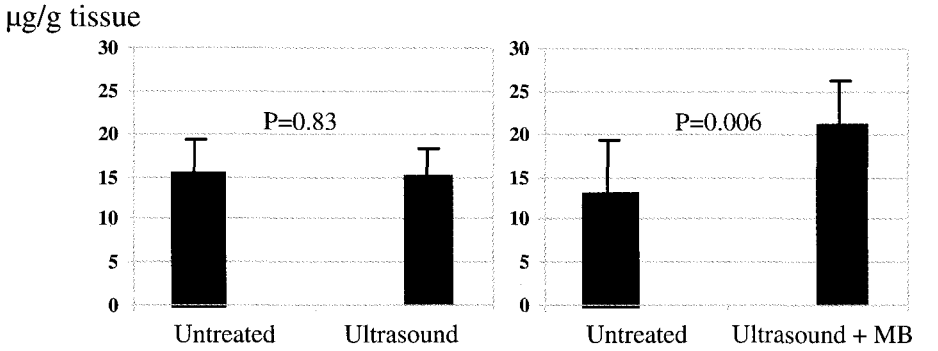


**Fig. 18.** Control pigs and pigs treated with ultrasound alone showed no difference in Evan's blue uptake. There was a significant difference in uptake when microbubbles were used in conjunction with ultrasound. Adapted from Porter *et al., J Am Soc Echocardiogr.*

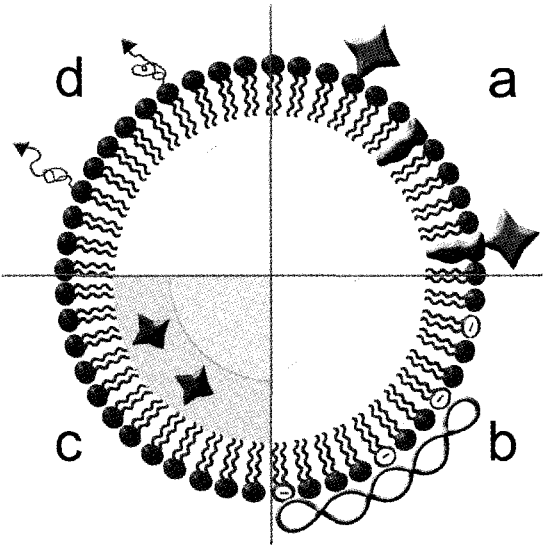


**Fig. 19.** Different ways that bubbles or droplets may be able to transport drugs. Drugs may be (a) bound or embedded in the lipid membrane, (b) bound to the surface charges of the phospholipid membrance (c) buried in the oil in a droplet (d) targeting ligands can be incorporated onto the membrance.

This technology of activation with ultrasound and microbubbles has the potential to also be used in the drug discovery process. By exposing cultured neurons to drugs, ultrasound and bubbles, high concentrations of the drug may be able to deliver to the cells without damaging them. This can potentially be used to screen neurons for new therapeutic compounds.

Potential CNS diseases amenable to treatment with submicron bubble delivery and classes of drugs

| Disease | Drugs |
|---|---|
| Alzheimer's Disease and other neurodegenerative disease, seizures and psychiatric disorders | Low molecular weight therapeutics with poor delivery to CNS, proteins, gene-based therapeutics. |
| Primary and Secondary (metastases) Brain Tumors | Low molecular weight therapeutics with poor delivery to CNS, proteins, genetic drugs. Radiation sensitizers. |
| Stroke, brain ischemia | Cavitation nuclei to augment sonothrombolysis, either with or without use of thrombolytic agent. Delivery of oxygen with microbubbles. Improvement of cerebral perfusion with microbubble-enhanced sonication. Delivery of anti-oxidants and growth factors. |
| Infection, e.g., AIDS | Delivery of anti-infectives, anti-retrovirals to CNS. |

## 6. Drug Delivery

In the foregoing sections, we discussed activating the bubbles or using them in conjunction with ultrasound-mediated processes (e.g. microbubble mediated sonothrombolysis to enhance the local activity of the drug such as t-PA), or that the availability of a drug may be increased, e.g. by opening the blood brain barrier. In this section, we will discuss evaluating drug-carrying microbubbles for drug delivery.

### 6.1. *Targeted bubbles*

As preliminary studies to demonstrate feasibility of using targeted bubbles as potential drug delivery agents, two different targeted bubbles were prepared using a mixture of DPPC, DPPE-PEG5000 and DPPA, as well as different oils and perfluorocarbons using a mixture of DDFP and n-perfluorohexane. In one study, a bioconjugate ligand targeted to the $\alpha_{IIb}\beta_{III}$ integrin was synthesized by solid phase peptide methodology.[62] Briefly, the bioconjugate, lipids, biocompatible drug, perfluoropropane were combined into a mixture and bubbles prepared by shaking the vials at approximately 4200 rpm. The size of the targeted bubbles

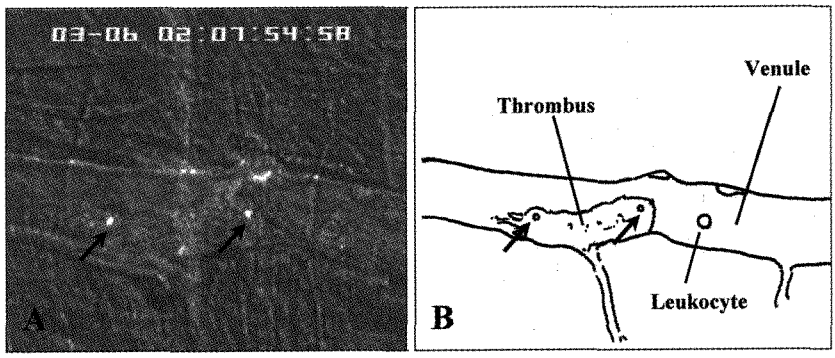


**Fig. 20.** Intravital microscopy demonstrating adherence of targeted microbubble to thrombus. Picture on the right is a graphic representation outlining the location of bound microbubbles on thrombus. Reproduced with permission from Schumann *et al., Investi Radiol.*

was approximately 2 $\mu$m, as measured by light obscuration measurements on a Particle Sizing Systems Model 470 sizer (Particle Sizing Systems, Santa Barbara, Calif.). Bubbles were injected into a mouse model where thrombi were previously formed in the cremasteric arterioles and venules. Fluorescent imaging revealed binding of the targeted bubbles to the thrombi in both arterioles and venules. Figure 20 demonstrates the utility of a targeted bubble.

Similarly, targeted bubbles were used in a HUVEC cell culture model. Briefly, bubbles with a targeting ligand directed to $\alpha_v\beta_3$ receptors on HUVEC cells were

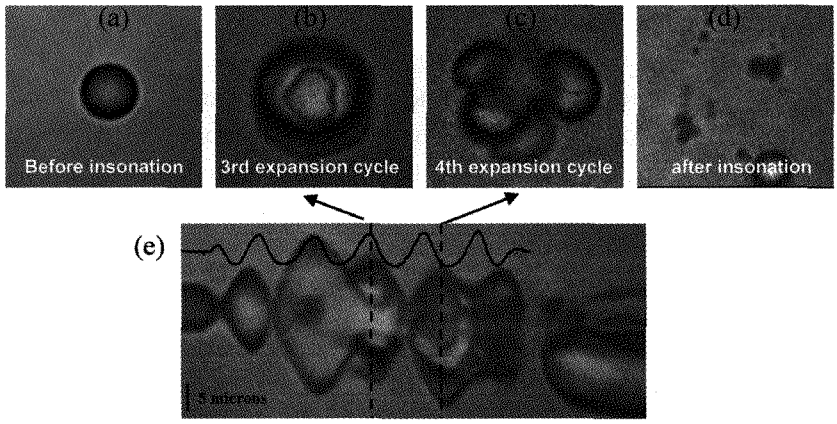


**Fig. 21.** Optical images of an AAL with 2-D frames occurring at times indicated on the streak image. AAL was insonated with a five-cycle pulse with peak negative pressure of 3.0 Mpa, at 1.5 MHz: (a) before insonation, radius is 4.5 $\mu$m; (b) 2-D image acquired during the third cycle; (c) 2-D image acquired during the fourth expansion cycle; (d) 2-D image acquired after insonation and subsequent fragmentation; (e) streak image, in which the horizontal axis is time with the entire image representing 5 $\mu$s, and the vertical axis indicates radial distance taken through the center of an AAL. Reproduced with permission of May *et al., IEEE Trans Ultrason, Ferroelect Freq Control.*

incubated followed by washing of the cells with buffer. Imaging of the cells revealed that the targeted bubbles bound avidly to the cells, whereas the untargeted bubbles (control) did not.[63]

Once targeted, bubbles were demonstrated to bind to the receptor determinants. Acoustic studies were performed *in vitro* to test the ability of the drug-carrying bubbles or acoustically active lipospheres (AALs) to cavitate in response to insonation.[64] Shown above is a streak image of an individual bubble in response to high mechanical index ultrasound. As can be seen, the bubble expands and contracts in response to the ultrasound.

The aforementioned studies have indicated that we have the ability to combine targeting bubbles with ultrasound-mediated delivery techniques.

## 6.2. *Targeted submicron-sized droplets*

Another paradigm being studied is liquid perfluorocarbon and oil-filled droplets for targeting and drug delivery. Droplets offer a smaller size regime (150 nm–400 nm) and a liquid core for drug loading. Just as with targeted bubbles, droplets are sensitive to radiation force motility by ultrasound beams,[65] although less compressible liquid-filled droplets require higher ultrasound intensities. An experiment was performed in cell culture using PC-3 human prostate cancer cells. The cells were grown on #1 coverslips and incubated with droplets containing a targeting ligand directed to $\alpha_6\beta_1$ receptors expressed on these tumor cells. As a delivery system, droplets were loaded with a fluorescently-labeled version of the chemotherapeutic paclitaxel. Cells were then exposed to 10 MHz ultrasound at an intensity of 200 mW/cm$^2$ for two min, followed by visualization on fluorescence microscopy. The figure below illustrates the results. The center image shows a low-magnification view of the cell monolayer, where the dotted line represents the region exposed to the circular focus of the ultrasound beam. Higher magnification images on the top and bottom show enlargements of regions with and without ultrasound exposure respectively. The leftmost pair of images is an epifluorescence and transillumination image of the same region, illustrating that minimal paclitaxel was transferred to the monolayer outside of the region of insonation. The transillumination image demonstrates that there are cells present in this area. The image pair on the right shows the monolayer in a region exposed to ultrasound, and one can clearly observe that the fluorescent paclitaxel has been transferred to the monolayer in this region.

The experiment described below demonstrates that ultrasound-mediated radiation force was able to site-selectively enhance the delivery of paclitaxel to a cell monolayer. Analysis showed that the fluorescence intensity of cells in the ultrasound-exposed region was approximately 100-fold greater than the cells in

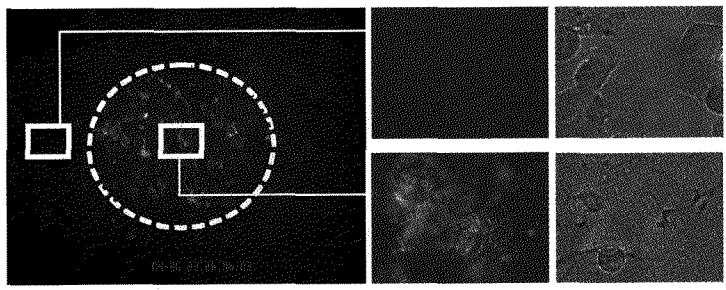

**Fig. 22.** Fluorescent images of a PC-3 cell monolayer administered fluorescent paclitaxel-containing droplets. Cells exposed to ultrasound (denoted by the dotted circle) received substantial delivery of the fluorescent chemotherapeutic, in contrast to cells not exposed to ultrasound. Images on the left show enlargements of the non-exposed (top) and exposed (bottom) areas respectively, in fluorescence and in white light. (Reference Dayton, PA, personal correspondence.)

the non-exposed region. Since the only fluorescent component present was the fluorescently-labeled paclitaxel, this intensity measurement correlates directly with the selectivity of ultrasound-mediated droplet delivery.

Droplets are currently being tested *in vivo* in the SCID (severe combined immunodeficiency) mouse model with human tumor xenografts of prostate cancer. Experiments to determine if ultrasound will improve efficacy are currently in progress. The agents have potential for a new, effective treatment of localized disease.

## 7. Gene Delivery

Cellular transfection of genes in the clinical setting has, to date, met with only anecdotal success. Lack of availability of safe, non-viral gene delivery vectors with high expression for localized delivery has impeded clinical development. Ultrasound-enhanced delivery of lipid-coated microbubbles bearing genes or other genetic materials e.g. anti-sense oligonucleotides, siRNA, etc. holds promise to a safe, effective delivery platform for localized gene delivery.[66–68]

The figure below shows results from an experiment using ultrasound to stimulate local DNA delivery to tumor. In this study, lyophilized liposomes were prepared to entrap the gene for interleukin 12 (IL-12). The liposomal system was then injected intravenously, followed by insonation of tumors previously implanted in mice thighs. Studies were performed in SCCVII squamous cell s.c. tumor bearing mice. As shown in the figure below, ultrasound applied to the tumor resulted in significant tumor regression, compared with control or treatment with IL-12 gene control excluding ultrasound.[69]

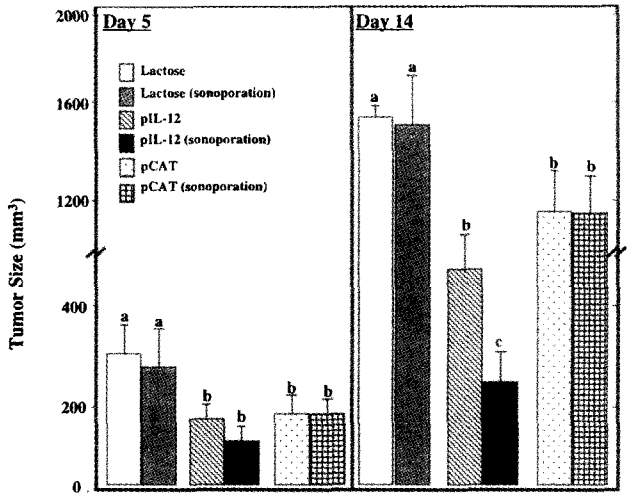


**Fig. 23.** Effect of ultrasound treatment on the inhibition of tumor growth, following systemic administration of IL-12 transfection complexes. SCCVII tumor-bearing mice were sonoporated for 2 min at 1.5 W/cm$^2$, followed by a single tail-vein injection of DOTMA:CHOL transfection complexes containing 10 $\mu$g IL-12 plasmid or CAT plasmid. Control animals received 10% lactose. Tumor size was measured 14 days after plasmid injection. Bars with different superscripts are statistically different ($P < 0.05$, mean $\pm$ s.d., $n = 5$), as determined by one-way ANOVA and Duncan's multiple range test. Reproduced with permission from Anwer *et al., Gene Ther.*

A number of animal studies have been performed, showing that microbubbles with ultrasound can be used for efficient gene delivery to the cardiovascular system.[70] The figure below shows data from an experiment using cationic lipid-coated perfluoropropane microbubbles, delivering the reporter gene for luciferase.

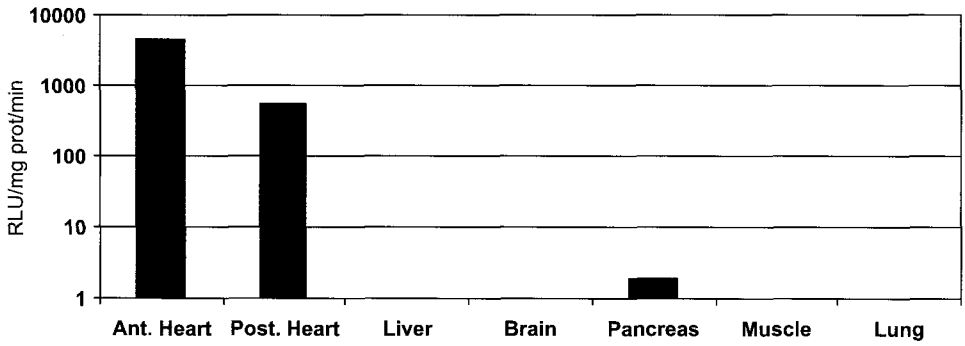


**Fig. 24.** Ultrasound-targeted microbubble disruption led to the expression of plasmid derived luciferase. The bars represent an average of 6 samples. Negligible activity was detected in the control organs, with highly specific tissue expression in the heart. Study performed in Sprague-Dawley rats. Adapted from Bekeredjian *et al., J Am Coll Cardiol.*

The gene carrying bubbles were administered i.v. and focused ultrasound was applied to the left ventricle of rats. High levels of luciferase expression in the anterior and posterior walls of the left ventricle coincided with the regions of ultrasound insonation, thus indicating that ultrasound-mediated delivery was effective.[71,72]

## 8. Oxygen Delivery

Lipid-coated microbubbles may have potential applications as blood substitutes for oxygen delivery. Rapid oxygen delivery to tissues is critical for normal functioning, in patients suffering from trauma. Perfluorocarbons are chemically inert hydrophobic molecules with the capacity to carry large amounts of oxygen. Liquid perfluorocarbons tested in clinical trials have been found to be safe, with side effects such as mild temperature increase and flu-like symptoms.[73] Compared with red blood cells or with liquid perfluorocarbon emulsions, perfluorocarbon gas microbubbles transport about 100 times as much oxygen per unit volume.[74] Since the longer chain length PFCs should give more stable microbubbles, and perfluoropentane (dodecafluoropentane, DDFP) is of as high a molecular weight as one can use; being still a gas at physiological temperature, DDFP is an excellent candidate for oxygen delivery. DDFP boils at approximately 28.5°C and can be injected as a liquid emulsion to volatilize in the blood.

Experimentally, the microbubbles have been visually observed with magnification light microscopy to increase and decrease in diameter as they imbibe oxygen when exposed to oxygen, and release oxygen under low oxygen tension conditions (Fig. 25).[75,76]

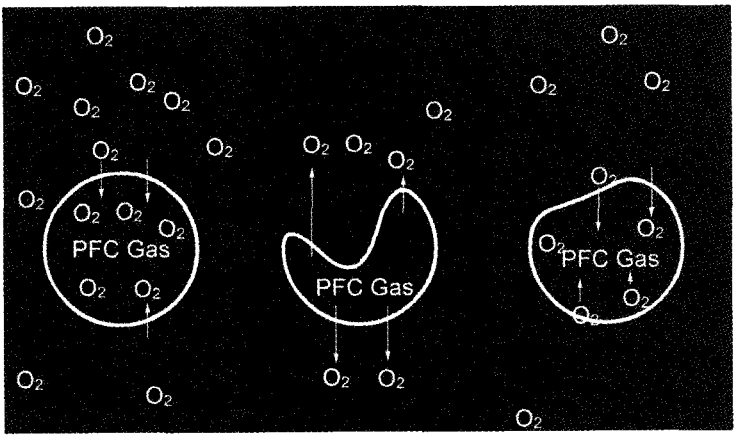


**Fig. 25.** The action of perfluorocarbon-filled, lipid-coated microbubbles in different oxygen environments.

The potential utility of lipid-coated DDFP as an oxygen carrier was recently shown by Lundgren *et al.*[77] at a scientific presentation at the American Heart Association. In this study, 10 pigs breathing room air were subjected to 50% blood volume loss*; 5 of the pigs received an intravenous infusion of 0.5 cc/kg, of 2% w/v DDFP emulsion, whereas 5 control pigs received the vehicle without DDFP. All of the treated pigs survived with normal weight gain and renal function. 4 out of 5 control pigs died, and the only surviving pig had severe renal damage.

Compared with other oxygen delivery technologies, lipid-coated DDFP emulsions may offer several advantages. Due to the high oxygen carrying capacity, only a small volume might be required for administration to a human subject. The studies described above[78,79] suggest that a volume of 30 to 35 cc should be sufficient to resuscitate a human subject from hemorrhagic shock, while breathing room air. The DDFP is inert and eliminated by respiration over a period of several hours. The gas gradually diffuses across the lipid membranes into the blood and is eliminated via the lungs. This is contrasted to liquid PFCs, e.g. perfluorooctylbromide, which have been extensively studied as blood substitutes.[80] The liquid PFCs are retained long-term by the reticuloendothelial system (RES) and require much larger volumes for the resuscitation of a hemorrhagic shock victim.[81]

Lipid-coated DDFP emulsions are not a blood substitute but a novel technology for oxygen delivery. The small requisite volume (e.g. 30 cc for 70 kg patient) may enable rapid resuscitation without volume overload and allows for subsequent treatment with whole blood. The small volume and characteristics of the lipid-coated DDFP emulsion may have advantages for resuscitation in critical environments like ischemia. As the small volume can be administered without hemodynamically compromising the subject, the technology might be exploited to minimize damage from ischemia, associated with conditions such as stroke, myocardial infarction and occlusive vascular disease. The prolonged retention of the liquid PFCs by the RES has proven problematic in the clinical development of these materials. The rapid clearance of gaseous PFCs from the body in several hours may prove helpful in the development of lipid-coated microbubbles for oxygen delivery.

## 9. Pulmonary Delivery

Lipid-coated microbubbles have the potential for delivery of genes and drugs to the lungs. Since the internal volume of the microbubbles is mainly gas, they have very low density. As such, these low density carriers may have unique properties for pulmonary delivery. A major problem with conventional aerosols is the precipitation onto the central airways, principally the oropharynx and trachea.[82] It is

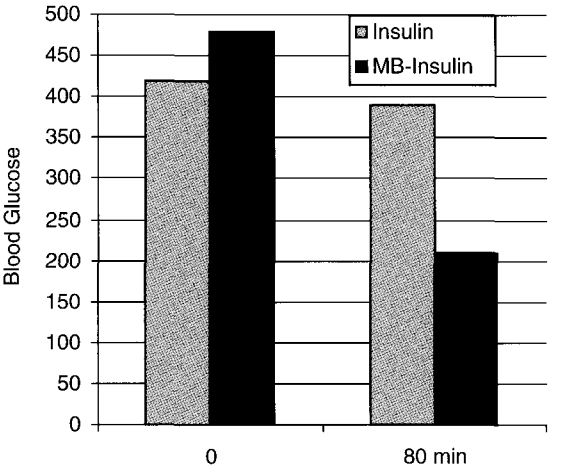


**Fig. 26.** Inhaled insulin v. inhaled microbubble encapsulated insulin.

advantageous, however, to deliver medicines into the terminal respiratory bronchioles to treat diseases such as asthma, and for the systemic delivery of medications via the lung. It is also desirable to reach the alveoli for the most efficient absorption of the medication. The low hydrodynamic index of the lipid-coated microbubbles appears to enable the particles to reach beyond the bronchi and potentially reach the alveoli.

Pulmonary delivery experiments were carried out in our laboratory to deliver both genes and drugs in mice. A standard nebulizer was used to deliver lipid-coated perfluoropropane microbubbles into a holding chamber. The mouse being treated was placed in this chamber for 15 min. The nebulizer reservoir contained enough materials for 10 min of delivery and the animals were held in the chamber for an additional 5 min.

Mice were treated with streptozotocin to create a diabetic condition. These mice were then treated with nebulized insulin either via the conventional formulation or via microbubbles. Conventional insulin showed no effect on blood sugar. The blood sugar levels were greatly reduced by the microbubble insulin in 80 min.

## 10. Conclusion

Lipid-coated, submicron-sized particles are used extensively in ultrasound imaging. The ability to activate these agents with ultrasound energy affords unique potential applications to these agents. Acoustic activation of micro- or submicron-sized bubbles affords potential as a less invasive, safer way of treating vascular thrombosis, and the ultimate utility of this technology will depend on results in the clinical development. The technology has the potential to dramatically improve

the therapeutic outcome in the treatment of stroke. Targeted ultrasound mediated delivery also affords potential in drug and gene delivery to treat cardiovascular disease, systemic disease and the central nervous system. In addition to the unique applications of these agents in concert with ultrasound energy, bubbles may have therapeutic applications in their own right for delivering oxygen and other biologically active gases. The low hydrodynamic radius of bubbles and the fusogenic properties of the bubbles themselves also increase the potential for pulmonary delivery.

## Special Thanks

The authors would like to thank Dr. Paul Dayton for his help in preparing this manuscript.

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# 12

# Nanocapsules: Preparation, Characterization and Therapeutic Applications

*Ruxandra Gref and Patrick Couvreur*

## 1. Introduction

Over the past decades, the new field of drug delivery has gained more and more attention by scientists, industry specialists and clinical doctors. It is now recognized that both the inherent value of an active molecule and the delivery system are crucial to achieve the desired pharmacological responses. Indeed, the administration of some newly developed powerful drug molecules is strongly limited by their inadequate biopharmaceutical profiles. Moreover, it is sometimes difficult to synthesize new drugs with adequate stability and permeability properties. In this case, the drug is unable to reach the adequate biological compartment. Therefore, the development of appropriate delivery systems for these compounds would be a step forward for their clinical exploitation. Not only could the entrapment of these drugs in nanoparticles protect them from the biological environment, it also facilitates their transport through biological barriers.

Nanoparticles are generally but not necessarily defined as submicronic ($<1$ $\mu$m) colloidal systems, made of polymers preferably biodegradable. Nanoparticles are at least ten times smaller than microparticles ($>1$ $\mu$m), which allows them to be administered intravenously without any risk of embolization. According to the process and the composition used in the preparation of nanoparticles, nanospheres

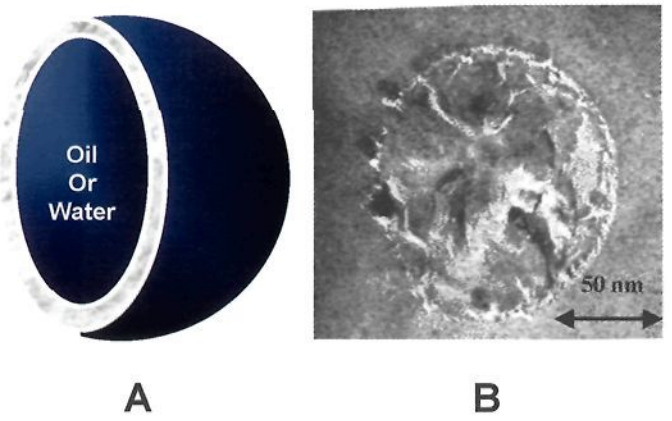


**Fig. 1.** (A) Schematic representation of the nanocapsule structure; (B) Morphological appearance of a nanocapsule with an oily core (transmission electron microscopy after freeze fracture).

or nanocapsules can be obtained. Nanospheres are matrix systems in which the drug is dispersed within the polymer throughout the particle. Contrarily, nanocapsules are vesicular or "reservoir" (heterogenous) systems, in which the drug is essentially confined to a cavity surrounded by a tiny polymeric membrane (Fig. 1). As in the case of nanospheres, depending on their physicochemical properties and composition, the drug may adsorb onto the surface as well as being included in the central core of nanocapsules. Therefore, drug localization is an important parameter in the characterization of nanocapsule preparations.

The nanocapsule core may be acqueous or composed of a lipophilic solvent, usually an oil. In order to achieve good drug loading, the core materials are chosen among the good solvents for the drug.[1] Expected advantages of confining the drug within a central cavity are: (a) burst effect may be avoided; (b) the drug is not in direct contact with tissues and therefore irritation at the site of administration could be reduced, and (c) the drug may be better protected from degradation both during storage and after administration. One of the advantages of nanocapsules over nanospheres is their low polymer content and a high loading capacity for lipophilic drugs.

Nanocapsules can either be obtained by interfacial polymerization of monomers or from preformed polymers. In the former, the molar mass of the coating polymer will depend on the preparation conditions and even on the drug used, whereas in the latter, it is determined at the outset. Polymerization of monomers may lead to a covalent linkage between the polymer and the drug. To date, all the methodologies described for preparing nanocapsules involve the preparation of emulsions. Oil-in-water (O/W) emulsions lead to the formation of nanocapsules with an oily core, suspended in water. Water-in-oil (W/O) emulsions lead to the

obtention of nanocapsules with an acqueous core, suspended in oil. More recently, nanocapsules with an acqueous core suspended in an acqueous medium were also obtained.

Nanocapsule technology and their pharmaceutical applications will be further discussed according to the method of obtaining the polymeric wall (polymerization *in situ* or preformed polymer) and whether the core is acqueous or oily.

## 2. Preparation

### 2.1. *Nanocapsules obtained by interfacial polymerization*

The advantage of obtaining nanocapsules by interfacial polymerization is that the polymer is formed *in situ*, allowing the polymer membrane to follow the contours of the inner phase of an O/W or W/O emulsion, thus entrapping drugs with high loadings. However, because reactive monomers are used, unwanted chemical reactions may occur between the drug and the monomer, before or during the polymerization process.

The preparation of nanocapsules by polymerization requires a fast polymerization of the monomers at the interface between the organic and the acqueous phase of the emulsions. Alkylcyanoacrylates, which polymerize within seconds, have been proposed for the preparation of both oil- and water-containing nanocapsules. Their polymerization is initiated by hydroxyl ions either from the equilibrium dissociation of water or by nucleophilic groups of any compound in the polymerization medium.[2]

#### 2.1.1. *Oil-containing nanocapsules*

The oil-containing nanocapsules are suitable for the encapsulation of the lipophilic and oil-soluble compounds. They are generally obtained by interfacial polymerization of alkylcyanoacrylates, after preparing a very fine oil-in-water emulsion with an additional water-miscible organic solvent such as ethanol or acetone.[3,4] These solvents serve as vehicles for the monomers, and also help to disperse the oil as very small droplets in the acqueous phase, which contains a hydrophilic surfactant. Indeed, as pointed out by Gallardo *et al.*,[5] the organic solvents must be completely water-miscible, so that the formation of small enough oil droplets occurs spontaneously, while the solvent is diffusing towards the acqueous phase and the water is diffusing toward the organic phase. Meanwhile, the polymerization of the monomer induced by the contact with hydroxyl ions from the water phase must be swift to allow efficient formation of the polymer envelope around the oil droplet, thus achieving effective encapsulation of drugs. Generally, particles with

sizes ranging between 250 and 300 nm, depending on the experimental conditions, were obtained.[5,6]

In a general procedure of nanocapsule preparation, the oil, the monomer, and the biologically active compound are dissolved together in the water-miscible organic solvent to prepare the organic phase.[3–9] This organic phase is then injected via a cannula, under strong stirring, into the acqueous phase containing water and a hydrophilic surfactant. The nanocapsules are formed to give a milky suspension immediately. The organic phase is then removed under reduced pressure and the nanocapsules are purified by ultracentrifugation. Depending on the density of the oil forming the core, nanocapsules will concentrate either as a pellet at the bottom of the ultracentrifuge tubes or as a floating layer at the top of the tubes.

A wide range of oils is suitable for the preparation of nanocapsules, including vegetable or mineral oils and pure compounds such as ethyl oleate and benzyl benzoate. The criteria for selection are the absence of toxicity, lack of affinity for the coating polymer, the absence of risk of degradation of the polymer, and a high capacity to dissolve the drug that is entrapped. Generally, Miglyol® is used to form the core of the nanocapsules.[3–7,9,10] Lipiodol® and benzyl benzoate have also been successfully used to form nanocapsules.[4] Soluble surfactants were chosen among Poloxamers,[3–9] Triton X100[9] and Tween 80.[9] In some cases, nanospheres formation together with nanocapsules were observed. Aprotic, fully water-soluble solvents such as acetone and acetonitrile lead to high-quality nanocapsule preparations, whereas protic water-miscible solvents including ethanol, *n*-butanol, and isopropanol promoted the formation of nanospheres during nanocapsule preparation.[5,9] It has been hypothesized that alcohols potentially initiate the polymerization reaction of alkylcyanoacrylates to form polymer nuclei or preformed polymers that may precipitate as nanospheres, when the organic phase is added to the acqueous phase.[5] Lowering the pH in the organic phase was shown to inhibit polymerization in this medium.[6]

Oil-containing nanocapsules have been used to encapsulate several types of biologically active compounds including both lipophilic molecules such as carbamazepine, indomethacin, lomustine, ethosuccimide, phenytoin,[1,10–14] and hydrophilic drugs such as peptides.[15–18] The lipophilic drugs were solubilized in the organic phase and were encapsulated during the preparation of the nanocapsules, usually using ethanol as the water-miscible organic solvent.[4,17] The encapsulation efficiency of lipophilic drugs was found to be related to their solubility in the encapsulated oil.[1] Quite surprisingly, hydrophilic compounds such as peptides have also been successfully encapsulated in oil-containing nanocapsules. Indeed, these highly water-soluble compounds do not tend to dissolve in oil. It has been suggested that the extremely rapid polymerization of the alkylcyanoacrylate occurring at the surface of the oil droplet limits the diffusion of the peptide towards the acqueous

phase, therefore leading to its entrapment in nanocapsules.[15] Another explanation is that surfactants may form inverse micelles in the oily phase, allowing some dissolution of hydrophilic compounds in this phase. Interestingly, in contrast to what has been observed with poly(alkylcyanoacrylate) nanospheres,[19] peptides do not react chemically with the alkylcyanoacrylate monomer during the preparation of nanocapsules when ethanol is used. The presence of a large excess of alcohol seems to prevent the hydroxyl and amino groups of the peptides from reacting with the monomer, thus retaining the biological activity of the entrapped peptides.[16–18,20,21] For example, encapsulated insulin was still recognized by the insulin receptor of hepatocytes after nanoencapsulation.[15,22]

### 2.1.2. *Nanocapsules containing an acqueous core*

Nanocapsules with an acqueous core are a recent technology developed for the efficient encapsulation of water-soluble compounds, which are generally difficult to include within nanospheres. They were obtained by interfacial polymerization, where the alkylcyanoacrylates monomers were added to a W/O emulsion.[23] Anionic polymerization of the cyanoacrylate in the oily phase was initiated at the interface by nucleophiles such as hydroxyl ions in the acqueous phase, leading to the formation of nanocapsules with an acqueous core. In a typical procedure (Fig. 2), an acqueous phase at pH 7.4, consisted of ethanol and water, was prepared.[23] This solution was emulsified in an organic phase containing Miglyol® and Montane®80. The slow addition (4 hrs) of the isobutylcyanoacrylate monomer in the organic phase under mechanical stirring allowed the polymerization to occur. This typical procedure leads to water droplets that are surrounded by a polymer core. The

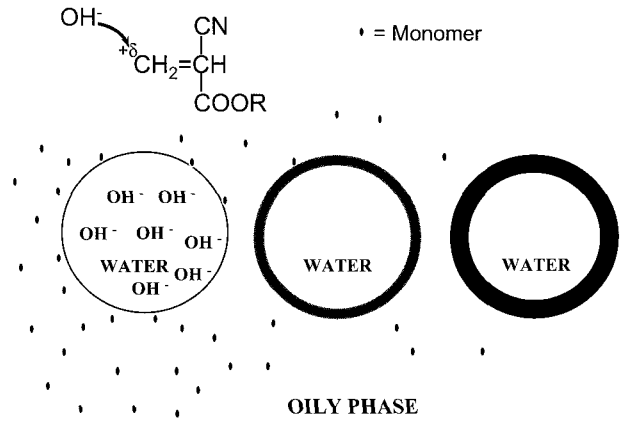


**Fig. 2.** Schematic representation of the interfacial polymerization of cyanoacrylic monomers leading to the formation of nanocapsules with an acqueous core.

resuspension of the nanocapsules with a mean diameter approximately 350 nm in a water phase has been achieved by the ultracentrifugation of the oily suspension, with an excess of demineralized water containing a surfactant. After removal of the upper oily phase, the nanocapsules pellet was resuspended in water.

These nanocapsules are very useful for the encapsulation of hydrophilic compounds such as oligonucleotides and peptides. In this case, these macromolecules are dissolved in the acqueous phase before the interfacial polymerization process takes place. For example, encapsulation efficiencies of 50% with an oligothymidylate (phosphodiester) and of 81% with a full phosphorothioate oligonucleotide (directed against EWS Fli-chimeric RNA) were obtained.[23,24] These entrapment differences were attributed to possible interactions of the oligonucleotides with the oily phase, Montane®80, or to the possible location of the oligonucleotide at the water-oil interface which could become saturated.[24]

The localization of the oligonucleotide (within the acqueous core or adsorbed on the surface) has been investigated through fluorescence quenching experiments using fluorescein-labeled oligonucleotide and potassium iodine as an external quencher.[23] It has been shown that fluorescent oligonucleotides were located in the acqueous core of the nanocapsules, surrounded by a polymeric wall, inaccessible to the quencher. On the contrary, when the fluorescent-oligonucleotides were free in solution, the fluorophores were highly accessible and strong quenching occurred. Similar quenching could be obtained with nanoencapsulated oligonucleotides only after the hydrolysis of the polymer wall, thus releasing the oligonucleotides.

Zeta potential experiments have confirmed the localization of oligonucleotide in the acqueous core of the capsule.[25] Moreover, nanoencapsulated oligonucleotides were protected against degradation by serum nucleases.[25,26] Phosphorothioate oligonucleotides directed against EWS Fli-1 chimeric RNA encapsulated within poly(alkylcyanoacrylate) nanocapsules were tested *in vivo* for their efficacy against the experimental Ewing sarcoma in mice after intratumoral administration.[24] Intratumoral injection of antisense-loaded nanocapsules led to a significant inhibition of tumor growth, whereas no antisense effect could be detected with the free oligonucleotide. These results were explained on the basis of a good protection of the oligonucleotide in the nanocapsules, which may act as a controled release system of oligonucleotide within the tumor.

Salmon calcitonin was also successfully entrapped within poly (butylcyanoacrylate) nanocapsules of 300 nm in diameter.[27] When the diameter was reduced to 50 nm, the encapsulation efficiencies decreased from 50 to 30%. After storage at room temperature or at 4°C, the nanocapsules retained their size for at least 34 months. The encapsulated calcitonin remained stable at 4°C for one year.

Polyalkylcyanoacrylate nanocapsules were also prepared by interfacial polymerization, using a microemulsion instead of an emulsion as the template.

Microemulsions are spontaneously forming, thermodynamically stable dispersed systems having a uniform droplet size of less than 200 nm. As such, they represent an interesting system that may be exploited for the preparation of nanocapsules too. Practically, a pseudo-ternary phase diagram of a mixture of medium chain glycerides (caprylic/capric triglycerides and mono-, diglycerides), a mixture of surfactants (polysorbate 80 and sorbitan monooleate) and water was constructed. Microemulsion domains were characterized by conductivity and viscosity to select systems suitable for the interfacial polymerization of ethyl-2-cyanoacrylate. Nanocapsules of 150 nm were obtained in those conditions and they were found to be able to encapsulate significant amounts of insulin.[28] Size of the capsules may be controled, depending on different formulation variables.[29] Factors influencing the encapsulation of hydrophilic compounds have been identified too.[30]

## 2.2. *Nanocapsules obtained from preformed polymers*

The preparation of nanocapsules from preformed polymers avoids some drawbacks of the interfacial polymerization process, such as the lack of control of the polymer molar masses and polydispersity, the presence of residual monomer in the preparation, and the possibility of drug inactivation.[31] An interfacial deposition process to prepare nanocapsules, also known as nanoprecipitation, has been developed.[32,33] In this simple and reproducible method, a water-miscible organic phase such as an alcohol or a ketone containing oil (with or without lipophilic surfactant) is mixed with an acqueous phase containing a hydrophilic surfactant. The preformed polymer, insoluble in both the oily and the acqueous phase, is solubilized in the organic phase. After the addition of the organic phase to the acqueous phase, the polymer diffuses with the organic solvent towards the acqueous phase and is stranded at the interface between oil and water. The driving force for nanocapsule formation is the rapid diffusion of the organic solvent in the acqueous phase, inducing interfacial nanoprecipitation of the polymer surrounding the droplets of the oily phase. Synthetic polymers such as poly(D,L-lactide), poly($\varepsilon$-caprolactone) and poly(alkylcyanoacrylate) are most frequently employed for nanocapsule formation.[32] Arabic gum, gelatin, ethylcellulose or hydroxypropylmethylcellulose phthalate were also successfully used.[32] The size of nanocapsules is usually found between 100 and 500 nm, and it depends on several factors, namely, the chemical nature and the concentration of the polymer and the encapsulated drug, the amount of surfactants, the ratio of organic solvent to water, the concentration of oil in the organic solution, and the speed of diffusion of the organic phase in the acqueous phase. In general, the lower the interfacial tension and the viscosity of the oil, the smaller the nanocapsules are formed.[34]

Both lipophilic and hydrophilic surfactants are used in the preparation of nanocapsules by this technique. However, not all the surfactants that are technically suitable are acceptable for parenteral administration; as such, the choice has to be made with the administration route in mind. Generally, the lipophilic surfactant is a natural lecithin of relatively low phosphatidylcholine content, whereas the hydrophilic one is ionic (i.e. lauryl sulphate, quaternary ammonium), or more commonly nonionic (i.e. poly(oxyethylene)-poly(propropylene) glycol).

Poly(ethylene glycol)-coated nanocapsules were also prepared by nanoprecipitation, using preformed diblock poly(lactide)-poly(ethylene glycol) copolymers or blends of these copolymers with the homopolymer poly(lactide.)[35–38] However, the most physically stable nanocapsules were those prepared with poly(lactide)-poly(ethylene glycol) copolymer alone. RU 58668, a promising pure antiestrogen, was entrapped into poly(ethylene glycol)-coated nanospheres and into nanocapsules with a similar coating.[37] A series of preformed diblock polyester-poly(ethylene glycol) copolymers were used for the design of these nanoparticles, both the molar masses of the poly(ethylene glycol) blocks and the nature of the hydrophobic polyester blocks being varied. Nanospheres which had a smaller size (≈110 nm), compared with nanocapsules (≈250 nm), were however able to incorporate larger amounts of the antioestrogen than the nanocapsules counterpart.

In an alternative method named solvent displacement method, an O/W emulsion was formed.[39] The organic phase contained the polymer, the oil and the drug, and the acqueous solution contained a stabilizing agent. In this procedure, the organic solvent was displaced into the external phase by the addition of an excess of water. This technique has several advantages such as the small quantities of solvents used, the good control of the size of the nanocapsules (80–900 nm), and the control of the thickness of the polymeric wall by monitoring the polymer concentrations.[40] However, large amounts of water have to be removed at the end of the process.

Two formulation processes which bring lipids into play should also be mentioned. The first methodology is based on the inversion phase of an emulsion to prepare original lipidic nanocapsules. These capsules, interestingly obtained as a suspension in saline water, were constituted by medium chain triglycerides and hydrophilic/lipophilic surfactants. According to the authors, the formulation method has been developed to avoid the use of organic solvent or the high quantity of surfactants and co-surfactants, due to the potential toxicity of their residues after human administration. Their original structure was found to be a hybrid between polymeric nanocapsules and liposomes as their oily core is being surrounded by a tensioactive rigid membrane.[41–43]

In another process, cisplatin lipid-based nanocapsules have been prepared by the repeated freezing and thawing of an equimolar dispersion of phosphatidylserine (PS) and phosphatidylcholine (PC) in a concentrated acqueous solution of cisplatin. Here, the molecular architecture of these novel nanostructures was elucidated by solid-state NMR techniques. $^{15}N$ NMR and $^{2}H$ NMR spectra of nanocapsules containing $^{15}N$- and $^{2}H$-labeled cisplatin respectively, demonstrated that the core of the nanocapsules consists of solid cisplatin devoid of free water. Magic-angle spinning $^{15}N$ NMR showed that approximately 90% of the cisplatin in the core is present as the dichloro species. The remaining 10% was accounted for by a newly discovered dinuclear Pt compound that was identified as the positively charged chloride-bridged dimer of cisplatin. NMR techniques, sensitive to lipid organization $^{31}P$ NMR and $^{2}H$ NMR, revealed that the cisplatin core is coated by phospholipids in a bilayer configuration and that the interaction between solid core and bilayer coat exerts a strong ordering effect on the phospholipid molecules. Compared with phospholipids in liposomal membranes, the motion of the phospholipid headgroups is restricted and the ordering of the acyl chains is increased, particularly in PS.[44] Analysis of the mechanism of the nanocapsule formation suggests that the method may be generalized to include other drugs showing low water solubility and lipophilicity.[45]

## 3. Characterization

Size evaluation of nanocapsules is most frequently done by photon correlation spectroscopy, transmission electron microscopy, and scanning electron microscopy, without or after freeze-fracture.[33,39,46] At present, transmission electron microscopy performed after freeze-fracture has given the most useful information about nanocapsule structure, highlighting the polymer envelope and the inner cavity, and allowing the wall thickness to be estimated.[1,7,47] Thus, polymer coatings were estimated to be around 5 nm, depending on the monomer concentration.[47] Freeze-fracture (Fig. 1) has also allowed the visualization of different possible organizations of lipophilic surfactant, which can form vesicles, micelles, bilayers, or monolayers, depending on its concentration.[33] The spherical shape of the nanocapsules was confirmed by atomic force microscopy.[39] Most images of nanocapsules have been obtained by transmission electron microscopy performed on negatively stained preparations, allowing to gain information about nanocapsule morphology and integrity[1,47] (Fig. 3A). Nanocapsules embedded in a suitable resin were cut into thin slices.[48] They were observed using electron microscopy, the contrast being created by encapsulation of a colloidal gold-labeled molecule during nanocapsule preparation. In this manner, both polymer envelope and the internal cavity were distinguished easily (Fig. 3B).

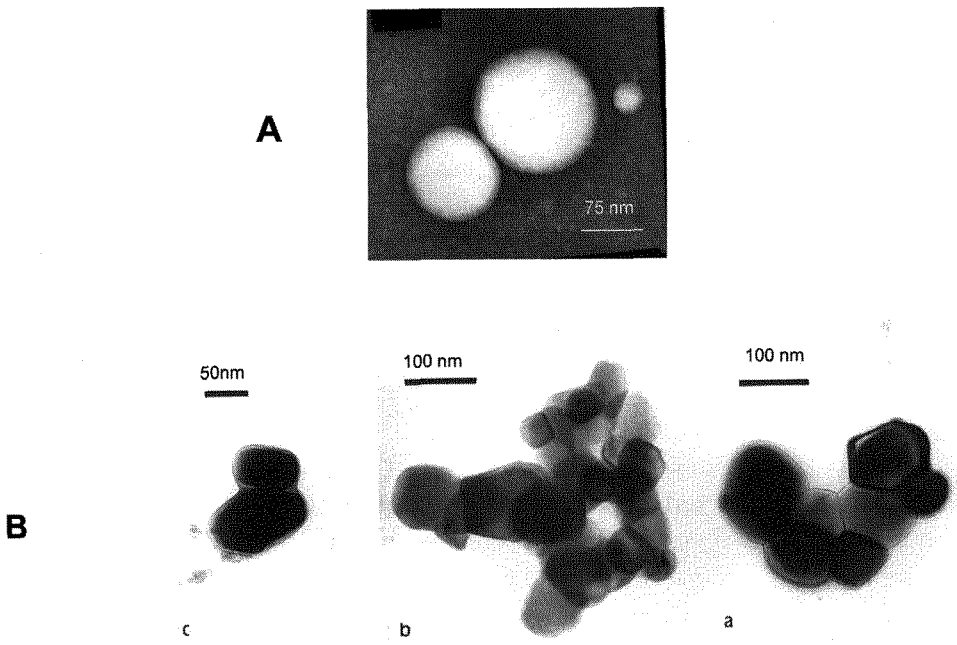


**Fig. 3.** (A) Morphological appearance of poly(lactic acid-co-glycolic) nanocapsules using the transmission electron microscopy. (B) Labeling insulin with gold allows to distinguish the localization of this molecule into the internal core of poly(isobutyl cyanoacrylate) nanocapsules; Transmission Electron Microscopy.

Zeta potential measurements are also very useful for the chraracterization of the nanocapsules. Surfactants and polymer are the major components that can affect this parameter. Many polymers such as poly (D,Llactide), poly($\varepsilon$-caprolactone) and lecithins impart a negative charge to the surface, whereas nonionic surfactants such as Poloxamer tend to reduce the absolute value of zeta potential.[34] Calvo *et al.*[49] described nanocapsules coated with positively charged polysaccharide chitosan. Their surface charge depended mainly on the viscosity of the chitosan solution used for coating. Positive values up to 46 mV were also observed with diethylaminoethyl-dextran coated nanocapsules.[8] Generally, Zeta potential values above 30 mV (positive or negative values) lead to more stable nanocapsule suspensions, because repulsion between the particles prevented their aggregation. In contrast to observations with nanospheres, the negative Zeta potential of the nanocapsules was not completely masked by the presence of neutral poly(ethylene glycol) chains at the surface.[63] This was due to the presence of lecithin in the poly(ethylene glycol) "brush", which remained necessary for nanocapsule stability. It was further highlighted that the presence of such a "brush" could reduce complement activation, an important step in the recognition of particles by macrophages.[50,51]

Centrifugation in a density gradient was used to confirm the existence of nanocapsules by comparing with the colloidal carriers prepared without polymer or oil. For example, isopycnic centrifugation in a density gradient of Percoll was used in the case of nanocapsules with a Miglyol core and a coating of poly(alkylcyanoacrylate) or poly(D,L lactide).[39] The density of the nanocapsules was found to be intermediate between that of nanospheres and that of emulsions. These studies also demonstrated that the density of nanocapsules and the band thickness increased when the quantity of polymer increased. No contamination of nanocapsules with nanospheres was observed. However, Mosqueira *et al.*[34] performed similar experiments and observed that nanocapsule preparations obtained by nanoprecipitation contained small amounts of nanospheres, as it has previously been described by Gallardo *et al.*[5] for nanocapsules prepared by interfacial polymerization. When lecithin was present in excess as lipophilic surfactant, liposomes were also detected in the nanocapsule preparations. Liposomes could not be distinguished from nanocapsules on the basis of density differences, but have been detected by electron microscopy[52] and by the encapsulation of an acqueous tracer.[34]

## 4. Drug Release

Release of encapsulated drugs from nanocapsules made of preformed polymers, appears only to be controled by the partition coefficient of the drug between the oily core and the acqueous external medium, and the relative volumes of these two phases. Except for macromolecules, the rate of diffusion of the drug through the thin polymeric coating does not seem to be a limiting factor, nor does the nature of the polymeric wall. This clearly suggests that the polymer membrane may be porous rather than a continuous film barrier to diffusional release. The nature of the external acqueous phase is of prime importance in the release. For example, indomethacin release was faster and more complete in the presence of albumin, which acts as an acceptor in the acqueous phase.[11,52] Similarly, release of halofantrine, a highly lipophilic drug, was only observed in the presence of serum, because the drug has a high affinity for lipoproteins.[36] The presence of a hydrophilic poly(ethylene glycol) "brush" at the nanocapsule surface was also shown to play a role in drug release. Release of halofantrine and primaquine from such surface-modified nanocapsules was reduced, compared with conventional nanocapsules.[36,53]

In conclusion, it may be considered a challenge to develop nanocapsule systems with release profiles, which may be controled not only by the partitioning coefficient, but also by the nature or morphology (i.e. thickness or porosity) of the surrounding membrane.

## 5. Applications

Nanocapsules have been proposed as drug delivery systems for several drugs by different routes of administration such as oral, ocular or parenteral. Drug-loaded nanocapsules were used to improve the stability of the drug either in biological fluids, or simply in the formulation. Another goal was to reduce the toxicity of some drugs known for their undesirable side effects.

### 5.1. *Oral route*

Challenging aspects related to oral administration deal with the entrapment of unstable molecules, such as peptides or that of anti-inflammatory compounds that cause local side effects on the mucosae. Pioneering studies in the mid 1980s dealt with indomethacin and insulin entrapment.

Indomethacin, an anti inflammatory drug, has been successfully encapsulated in the polyalkylcyanoacrylate nanocapsules with the aim of reducing its side effects on the gastric and intestinal mucosa.[11] The drug retained its biological activity after nanoencapsulation. Moreover, nanoencapsulated formulations allowed a dramatic reduction of the ulcerative side effects usually induced by indomethacin on the mucosae.[54] This protection was attributed to the combined effect of the sustained release of indomethacin from the nanocapsules, with a significant reduction of the direct contact between drug and the mucosae. In the case of nanocapsules obtained by nanoprecipitation using polyesters, the release kinetics in media mimicking pH of the gut were more sensitive to changes in drug partitioning related to the change of pH, than to the type of polymer used.[55,56] Drug release from nanocapsules was accelerated in the presence of digestive enzymes such as proteases and esterases. This was correlated with a decrease in polymer molecular weight.[55,56] Diclofenac and indomethacin, two major nonsteroidal anti inflammatory agents, have been encapsulated in poly(lactic acid) nanocapsules obtained by nanoprecipitation, with the aim of reducing their side effects on the gastric mucosa.[54,57,58] The side effects of both drugs were completely modified and reduced by the encapsulation in nanocapsules.[54] As in the case of nanocapsules produced by interfacial polymerization, a marked protective effect on the gastrointestinal mucosa, as compared with the ulcerative effect observed with the drug solutions, was observed.

Insulin-loaded nanocapsules yielded promising pharmacological results.[16,21] When given orally to diabetic rats and dogs, single administration produced a reduction in glycemia after an unusually long lag of several days, and this hypoglycemia was sustained for up to 20 days.[16,20,21,59] It was suggested that nanocapsules could release insulin slowly from a depot within the body. The nanocapsules seemed to be involved in carrying the insulin near the intestinal

epithelium where they were absorbed and translocated as intact nanocapsules to the blood vessels.[48,59–61] However, Lowe and Temple[16] reported that insulin adsorption from orally administered nanocapsules reached a maximum of absorption, 15 min after administration and any trace of insulin in blood was detected after a few hours. Sai *et al.*[62] have proposed the use of insulin-loaded nanocapsules as a new prophylactic tool to prevent diabetes. They showed in a model of non-obese diabetic mice that prophylactic injection of such nanocapsules reduced the incidence of diabetes.

Anti infectious agents such as atovaquone and rifabutin, two compounds active against the opportunistic parasite *Toxoplasma gondii*, were successfully entrapped in poly(lactide) nanocapsules formed by nanoprecipitation. These drugs have a poor bioavailability because of their insolubility in water. Nanoencapsulation is allowed to decrease in the brain parasitic burden in a higher extent than the same amount of free drug.[63]

Chitosan-coated nanocapsules were particularly interesting for oral administration, probably because their positive charge allow them to stick efficiently along the gastro-intestinal mucosa, with a further possible diffusion through the epithelium , thus providing a continuous drug delivery into the blood stream.[64,65] When the peptide salmon calcitonin was entrapped into these nanocapsules, long-lasting hypocalcemia effects were observed, following oral administration to rats.[66] In contrast, calcitonin control emulsions led to negligible responses.

## 5.2. *Parenteral route*

As far as the parenteral route is concerned, nanocapsules could be useful for the formulation of poorly soluble drugs, and for controling the drug biodistribution according to the properties of the carrier. In this view, indomethacin and diclofenac were entrapped in nanocapsules, but diclofenac in solution or in nanocapsules showed similar plasma concentration profiles. After intravenous administration, encapsulated indomethacin showed even lower plasma concentrations than the free drug because of enhanced hepatic uptake of loaded nanocapsules.[57] One possible explanation for the absence of the modification of the pharmacokinetics and biodistribution profiles of the encapsulated drugs probably results from the rapid rate of release of these drugs into the circulation, due to the high blood dilution and/or the presence of plasma proteins. Subcutaneous injection did not lead to a slow release of the drug either. Nevertheless, after intramuscular administration, the nanocapsules containing diclofenac showed a significantly reduced inflammation at the site of injection, compared with the free drug in solution.[67] Similarly, darodipine nanocapsules provided a prolonged antihypertensive effect compared with free drug which lasted for at least 24 hrs.[68]

Nanocapsules prepared by interfacial polymerization of the isobutylcyanoacrylate monomers were retained longer at the injection site after intramuscular administration than the other types of carriers such as emulsions or liposomes.[69] Moreover, they were taken up to a significant extent by the regional lymph nodes, likely owing to the phagocytosis by macrophages. These observations open up the possibility of delivering cytostatic drugs and immunomodulators to the lymph node metastases.

When administered intravenously, nanocapsules made by interfacial polymerization or by nanoprecipitation were taken up rapidly by organs of the mononuclear phagocyte system, mainly the liver.[70] To take advantage of this particular tissue distribution, nanocapsules containing muramyltripeptide cholesterol (MTP-Chol) were designed.[71,72] This immunostimulating agent, able to activate the macrophages and to stimulate their innate defense functions against tumor cells, is a useful agent to treat metastatic cancer. *In vitro* studies with rat alveolar macrophages have shown that nanocapsules prepared from poly(D,Llactic acid) containing MTP-Chol were more efficient activators than the free drug. This was attributed to the intracellular delivery of the nanoencapsulated immunomodulator after cell phagocytosis; an intermediate transfer of the drug to serum proteins was another suggested mechanism.[73] *In vivo*, this type of nanocapsules is allowed to obtain significant antimetastatic effects in a model of liver metastases.[74]

For other types of applications, to avoid the rapid clearance by the mononuclear phagocyte system, nanocapsules coated with poly(ethylene glycol) with a molar mass of 20,000 g/mole were developed. An antimalarial drug, halofantrine, was entrapped with the aim of obtaining a well-tolerated injectable form for the treatment of this severe intravascular disease.[36] In mice, at an advanced stage of infection with *Plasmodium berghei*, the area under the curve for plasma halofantrine was increased six-fold, compared with the free drug when the molecule was presented as nanocapsules. Moreover, the toxicity of halofantrine was reduced by incorporation into the nanocapsules. Up to 100 mg/kg could be administered intravenously without toxicity, yet all mice injected with this dose of free halofantrine died instantaneously. However, *in vivo*, only small differences were observed in terms of the therapeutic activity between poly(ethylene glycol) coated nanocapsules and the uncoated ones. This was explained by the possible saturation of the phagocytic capacity of the liver in severely infected mice, as a result of the uptake of parasitized erythrocytes.[75] Moreover, it was emphasized that the amount of serum lipoproteins, which acted as acceptors for halofantrine released from nanocapsules, is reduced during the disease.

Poly(ethylene glycol) coated nanocapsules were also used to deliver lipophilic drugs to the solid tumors. In this case, the vascular endothelium is known to be more permeable, thus allowing the extravasation of small-sized colloidal particles. This specific distribution of colloids into tumoral sites is known as the enhanced

permeability and retention effect (EPR effect). The efficacy of this strategy has been demonstrated using a photosensitizer, meta-tetra (hydroxyphenyl) chlorine, encapsulated in nanocapsules designed from diblock poly(D,L lactide)-poly(ethylene glycol) copolymers.[35]

## 5.3. *Ocular delivery*

The major problems encountered when delivering drugs to the eyes are the poor permeability of the corneal epithelium and the rapid clearance because of tear turnover and lacrimal drainage. Nanocapsule formulations were developed with the aim of improving drug efficacy by retaining it at the level of the ocular tissue, thus reducing the number of administrations.[76,77]

Betaxolol-loaded poly(isobutylcyanoacrylate) nanocapsules made by interfacial polymerization were prepared for the treatment of glaucoma. Only a marginal decrease in the intraocular pressure was observed with this type of formulation, compared with the activity obtained with the commercial form (single solution) or by other carriers.[13] More promising results have been obtained with pilocarpine.[14] In this case, sustained drug release was obtained when incorporating the pilocarpine loaded nanocapsules into a Pluronic gel. Thus, a significant increase in the bioavailability of the drug was achieved.

Ganciclovir is an antiviral drug used for the treatment of cytomegalovirus infections. In the clinical practice, two to three intravitreal injections per week are needed to overcome the rapid clearance of the drug from the eyes. Ganciclovir encapsulation in poly(ethylcyanoacrylate) nanocapsules made by interfacial polymerization provided a sustained release of the drug over four days.[10] Moreover, after intravitreal injection of the nanocapsules, the drug could still be detected in the eyes at a therapeutic level after ten days. Significant amounts of ganciclovir were found in the retina and in the vitreous humor which is considered as beneficial in the treatment of cytomegalovirus retinitis. On the contrary, after administration of single solutions of the drug free, the maximum concentration of ganciclovir was reached in less than one day and no drug could be detected later. However, despite these beneficial results, some toxicity (opacification of the lens and vitreous humor turbidity) was found as a result of the nanocapsules.

Antiglaucomatous agents such as carteolol and betaxolol were also encapsulated in nanocapsules prepared from preformed polymers, but they only showed a reduction of the noncorneal absorption (systemic circulation), leading to lesser side effects as compared with the free drug.[13,78,79] Encapsulation in nanocapsules produced an improved pharmacological effect characterized by a more important reduction of the intraocular pressure, compared with the free drug treatment, as well as with the same treatment but delivered by nanospheres; reduced

cardiovascular systemic side effects were also observed with the nanocapsules.[13,78] In the case of betaxolol, the nature of the polymer making up the nanocapsule wall was found to play a major role in the pharmacological responses.[78,80] Thus, poly(ε-caprolactone) walls were more efficient than poly(isobutylcyanocrylate) or poly(lactide-co-glycolide) ones. Indeed, as shown by the confocal microscopy, poly(ε-caprolactone) nanocapsules could specifically penetrate the corneal epithelium by an endocytic process, without causing any damage to the cells. In contrast, poly(isobutylcyanoacrylate) nanoparticles produced a cellular lysis.[81] As no differences in penetration were observed between nanospheres and nanocapsules, the presence of an oily core did not seem to influence activity of the formulation. Coating the negatively charged surface of poly(ε-caprolactone) nanocapsules with chitosan, a cationic polymer, provided the best corneal drug penetration, together with preventing the degradation caused by the adsorption of lysozyme, a positively charged enzyme found in tear fluid.[82] This was explained by the higher penetration of the nanocapsules into the corneal epithelial cells and by the mucoadhesion of these positively charged particles onto the negatively charged membranes. Additionally, a specific effect of chitosan on the tight junctions has been mentioned.[83]

Encouraging results were also obtained with nanocapsules containing the immunosuppressive peptide cyclosporin A.[84] This drug was efficiently entrapped in poly(ε-caprolactone) nanocapsules, leading to a five-fold increase of the cyclosporin A corneal concentrations, compared with an oily solution of the drug. Again, chitosan-overcoated nanocapsules were able to provide a selective and prolonged delivery of cyclosporine A to the ocular mucosae, without compromising the inner ocular tissues and avoiding systemic absorption.[84] The mechanism that explains the increased ocular penetration was understood as the combination of an improved interaction with the corneal epithelium, followed by the penetration of the particles into the corneal epithelium.[85] In the case of indomethacin associated with chitosane-coated nanocapsules, the use of confocal microscopy established the fact that the nanocapsules penetrated through the corneal epithelium following a transcellular pathway.[85,86]

## 6. Conclusion

As discussed in this chapter, there are now various technologies for the preparation of nanocapsules. These methods which obey a wide variety of principles may either start from a monomer or from a preformed polymer. They employ macromolecular materials of synthetic or natural origin and they allow the design of nanocapsules with either an acqueous or an oily core. Thus, they can efficiently entrap almost every molecule. The most significant advantage of nanocapsules over nanospheres is that the drug to polymer ratio is generally much higher, which allows the use of

lesser polymer to deliver the same amount of drug to the cells and tissues. This is, from a toxicological point of view, a substantial advantage of this type of technology. On the contrary, drug release from nanocapsules is mainly dependent on the partitioning coefficient of the biologically active compound between the nanocapsule core and the biological receptor medium. If the nanocapsule thin polymer membrane may be a barrier for the diffusion of macromolecules, it is not the case for small organic molecules. Thus, to control the drug release kinetic from nanocapsules, it is likely to remain the primary challenge to be resolved with this kind of technology in the next few years.

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# 13

# Dendrimers as Nanoparticulate Drug Carriers

*Sönke Svenson and Donald A. Tomalia*

## 1. Introduction

The development of molecular nanostructures with well-defined particle size and shape is of eminent interest in biomedical applications such as the delivery of active pharmaceuticals, imaging agents, or gene transfection. For example, constructs utilized as carriers in drug delivery generally should be in the nanometer range and uniform in size to enhance their ability to cross cell membranes and reduce the risk of undesired clearance from the body through the liver or spleen. Two traditional routes to produce particles that will meet some of these requirements have been widely investigated. The first route takes advantage of the ability of amphiphilic molecules (i.e. molecules consisting of a hydrophilic and hydrophobic moiety) to self-assemble in water above a system-specific critical micelle concentration (CMC) to form micelles. Size and shape of these micelles depend on the geometry of the constituent monomers, intermolecular interactions, and conditions of the bulk solution (i.e. concentration, ionic strength, pH, and temperature). Spherical micelles are monodisperse in size; however, they are highly dynamic in nature with monomer exchange rates in millisecond to microsecond time ranges. Micelles have the ability to encapsulate and carry lipophilic actives within their hydrocarbon cores. Depending on the specific system, some micelles either spontaneously rearrange to form liposomes after a minor change of solution conditions, or when they are exposed to external energy input such as agitation, sonication, or extrusion through a filter

membrane. Liposomes consist of bilayer lipid membranes (BLM) enclosing an aqueous core, which can be utilized to carry hydrophilic actives. Furthermore, liposomes with multilamellar membranes provide cargo space for lipophilic actives as well. However, most liposomes are considered energetically metastable, and will eventually rearrange to form planar bilayers.[1,2] The second route relies on engineering the well-defined particles through processing protocols. Examples for this approach include (i) shearing or homogenization of oil-in-water (o/w) emulsions or w/o/w double emulsions to produce stable and monodisperse droplets, (ii) extrusion of polymer strands or viscous gels through nozzles of defined size to manufacture stable and monodisperse micro and nanospheres, (iii) layer-by-layer (LbL) deposition of polyelectrolytes and other polymeric molecules around colloidal cores, resulting in the formation of monodisperse nanocapsules after the removal of the templating core, and (iv) controlled precipitation from a solution into an anti-solvent, including supercritical fluids. Size, degree of monodispersity, and stability of these structures depend on the systems that are being used in these applications.[3] These systems and their utilization in drug delivery are being discussed in detail in other chapters of this book.

Currently, a new third route to create very well-defined, monodisperse, stable molecular level nanostructures is being studied based on the "dendritic state" architecture.[4] Dendritic architecture is undoubtedly one of the most pervasive topologies observed throughout biological systems at virtually all dimensional length scales. This architecture is found at the meter scale in tree branching and roots, on the centimeter and millimeter scales in circulatory topologies in the human anatomy such as lungs, kidney, liver, and spleen, and on the micrometer scale in cerebral neurons. On the nanometer level, key examples of dendritic structures include glycogen, amylopectin, and proteoglycans. Amylopectins and glycogen are critical molecular level constructs involved in energy storage in plants and animals, while proteoglycans are an important constituent of connective tissue, determining its viscoelastic properties. Upon the analysis of these ubiquitous dendritic patterns, it is evident that these highly branched architectures offer unique interfacial and functional performance advantages. The objective of this review is to study the use of dendrimers in drug delivery applications. Four main properties of dendrimers will be discussed: (i) nanoscale container properties (i.e. encapsulation and transport of a drug), (ii) nano-scaffolding properties (i.e. surface adsorption or attachment of a drug and/or targeting ligand), (iii) dendrimers as drugs, and (iv) biocompatibility of dendrimers. In addition, routes of application currently investigated will be presented. Particular emphasis will be placed on poly (amidoamine) (PAMAM) dendrimers, the first and most extensively studied family of dendrimers.[4c,5]

## 2. Nanoscale Containers — Micelles, Dendritic Boxes, Dendrophanes, and Dendroclefts

Dendrimers may be visualized as consisting of three critical architectural domains: (i) the multivalent surface, containing a larger number of potentially reactive/passive sites (nano-scaffolding), (ii) the interior shells (i.e. branch cell layers defined by dendrons) surrounding the core, and (iii) the core to which the dendrons are attached. The two latter domains represent well-defined nano-environments, which are protected from the outside by the dendrimer surface (nanoscale containers) in the case of higher generation dendrimers. These domains can be tailored for a specific purpose. The interior is well-suited for host-guest interaction and the encapsulation of guest molecules.

### 2.1. *Dendritic micelles*

Tomalia and coworkers demonstrated by electron microscopy observation that sodium carboxylated PAMAM dendrimers possess topologies reminiscent of regular classical micelles.[4] It was also noted from electron micrographs that a large population of individual dendrimers possessed a hollow core. Supporting these observations, Turro and colleagues designed a hydrophobic 12-carbon atom alkylene chain into the core of a homologous series of PAMAM dendrimers (G = 2, 3, and 4) to mimic the hydrophobic and hydrophilic core-shell topology of a regular micelle. The hosting properties of this series towards a hydrophobic dye as a guest molecule were then compared with a PAMAM dendrimer series possessing non-hydrophobic cores (e.g. $NH_3$ and ethylenediamine). Dramatically enhanced emission of the hydrophobic dye was noted in aqueous solution in the presence of hydrophobic versus hydrophilic cored dendrimers.[6a] Less polar dendrimers (i.e. dendrimers containing aryl groups or other hydrophobic moieties as building elements), behave as inverse micelles.[6b] A critical property difference relative to micelles is the increased density of surface groups with higher generations. At some generational level, the surface groups will reach the so-called "de Gennes dense packing" limit and seal the interior from the bulk solution (Fig. 1).[7–9] The limit depends on the strength of intramolecular interactions between adjacent surface groups, and therefore, on the condition of the bulk solution (i.e. pH, polarity and temperature).

This nanoscale container feature, originally noted for PAMAM dendrimers by Tomalia *et al.* and referred to as "unimolecular encapsulation", can be utilized to tailor the encapsulation and release properties of dendrimers in drug delivery applications.[9,10] For example, adding up to a limiting amount of *X* mmol of either 2,4-dichlorophenoxyacetic acid or aspirin (acetylsalicylic acid) to 1 mmol of

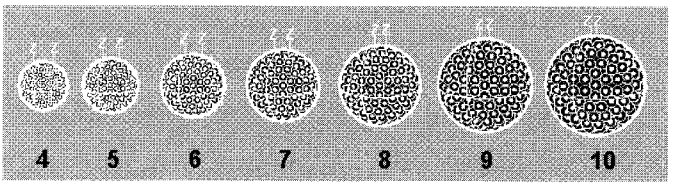


**Fig. 1.** Periodic properties of PAMAM dendrimers generations G = 4–10, depicting the decreasing distances between surface charges (Z-Z). The "de Gennes dense packing" appears at G = 8. Dendrimers G = 4–6 display "nanoscale container" properties, the larger analogues G = 7–10 display "nano-scaffolding" properties.

STARBURST® carbomethoxy-terminated PAMAM dendrimers generations 0.5–5.5 produced spin-lattice relaxation times ($T_1$) much lower than the values of these guest molecules in solvent without dendrimer. The new relaxation times decreased for generations 0.5–3.5, but remained constant for generations 3.5 to 5.5. The maximum concentration $X$ varied uniformly from 12 (generation 0.5) to 68 (generation 5.5). On the basis of these maximum concentrations, the guest-to-host ratios were shown to be ~4:1 by weight and ~3:1 based on a molar comparison of dendrimer guest carboxylic acid-to-interior tertiary nitrogen moieties for generations 2.5–5.5. Exceeding the maximum concentration $X$ resulted in the appearance of a second relaxation time, $T_{1'}$, characteristic of the guest molecules in bulk solvent phase.[10]

## 2.2. *Dendritic box (Nano container)*

Surface-modification of G = 5 poly(propyleneimine) (PPI) dendrimers with Boc-protected amino acids induced dendrimer encapsulation properties by the formation of dense, hydrogen-bonded surface shells with solid-state character ("dendritic box").[8] Small guest-molecules were captured in such dendrimer interiors and were unable to escape even after extensive dialysis. The maximum amount of entrapped guest molecules was directly proportional to the shape and size of the guest molecules, as well as to the amount, shape and size of the available internal dendrimer cavities. Four large guest-molecules (i.e. Rose Bengal) and 8–10 small guest-molecules (i.e. p-nitrobenzoic acid) could be simultaneously encapsulated within PPI dendrimers containing four large and twelve smaller cavities. Remarkably, this dendritic box could be opened under controlled conditions to release either some or all of the entrapped guest molecules. For example, partial hydrolysis of the hydrogen-bonded Boc-shell liberated only small guest-molecules, whereas total hydrolysis released all sizes of entrapped molecules.[8,11,12]

Although the "dendritic box" concept demonstrates the unique shape-dependent cargo space that can be found in certain dendrimers, other parameters have to be considered as well for delivering and releasing therapeutic drugs

under physiological conditions. From a thermodynamic perspective, free guest-molecules (i.e. drugs) can be distinguished from those encapsulated or bound in a complex by finite energy barriers related to the ease of entry and departure to the dendrimer cavities. If the drug molecule is incompatible with either the dimension or hydrophilic/lipophilic character of the dendrimer cavity, a complex might not form, or the guest might only be partially encapsulated within the dendrimer host. A hydrophobic drug would be expected to associate with a dendrimer core to achieve maximum contact with its hydrophobic domain. In addition, the hydrophobic character of this guest molecule would be expected to isolate itself from the dendrimer surface and the interface to the bulk solution to afford minimum contact with polar and aqueous domains (i.e. physiological media). Notably, the hydrophobic and hydrophilic properties, as well as other non-covalent binding properties of these spatial binding-sites are expected to strongly influence these guest–host relationships. Analysis of a typical symmetrically branched dendrimer makes it apparent that there are other subtle and yet important parameters that could control the interior space of a dendrimer and influence the guest–host interactions. These include components such as branching angles, branching symmetry rotational angles, and the length of a repeat-unit segment.[13] Of equal importance are the properties of the core. Within a homologous PAMAM dendrimer series, the effect of changing the length scale of the core on dendrimer guest–host properties was studied. Specifically, a series of polyhydroxy-surfaced PAMAM dendrimers with core molecules differing in length by one carbon atom ($NH_2$-$C_n$-$NH_2$ with $n$ = 2–6) were synthesized. Three aromatic carboxylic acids, differing systematically by one aromatic ring (benzoic acid, 1-naphthoic acid, 9-anthracene carboxylic acid), were examined as guest-molecule probes. Two sets of dendrimers, possessing 24 and 48 surface hydroxy groups, were investigated.[14] The observed trends can be summarized as follows: (i) in general, all dendritic hosts accommodated larger amounts of the smaller guest-molecule (i.e. molar uptake benzoic > 1-naphthoic > 9-anthracene carboxylic acid). This observation was particularly significant for the more congested dendrimer surface having 48 surface OH-groups. (ii) Uptake maxima values specific to both the core size and the specific guest-probe were noted. This observation might be related to the combination of shape and lipophilicity manifested by the guest probe. (iii) A decrease in the molar uptake was measured for all probes as the core was enhanced beyond an ideal dimension (i.e. 5–6 carbons). It is therefore obvious that both core size and surface congestion dramatically affect the cargo-space of the dendrimer host. Furthermore, it is apparent that size and shape of the guest probe can significantly affect the maximum loading as a function of core size. Finally, it should also be noted that for the dendrimers G = 2 (24-OH) and G = 3 (48-OH), the guest probes had desirable release properties from the host as a function of time, when re-dissolved in water. Performing these same experiments using a dendrimer

with more densely packed surface groups (i.e. G = 4 with 96 surface OH-groups) appeared to produce dendritic box behavior. Although guest molecules could be encapsulated within the core, the release from the host was delayed as determined by analysis after extensive dialysis.[14] Structure-property relationships in dendritic encapsulation have been studied extensively, mainly using photoactive and redox-active model dendrimers to gain a better understanding of the structural effects that cores and branches have on encapsulation.[15–17]

### 2.3. *Dendrophanes and dendroclefts*

Specific binding of guest molecules to the dendrimer core can affect the loading capacity by enhancing specific interactions between the core and guest (i.e. hydrophobic and polar interactions). Dendrimers specifically tailored to bind hydrophobic guests to the core have been created by Diederich and coworkers and coined "dendrophanes". These water-soluble dendrophanes are built around a cyclophane core, and can bind aromatic compounds, presumably *via* p–p interactions. Dendrophanes were shown to be excellent carriers of steroids.[18,19] The same group synthesized dendrimers tailored to bind more polar bioactive compounds to the core, coined "dendroclefts".[20,21] In another approach, the surface amines of PAMAM dendrimers were modified with tris(hydroxymethyl)aminomethane (TRIS) to create water-soluble dendrimers capable of binding carboxylic aromatic, antibacterial compounds, which could be released by lowering the pH.[14] An alternative approach to creating dendritic hosts with highly selective guest recognition utilized the principle of "molecular imprinting".[22] A dendrimer consisting of a porphyrin core and a surface containing terminal double bonds was polymerized into a polydendritic network. Subsequently, the base-labile ester bonds between cores and dendritic wedges were cleaved, releasing the porphyrin core from the dendritic polymer. This polymer was capable of selectively binding porphyrins with association constants of $1.4 \times 10^5\ M^{-1}$. Very recently, an impressive approach has been presented, using tandem mass spectrometry, i.e. the combination of electrospray ionization (ESI) and collision-induced dissociation (CID) mass spectrometers connected in series, to investigate the dynamic behavior of host-guest dendrimer complexes.[23] This approach offers the potential to provide better insights into these constructs.

## 3. Dendrimers in Drug Delivery

Dendrimers have been utilized to carry a variety of small molecule pharmaceuticals with the purpose to enhance their solubility and therefore bioavailability, and to utilize the passive and active targeting properties of dendrimers, either through the

"Enhanced Permeability and Retention" (EPR)[24] effect or specific targeting ligands. Some aspects of dendrimers in drug delivery have been reviewed recently.[13,25–27] In the following, selected examples of important drug delivery aspects will be presented.

## 3.1. *Cisplatin*

Encapsulation of the well-known anticancer drug cisplatin within PAMAM dendrimers gives complexes that exhibit slower release, higher accumulation in solid tumors, and lower toxicity compared with free cisplatin.[28,29] Cisplatin is an antitumor drug that exerts its effects by forming stable DNA-cisplatin complexes through intrastrand cross-links, resulting in an alteration of the DNA structure that prevents replication and activates cell repair mechanisms. The cell detects defective DNA and initiates apoptosis. Cisplatin is effective in treating several cancers such as ovarian, head and neck, and lung cancers, as well as melanomas, lymphomas, osteosarcomas, bladder, cervical, bronchogenic, and oropharyngeal carcinomas. Unfortunately, cisplatin has many adverse side effects to the body, the most important being nephrotoxicity and cytotoxicity to non-cancerous tissue, because of the non-selective interaction between cisplatin and DNA. In addition, the therapeutic effect of cisplatin is limited by its poor water solubility (1 mg/mL), low lipophilicity, and the development of resistance to cisplatin drugs. Although numerous cisplatin derivatives have undergone preclinical and clinical testing, only cisplatin and its derivatives carboplatin and oxaliplatin have been approved for routine clinical use (Fig. 2).[30]

Preliminary studies gave cisplatin loadings of 15–25 wt% for PAMAM dendrimers generation 3.5 (size ~ 3.5 nm; MW ~ 13 kDa). In comparison, the cisplatin loading of linear poly(amidoamines) and linear N-(2-hydroxypropyl) methacrylamide (HPMA; MW 25–31 kDa) was found to be 5–10 wt% and 3–8 wt%, respectively. HPMA-cisplatin complexes are currently in clinical trials.[31] The cisplatin-dendrimer complex could be visualized by Atomic Force Microscopy (AFM; carbon nanotip) as shown in Fig. 3.

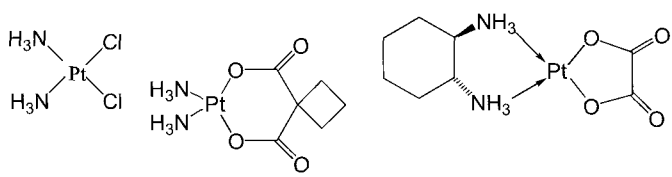


**Fig. 2.** Chemical structures of the platinum drugs cisplatin (PLATINOL®), carboplatin (PARAPLATIN®), and oxaliplatin (ELOXATIN™).

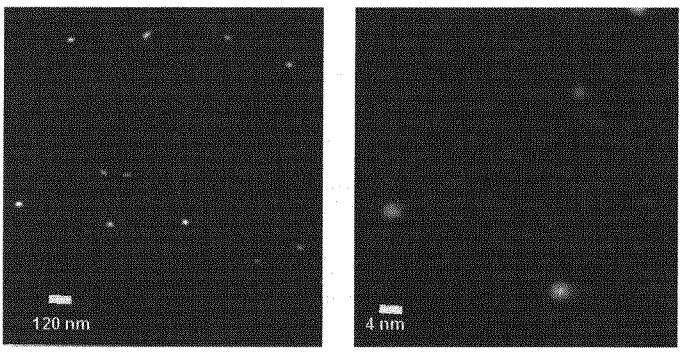


**Fig. 3.** AFM images of cisplatin-dendrimer complexes at 120 (left) and 4 nm (right) magnification.

**Table 1** AUC value ($\mu$g Pt/mL blood or $\mu$g Pt/organ) over 48 hours; 5 mice/data point.

| Organ | Cisplatin | Cisplatin-dendrimer Complex |
|---|---|---|
| Tumor | 5.3 | 25.4 |
| Blood | 9.4 | 10.7 |
| Liver | 51.6 | 17.0 |
| Kidney | 57.6 | 138.1 |

The tumor activity of the cisplatin-dendrimer formulation was studied using B16F10 cells. These cells were injected into C57 mice subcutaneously (s.c.) to provide a solid tumor model. After approximately 12 days, when the tumors had developed to a mean area of 50–100 $mm^2$, the animals were injected i.v. with a single dose of either cisplatin or cisplatin-dendrimer complex (1 mg/kg cisplatin for both formulations). At certain time points within 48 hours, animals were culled and blood and tissue samples were taken. Compared with cisplatin alone, the cisplatin-dendrimer complex was found to accumulate preferentially in the tumor site relatively quickly after the injection. The tumor area under the curve (AUC) for the complex was 5 times higher than that of free cisplatin, while that in the kidney only increased 2.4 times, and accumulation in the liver was reduced (Table 1).[29]

Another recent study revealed a sufficient stability of cisplatin-dendrimer complexes, with a 20% release of cisplatin over the first 8 hours, and an additional 60% release within 150 hours. *In vivo* animal efficacy of the platinate was demonstrated using B16F10 tumor cells that are subcutaneous implanted into mice. The tumor was allowed to grow for 7 days prior to treatment with two doses of drug on day 7 and day 14, providing equal cisplatin (5 mg/kg) doses in both the dendrimer-cisplatin complex and free cisplatin. A tumor weight reduction of ~40% above that observed for the free drug was found in this study.

## 3.2. *Silver salts*

The encapsulation of silver salts within PAMAM dendrimers produced conjugates, exhibiting slow silver release rates and antimicrobial activity against various Gram positive bacteria.[32] PAMAM dendrimers, generation four with ethylenediamine (EDA) core and tris(2-hydroxymethyl)amidomethane (TRIS) OH-surface and generation five, EDA core with carboxylate $COO^-$ surface, were used. Silver containing PAMAM complexes were prepared by adding aqueous solutions of the dendrimers to the calculated amount of silver acetate powder. Although $CH_3COOAg$ is hardly soluble in water, it quickly dissolved in the PAMAM solutions. This enhancement is due to the combined action of the silver carboxylate salt formation and/or to the complex formation with the internal dendrimer nitrogens. This procedure resulted in slightly yellow dendrimer-complex/salt solutions that very slowly photolyzed when exposed to light, into dark brown, metallic silver, containing dendrimer-silver nanocomposite solutions. Final sample concentrations were confirmed by atomic absorption spectroscopy. For antimicrobial testing, the standard agar overlay method was used. In this test, dendrimer-silver compounds were examined for diffusible antimicrobial activity by placing a 10-$\mu$L sample of each solution onto a 6-mm filter paper disk and applying the disk to a dilute population of the test organisms, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The silver-dendrimer complexes displayed antimicrobial activity, comparable to or better than those of silver nitrate solutions. Interestingly, increased antimicrobial activity was observed with dendrimer carboxylate salts, which was attributed to the very high local concentration (256 carboxylate groups around a 5.4 nm diameter sphere) of nanoscopic size silver composite particles that are accessible for microorganisms. The antimicrobial activity was smaller when internal silver complexes were applied instead of silver adducts to the surface, indicating that the accessibility of the silver is an important factor.

## 3.3. *Adriamycin, methotrexate, and 5-fluorouracil*

The anticancer drugs, adriamycin and methotrexate, were encapsulated into generations 3 and 4 PAMAM dendrimers which had poly(ethylene glycol) monomethyl ether chains with molecular weights of 550 and 2000 Da attached to their surfaces via urethane bonds (Fig. 4). The encapsulation efficiency was dependent on the PEG chain length and the size of the dendrimer, with the highest encapsulation efficiencies (on average, 6.5 adriamycin molecules and 26 methotrexate molecules per dendrimer) found for the G = 4 PAMAM terminated with $PEG_{2000}$ chains. The drug release from this dendrimer was sustained at low ionic strength, again reflecting PEG chain length and dendrimer size, but fast in isotonic solution.[33] In a related study, it was reported that the surface coverage of PAMAM dendrimers with

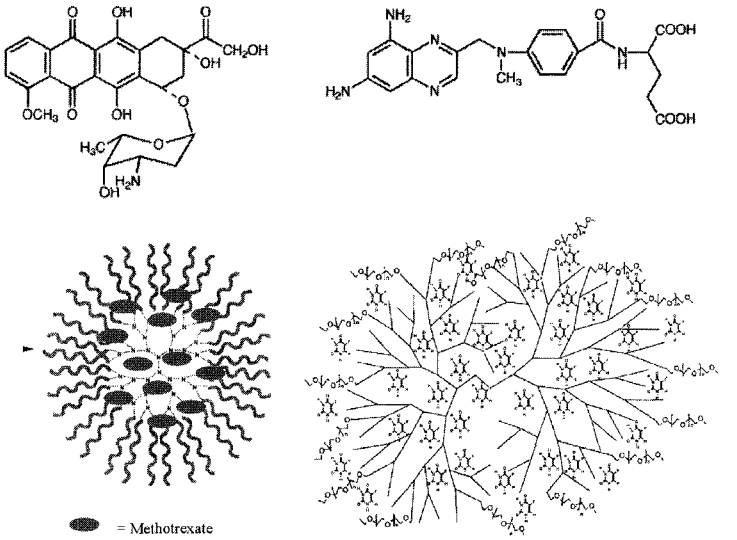


**Fig. 4.** Above: Structures of anticancer drugs adriamycin (left) and methotrexate (right). Below: Schematic presentations of the encapsulation of methotrexate (left) and 5-fluorouracil (right) into PAMAM dendrimers.

$PEG_{2000}$ chains had little influence on the encapsulation efficiency of methotrexate, but affected the release rate.[34]

A similar construct between PEG chains and PAMAM was utilized to deliver the anticancer drug 5-fluorouracil. Encapsulation of 5-fluorouracil into G = 4 PAMAM dendrimers with carboxymethyl $PEG_{5000}$ surface chains revealed reasonable drug loading, a reduced release rate, and reduced hemolytic toxicity compared to the non-PEGylated dendrimer (Fig. 4).[35]

## 3.4. *Etoposide, mefenamic acid, diclofenac, and venlafaxine*

The combination between dendrimers and hydrophilic and/or hydrophobic polymer chains has recently been extended to solubilize the hydrophobic anticancer drug etoposide. A star polymer composed of amphiphilic block copolymer arms has been synthesized and characterized. The core of the star polymer was a generation two PAMAM-OH dendrimer, the inner block of the arm a lipophilic poly($\epsilon$-caprolactone) (PCL) and the outer block of the arm a hydrophilic $PEG_{5000}$. The star-PCL polymer was synthesized first by ring-opening polymerization of $\epsilon$-caprolactone with the PAMAM-OH dendrimer as initiator. The PEG polymer was then attached to the PCL terminus by an ester-forming reaction. Characterization with SEC, 1-H NMR, FTIR, TGA, and DSC confirmed the star structure of the polymers. A loading capacity of up to 22% (w/w) was achieved with etoposide.

A cytotoxicity assay demonstrated that the star-PCL-PEG copolymer was nontoxic in cell culture.[36]

Citric acid-poly(ethylene glycol)-citric acid (CPEGC) triblock dendrimers generations 1–3 were applied to encapsulate small molecule drugs such as mefenamic acid and diclofenac. The formulations were stored at room temperature for up to ten months and remained stable with no reported release of the drugs.[37]

The attachment of the novel third-generation antidepressant venlafaxine onto anionic PAMAM dendrimers (G = 2.5) via a hydrolyzable ester bond and the incorporation of this drug-dendrimer complex into a semi-interpenetrating network of an acrylamide hydrogel has been studied as a novel drug delivery formulation to avoid the currently necessary multiple daily administration of the antidepressant. The effect of PEG concentration and molecular weight was studied to find optimal release conditions.[38]

## 3.5. *Ibuprofen, indomethacin, nifedipine, naproxen, paclitaxel, and methylprednisolone*

The anti-inflammatory drug ibuprofen was used as a model compound to study its complexation and encapsulation into generations 3 and 4 PAMAM dendrimers and a hyperbranched polyester, having approximately 128 surface OH-groups. It was found that up to 78 ibuprofen molecules were complexed by the PAMAM dendrimers through electrostatic interactions between the dendrimer amines and the carboxyl group of the drug. In contrast, up to 24 drug molecules were encapsulated into the hyperbranched polyol.[39] The drug was successfully transported into A549 human lung epithelial carcinoma cells by the dendrimers. The PAMAM dendrimers with either amino or hydroxy surfaces entered the cells faster (in approximately 1 hr) than the hyperbranched polyol (approximately 2 hrs). However, both entries were faster than the pure drug. The anti-inflammatory effect of ibuprofen-dendrimer complexes was demonstrated by more rapid suppression of COX-2 mRNA levels than that achieved by the pure drug.[40]

The non-steroidal anti-inflammatory drug (NSAID) indomethacin is practically insoluble in water and only sparingly soluble in alcohol. Encapsulation of indomethacin into generation 4 PAMAM dendrimers with amino, hydroxy, and carboxylate surfaces remarkably enhanced the drug solubility in water, and therefore, its bioavailability (Fig. 5).[41] The encapsulation efficiency of indomethacin into PAMAM dendrimers is dependent on the dendrimer size (G6 > G5 > G4 > G3) and the surface functionalization, (NH2 > PEG = PYR > AE) (Fig. 6).[42]

The effect of PAMAM dendrimer generation size and surface functional group on the aqueous solubility, and therefore, bioavailability of the calcium channel blocking agent nifedipine has been studied using PAMAM dendrimers with EDA

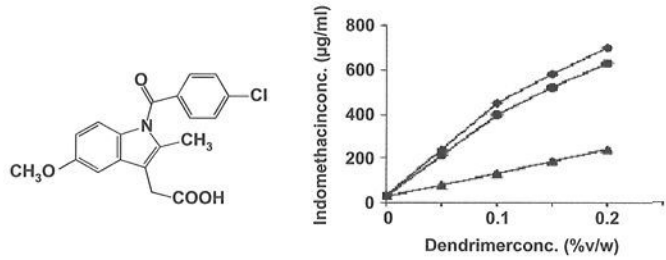


**Fig. 5.** Molecular structure of indomethacin and its solubility profiles in the presence of differing concentrations of G4-$NH_2$, (♦) G4-OH (•), and G4.5-COOH (▲) PAMAM dendrimers at pH 7 ($n = 3$, R.S.D. $\leq 5\%$).

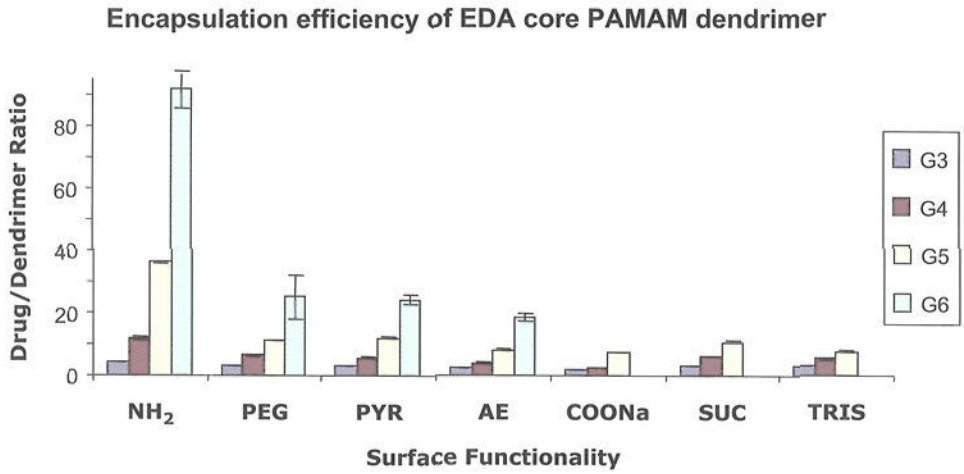


**Fig. 6.** Encapsulation efficiency into PAMAM dendrimers generations 3–6 with amino ($NH_2$), poly(ethylene glycol) (PEG), carbomethoxypyrrolidinone (PYR), amidoethanol (AE), sodium carboxylate (COONa), succinamic acid (SUC), and tris(hydroxymethyl)-aminomethane (TRIS) surface groups.

core and amino surface (G = 0, 1, 2, 3) or ester surface (G = 0.5, 1.5, 2.5) at pH 4, 7 and 10. The solubility enhancement of nifedipine was higher in the presence of ester-terminated dendrimers than their amino-terminated analogues, possessing the same number of surface groups. The nifedipine solubility expectedly increased with the size of the dendrimers. For pH 7, the sequence G2.5 > G3 > G1.5 > G2 ≥ G0.5 > G1 > G0 was reported.[43]

In another approach, the non-steroidal anti-inflammatory drug naproxen was covalently attached to unsymmetrical poly(arylester) dendrimers to prepare a complex with enhanced water solubility of the drug and access for hydrolytic cleavage

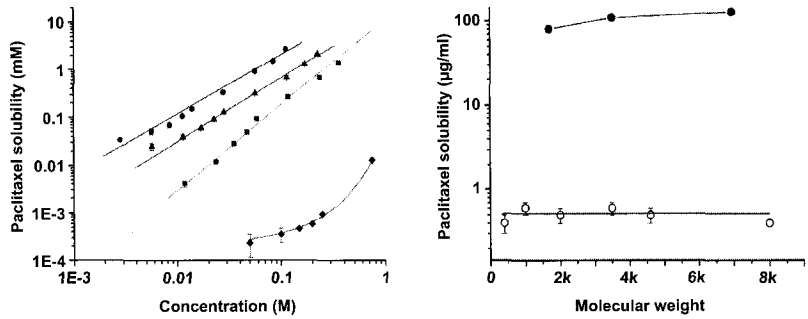


**Fig. 7.** Aqueous paclitaxel solubility as a function of the polyglycerol dendrimer concentration (mean ± SD, $n = 3$); G5 (circle), G4 (triangle), G3 (square), and PEG400 (diamond) (left). Molecular weight dependency of dendrimers (closed circle) and PEG (open circle) on the aqueous paclitaxel solubility. The concentration of dendrimers and PEG was 10 wt%. (Reproduced with permission from Ref. 45. Copyright 2004 American Chemical Society.)

of the bond between drug and carrier. Detailed results on the biological evaluation of these complexes have not been reported.[44]

The anticancer drug paclitaxel, which is being used to treat metastatic breast and ovarian cancers and Kaposi's sarcoma, has poor water solubility. To enhance its bioavailability, paclitaxel has been encapsulated into polyglycerol dendrimers, resulting in a 10,000-fold improved water solubility compared with the pure drug, which is much higher than that found for PEG400, a commonly used linear chain cosolvent or hydrotropic agent (Fig. 7). The drug release rate was a function of the dendrimer generation.[45]

Generation 4 PAMAM dendrimers with hydroxy surface have been utilized to improve the bioavailability of the corticosteroid methylprednisolone, which decreases inflammation by stabilizing leukocyte lysosomal membrane. By connecting the drug to the dendrimer using glutaric acid as the spacer, a payload of 32 wt% was achieved. The drug-dendrimer complex was taken up by A549 human lung epithelial carcinoma cells and mostly localized in the cytosol. The complex showed a pharmacological activity comparable to the free drug as measured by the inhibition of the prostaglandin secretion.[46]

## 3.6. *Doxorubicin and camptothecin — self-immolative dendritic prodrugs*

An exciting new approach to dendritic drug delivery involves the utilization of a drug as a part of the dendritic molecule. Self-immolative dendrimers have recently been developed and introduced as a potential platform for a multi-prodrug. These unique structural dendrimers can release all of their outer branch units through

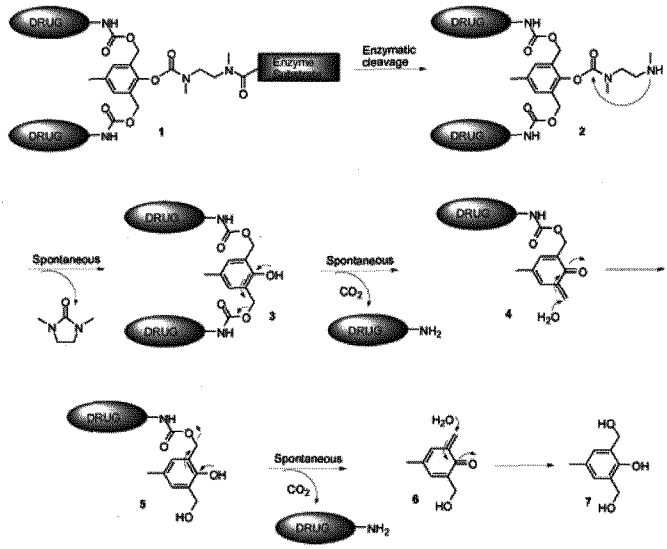


**Fig. 8.** Mechanism of dimeric prodrug activation by a single enzymatic cleavage. (Reproduced with permission from Ref. 47. Copyright 2004 American Chemical Society.)

a self-immolative chain fragmentation, initiated by a single cleavage at the dendrimer's core. Incorporation of drug molecules as these outer branch units and an enzyme substrate as the trigger can generate a multi-prodrug unit that will be activated with a single enzymatic cleavage (Fig. 8). The first generation of dendritic prodrugs with doxorubicin and camptothecin as branch units and retro-Michael focal trigger, which can be cleaved by the catalytic antibody 38C2, has been reported. Bioactivation of the dendritic prodrugs was evaluated in cell-growth inhibition assay with the Molt-3 leukemia cell line in the presence and absence of antibody 38C2. A remarkable increase in toxicity was observed. Dependent on the linker molecule, different numbers of drug molecules can be released in one single activation step.[47,48]

In a more "classical" approach to deliver doxorubicin, two polyester-based dendrimers (generation 4 with trisphenolic core) were synthesized, one carrying a hydroxy surface, the other a tri(ethylene glycol) monomethyl ether surface. These dendrimers were compared with a 3-arm poly(ethylene oxide) star polymer, carrying G = 2 dendritic polyester units at the surface. The star polymer gave the most promising results regarding cytotoxicity and systemic circulatory half-life (72 hrs). Therefore, the anticancer drug doxorubicin was covalently bound to this carrier via an acid-labile hydrazone linkage. The cytotoxicity of doxorubicin was significantly reduced (80–98%) and the drug was successfully taken up by several cancer cell lines.[49]

## 3.7. *Photodynamic therapy (PDT) and boron neutron capture therapy (BNCT)*

Dendrimers have been used to optimize the antitumor effect in photodynamic therapy (PDT) and boron neutron capture therapy (BNCT). One of the newest developments in the dendrimer field is their application to photodynamic therapy (PDT). This cancer treatment involves the administration of a light-activated photosensitizing moiety that selectively concentrates in diseased tissue. Subsequent activation of the photosensitizer leads to the generation of reactive oxygen, primarily singlet oxygen, that damages intracellular components such as lipids and amino acid residues through oxidation, ultimately leading to cell death by apoptosis. Disadvantages of currently used photosensitizers include skin phototoxicity, poor selectivity for tumor tissue, poor water solubility, and difficulties in the treatment of solid tumors because of the impermeability of the skin and tissues to the visible light required to excite the chromophores.

In one set of studies, dendrimers have been constructed around a light harvesting core (i.e. a porphyrin).[50] To reduce the toxicity under non-irradiative conditions (dark toxicity) and to prevent aggregation, and consequently, self-quenching of the porphyrin cores, these dendrimers have been further encapsulated into micelles. For example, poly(ethylene glycol)-*b*-poly(aspartic acid) and PEG-*b*-poly(L-lysine) micelles have been studied in this regard. These micelles are stable under physiological conditions pH 6.2 to 7.4. However, they disintegrate in the acidic intracellular endosomal compartment (pH $\sim$ 5.0).[51,52] Alternatively, the photosensitizer 5-aminolevulinic acid has been attached to the surface of dendrimers and studied as an agent for PDT of tumorigenic keratinocytes.[53] Photosensitive dyes have been incorporated into dendrimers and utilized in PDT devices. For example, uptake, toxicity, and the mechanism of photosensitization of the dye pheophorbide a (pheo) was compared with its complex with diaminobutane poly(propylene imine) (DAB) dendrimers in human leukemia cells *in vitro*.[54]

The second therapy, boron neutron capture therapy, is a cancer treatment based on a nuclear capture reaction. When $^{10}B$ is irradiated with low energy or thermal neutrons, highly energetic $\alpha$-particles and $^{7}Li$ ions are produced, that are toxic to tumor cells. To achieve the desired effects, it is necessary to deliver $^{10}B$ to tumor cells at a concentration of at least $10^9$ atoms per cell. High levels of boron accumulation in tumor tissue can be achieved by using boronated antibodies that are targeted towards tumor antigens. However, this approach can impair the solubility and targeting efficiency of the antibodies.

One study, involving intratumoral injection of a conjugation between a generation 5 PAMAM dendrimer carrying 1100 boron atoms and cetuximab, a monoclonal antibody specific for the EGF receptor, showed that the conjugate was present

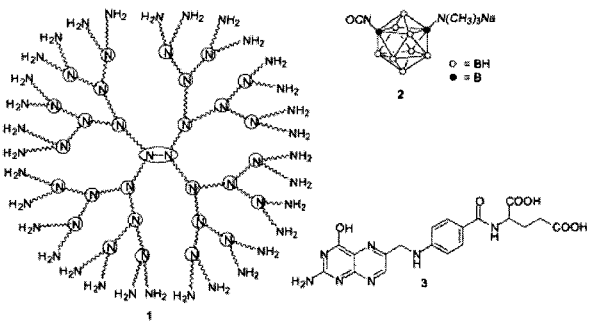

**Fig. 9.** Schematic presentation of an EDA core G = 3 PAMAM dendrimer (1), the boron carrier $Na(CH_3)_3NB_{10}H_8NCO$ (2), and the targeting ligand folic acid (3). (Reproduced with permission from Ref. 56. Copyright 2003 American Chemical Society.)

at an almost 10-fold higher concentration in brain tumors than in normal brain tissue.[55] To reduce the liver uptake observed for boronated PAMAM dendrimer conjugates, PEG chains were attached onto the dendrimer surface, in addition to the borane clusters, to provide steric shielding. As compared with a dendrimer without PEG chains, the amount of liver uptake was found to be less for PEG-conjugated dendrimers with an average of 1.0–1.5 chains of $PEG_{2000}$, but higher for dendrimers with 11 chains of $PEG_{550}$. Folic acid moieties were also conjugated to the ends of the PEG chains to enhance the uptake of the dendrimers by tumors overexpressing folate receptors. Although this strategy was successful in enhancing localization of the molecules to tumors in mice bearing 24JK-FBP tumors expressing the folate receptor, it also led to an increase in the uptake of the dendrimers by the liver and kidneys.[56]

## 4. Nano-Scaffolds for Targeting Ligands

The surface of dendrimers provides an excellent platform for the attachment of cell-specific ligands, solubility modifiers, stealth molecules, reducing the interaction with macromolecules from the body defense system, and imaging tags. The ability to attach any or all of these molecules in a well-defined and controllable manner onto a robust dendritic surface, clearly differentiates dendrimers from other carriers such as micelles, liposomes, emulsion droplets, and engineered particles.

### 4.1. *Folic acid*

One example of cell-specific dendritic carriers is a dendrimer modified with folic acid. The membrane-associated high affinity folate receptor (hFR) is a folate binding protein that is overexpressed on the surface of a variety of cancer cells, and

therefore, folate-modified dendrimers would be expected to internalize into these cells preferentially over normal cells via receptor-mediated endocytosis. Folate-dendrimer conjugates have been shown to be well-suited for targeted, cancer-specific drug delivery of cytotoxic substances.[56–59]

In a very recent study, branched poly(L-glutamic acid) chains were centered around PAMAM dendrimers generations 2 and 3 and poly(ethylene imine) (PEI) cores to create new biodegradable polymers with improved biodistribution and targeting ability. These constructs were surface-terminated with poly(ethylene glycol) chains to enhance their biocompatibility, and folic acid ligands to introduce cell-specific targeting. Cell binding studies have been performed using the epidermal carcinoma cell line, KB.[60]

## 4.2. *Carbohydrates*

In addition to folates, carbohydrates constitute another important class of biological recognition molecules, displaying a wide variety of spatial structures due to their branching possibility and anomericity. To achieve sufficiently high binding affinities between simple mono- and oligosaccharide ligands and cell membrane receptors, these ligands have to be presented to the receptors in a multivalent or cluster fashion.[61,62] The highly functionalized surface of dendrimers provides an excellent platform for such presentations. The design, synthesis, and biomedical use of glycodendrimers, as well as their application in diagnostic and for vaccinations, have been thoroughly reviewed recently.[63–69] For example, the Thomsen-Friedenreich carbohydrate antigen (T-antigen), $\beta$-Gal-(1-3)-$\alpha$-GalNAc, which has been well documented as an important antigen for the detection and immunotherapy of carcinomas, especially relevant to breast cancer, has been attached to the surface of PAMAM and other dendrimers.[70–72] An enhanced binding affinity was observed for all glycodendrimers. These constructs could have potential in blocking the metastatic sites of invasive tumor cells. A series of dendritic $\beta$-cyclodextrin derivatives, bearing multivalent mannosyl ligands, has been prepared and their binding efficiency towards the plant lectin concanavalin A (Con A) and a mammalian mannose-specific cell surface receptor from macrophages has been studied. The effects of glycodendritic architecture on binding efficiency, molecular inclusion, lectin-binding properties, and the consequence of complex formation using the anticancer drug docetaxel on biological recognition were investigated.[73] Di- to tetravalent dendritic galabiosides, carrying (Gal$\alpha$1-4Gal) moieties on their surfaces, were studied as inhibitors of pathogens based on bacterial species such as *E. coli* and *Streptococcus suis*. Attachment of dendritic galabiosides onto cell surfaces would be expected to inhibit the attachment of bacteria using the same sugar ligand-receptor interactions. The study revealed a clear enhancement of the binding affinity between

glycodendrons and cell surfaces, with an increasing number of sugar moieties.[74] In a similar approach, glycodendrons carrying two to four β-D-galactose moieties on their surface, while the dendron core was connected to a protein-degrading enzyme, were synthesized. These glycodendriproteins are expected to attach to the surface of bacteria, allowing the enzyme to degrade the bacterial adhesin, hence rendering the bacteria incapable of attaching to the cell surfaces.[75] Anionic PAMAM dendrimers (G = 3.5) were conjugated to D(+)-glucosamine and D(+)-glucoseamine 6-sulfate. These water-soluble conjugates not only revealed immuno-modulatory and antiangiogenic properties, but synergistically prevented scar tissue formation after glaucoma filtration surgery. In a validated and clinically relevant rabbit study, the long-term success rate was increased from 30 to 80% using these dendrimer-conjugates.[76]

## 4.3. *Antibodies and biotin-avidin binding*

Generation 5 PAMAM dendrimerendrimers with amino surface were conjugated to fluorescein isothiocyanate as a means to analyze cell binding and internalization. Two different antibodies, 60bca and J591, which bind to CD14 and prostate-specific membrane antigen (PSMA) respectively, were used as model targeting molecules. The binding of the antibody-conjugated dendrimers to antigen-expressing cells was evaluated by flow cytometry and confocal microscopy. The conjugates specifically bound to the antigen-expressing cells in a time- and dose-dependent fashion, with affinity similar to that of the free antibody (Fig. 10). Confocal microscopic analysis suggested at least some cellular internalization of the dendrimer conjugate. Dendrimer-antibody conjugates are, therefore, a suitable platform for targeted molecule delivery into antigen-expressing cells.[77]

Monolayers formed by generation 4 PAMAM dendrimers on a gold surface were functionalized with biotin and produced a biomolecular interface that was

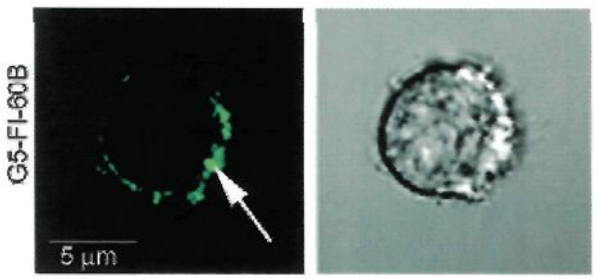


**Fig. 10.** Confocal microscopic analysis of HL60 cells, which were incubated (1 h at 4°C) with 12.5 nM G5 PAMAM carrying fluorescence dye and 60bca antibody on the surface. The cells were rinsed and confocal images were taken. The left and right panels represent the FITC fluorescence and light images taken in the same cell. The arrow indicates the binding of the conjugate on the cell surface at 4°C. (Reproduced with permission from Ref. 77. Copyright 2004 American Chemical Society.)

capable of binding high levels of avidin. Avidin binding as high as 88% coverage of the surface was observed despite conditions that should cause serious steric hindrance. These dendritic monolayers were utilized as a model to study protein-ligand interactions.[78]

### 4.4. *Penicillins*

The surfaces of PAMAM dendrimers, generations 0 to 3, were decorated with benzylpenicillin in an attempt to develop a new *in vitro* test to quantify IgE antibodies to specific $\beta$-lactam conjugates, with the goal of improving the existing methods for diagnosing allergy to this type of antibiotic. The monodispersity of dendrimers is advantageous over conventional peptide carrier conjugates such as human serum albumin (non-precise density of haptens in their structure) and poly-L-lysine (mixture of heterogeneous molecular weight peptides). Preliminary radioallergosorbent tests (RAST), using sera from patients allergic to penicillin, have confirmed the usefulness of penicilloylated dendrimers.[79]

Penicillin V was used as a model drug containing a carboxylic group and attached to the surface of PAMAM dendrimers generations 2.5 and 3, both containing 32 surface functionalities. The drug was complexed to the dendrimers via amide or ester bonds. It was found in tests using a single-strain bacterium, *Staphylococcus aureus*, that the bioavailability of the penicillin was unaltered after the drug was released from the complex through ester bond hydrolysis.[80]

## 5. Dendrimers as Nano-Drugs

Dendrimers have been studied as antitumor, antiviral and antibacterial drugs.[25] The most prominent and advanced example is the use of poly(lysine) dendrimers, modified with sulfonated naphthyl groups, as antiviral drugs against the *herpes simplex* virus.[81] Such a conjugate based on dendritic poly(lysine) scaffolding is VivaGel™, a topical agent currently under development by Starpharma Ltd., Melbourne, Australia, that can potentially prevent/reduce transmission of HIV and other sexually transmitted diseases (STDs). VivaGel™ (SPL 7013) is being offered as a water-based gel, with the purpose to prevent HIV from binding to cells in the body. The gel differs from physical barriers to STDs such as condoms, by exhibiting inhibitory activity against HIV and other STDs. In July 2003, following submission of an Investigational New Drug (IND) application, Starpharma gained clearance under U.S. FDA regulations to proceed with a Phase I clinical study to assess the safety of VivaGel™ in healthy human subjects. This phase 1 study, representing for the first time a dendrimer pharmaceutical tested in humans, compared 36 women who received either various intra-vaginal doses of VivaGel™ or a placebo gel daily for one week. The trial was double blinded so that the volunteers, principal

investigator and Starpharma did not know who was receiving placebo or VivaGel™. Study participants were assessed for possible irritant effects of the gel. Additionally, the women were assessed for any possible effect upon vaginal microflora (natural micro-organisms in the vagina) or absorption into the blood of the active ingredient of VivaGel™. A thorough review of the complete data revealed no evidence of irritation or inflammation. Preclinical development studies had demonstrated that VivaGel™ was 100% effective at preventing infection of primates exposed to a humanized strain of simian immunodeficiency virus (SHIV).[82] In earlier studies, it was found that PAMAM dendrimers covalently modified with naphthyl sulfonate residues on the surface, also exhibited antiviral activity against HIV. This dendrimer-based nano-drug inhibited early stage virus/cell adsorption and later stage viral replication, by interfering with reverse transcriptase and/or integrase enzyme activities.[83,84]

The general mode of action of antibacterial dendrimers is to adhere to and damage the anionic bacterial membrane, causing bacterial lysis.[25,85] PPI dendrimers with tertiary alkyl ammonium groups attached to the surface have been shown to be potent antibacterial biocides against Gram positive and Gram negative bacteria. The nature of the counterion is important, as tetraalkylammonium bromides were found to be more potent antibacterials over the corresponding chlorides.[86] Poly(lysine) dendrimers with mannosyl surface groups are effective inhibitors of the adhesion of *E. coli* to horse blood cells in a haemagglutination assay, making these structures promising antibacterial agents.[87] Chitosan-dendrimer hybrids have been found to be useful as antibacterial agents, carriers in drug delivery systems, and in other biomedical applications. Their behavior have been reviewed very recently.[88] Triazine-based antibiotics were loaded into dendrimer beads at high yields. The release of the antibiotic compounds from a single bead was sufficient to give a clear inhibition effect.[89] In many cases, dendritic constructs were more potent than analogous systems based on hyperbranched polymers.

The anti-prion activity of cationic phosphorus-containing dendrimers with tertiary amine surface groups has been evaluated. These molecules had a strong anti prion activity at non-toxic doses. They have been found to decrease the amount of pre-existing PrPSc from several prion starins, including the BSE strain. In addition, these dendrimers were able to reduce PrPSc accumulation in the spleen by more than 80%.[90]

## 6. Routes of Application

Most commonly, dendrimers are applied as parenteral injections, either directly into the tumor tissue or intravenous for systemic delivery. However, recent oral drug delivery studies using the human colon adenocarcinoma cell line, Caco-2,

have indicated that low generation PAMAM dendrimers cross cell membranes, presumably through a combination of two processes, i.e. paracellular transport and adsorptive endocytosis.[91] The P-glycoprotein (P-gp) efflux transporter does not effect dendrimers, and therefore, drug-dendrimer complexes are able to bypass the efflux transporter.[92]

Furthermore, recent work has shown that PAMAM dendrimers enhanced the bioavailability of indomethacin in transdermal delivery applications.[93] Similarly, the drug tamsulosin was used as a model to study transdermal delivery utilizing PAMAM dendrimers. The dendrimers were found to be weak penetration enhancers.[94] However, no dendrimer-driven effect was observed for the drugs ketoprofen and clonidine. As an explanation, dendrimer-triggered drug crystallization within the transdermal delivery matrix was discussed, allowing the formation of drug polymorphs that can or cannot facilitate transdermal delivery.[95]

Several PAMAM dendrimers (generations 1.5, 2–3.5 and 4) with amine, carboxylate and hydroxyl surface groups were studied for controlled ocular drug delivery. The duration of residence time was evaluated after solubilization of these dendrimers in buffered phosphate solutions containing 2 parts per thousand (w/v) of fluorescein. The New Zealand albino rabbit was used as an *in vivo* model for qualitative and quantitative assessment of ocular tolerance and retention time, after a single application of 25 $\mu$L of dendrimer solution to the eye. The same model was also used to determine the prolonged miotic or mydriatic activities of dendrimer solutions, some containing pilocarpine nitrate and some tropicamide, respectively. Residence time was longer for the solutions containing dendrimers with carboxylic and hydroxyl surface groups. No prolongation of remanence time was observed when dendrimer concentration (0.25–2%) increased. The remanence time of PAMAM dendrimer solutions on the cornea showed size and molecular weight dependency. This study allowed novel macromolecular carriers to be designed with prolonged drug residence time for the ophthalmic route.[96]

## 7. Biocompatibility of Dendrimers

Dendrimers have to exhibit low toxicity and be non-immunogenic in order to be widely used in biomedical applications. To date, the cytotoxicity of dendrimers has been primarily studied *in vitro*, however, a few *in vivo* studies have been published.[25] As observed for other cationic macromolecules, including liposomes and micelles, dendrimers with positively charged surface groups are prone to destabilize cell membranes and cause cell lysis. For example, *in vitro* cytotoxicity $IC_{50}$ measurements (i.e. the concentration where 50% of cell lysis is observed) for amino-terminated PAMAM dendrimers revealed significant cytotoxicity on human intestinal adenocarcinoma Caco-2 cells.[97,98] Furthermore, the cytotoxicity was found to be

generation-dependent, with higher generation dendrimers being the most toxic.[97,99] A similar generation dependence of amino-terminated PAMAM dendrimers was observed for the haemolytic effect, studied on a solution of rat blood cells.[100] However, some recent studies have shown that amino-terminated PAMAM dendrimers exhibit lower toxicity than more flexible amino-functionalized linear polymers perhaps due to lower adherence of the rigid globular dendrimers to cellular surfaces. The degree of substitution, as well as the type of amine functionality, is important, with primary amines being more toxic than secondary or tertiary amines.[99] Amino-terminated PPI and PAMAM dendrimers behave similarly with regard to cytotoxicity and haemolytic effects, including the generation-dependent increase of both.[100,101]

Comparative toxicity studies on anionic (carboxylate-terminated) and cationic (amino-terminated) PAMAM dendrimers using Caco-2 cells have shown a significantly lower cytotoxicity of the anionic compounds.[97] In fact, lower generation PAMAM dendrimers possessing carboxylate surface groups show neither haematotoxicity nor cytotoxicity at concentrations up to 2 mg/ml.[100] The biocompatability of dendrimers is not solely determined by the surface groups. Dendrimers containing an aromatic polyether core and anionic carboxylate surface groups have shown to be haemolytic on a solution of rat blood cells after 24 hrs. It is suggested that the aromatic interior of the dendrimer may cause haemolysis through hydrophobic membrane contact.[100]

One way to reduce the cytotoxicity of cationic dendrimers may reside in partial surface derivatization with chemically inert functionalities such as PEG or fatty acids. The cytotoxicity towards Caco-2 cells can be reduced significantly (from $IC_{50} \sim 0.13$ mM to >1 mM) after such a modification. This observation can be explained by the reduced overall positive charge of these surface-modified dendrimers. A partial derivatization with as few as six lipid chains or four PEG chains on a G4-PAMAM, respectively, was sufficient to lower the cytotoxicity substantially.[98] In studies conducted at Dendritic Nano Technologies, Inc. using Caco-2 and two other cell lines, it was found that besides (partial) PEGylation of the surface, surface modification with pyrrolidone, another biocompatible compound, can significantly reduce cytotoxicity to levels far better than those of currently available products.[102] In some cases, the cytotoxicity of PAMAM dendrimers could be reduced by additives such as fetal calf serum.[103]

Only a few systematic studies on the *in vivo* toxicity of dendrimers have been reported so far. Upon injection into mice, doses of 10 mg/kg of PAMAM dendrimers (up to G = 5), displaying either unmodified or modified amino-terminated surfaces, did not appear to be toxic.[81,104] Hydroxy- or methoxy-terminated dendrimers based on a polyester dendrimer scaffold have been shown to be of low toxicity both *in vitro* and *in vivo*. At very high concentrations (40 mg/ml), these polyester

dendrimers induced some inhibition of cell growth *in vitro*, but no increase in cell death was observed. Upon injection into mice, no acute or long-term toxicity problems were observed. The non-toxic properties make these new dendritic motifs very promising candidates for drug delivery devices.[49]

Initial immunogenicity studies performed on unmodified amino-terminated PAMAM dendrimers showed no or weak immunogenicity of the G3–G7 dendrimers. However, later studies indicated some immunogenicity of these dendrimers, which could be reduced by surface-modification utilizing PEG chains.[105]

## 8. Conclusions

The high level of control over the architecture of dendrimers, their size, shape, branching length and density, and their surface functionality, makes these compounds ideal carriers in drug delivery applications. The bioactive agents may either be encapsulated into the interior of the dendrimers or they may be chemically attached or physically adsorbed onto the dendrimer surface, with the option to tailor the properties of the carrier to the specific needs of the active material and its therapeutic applications. Furthermore, the high density of surface groups allows attachment of targeting groups as well as groups that modify the solution behavior or toxicity of dendrimers. Surface-modified dendrimers themselves may act as nano-drugs against tumors, bacteria and viruses. This review of drug delivery applications of dendrimers clearly illustrates the potential of this new "fourth architectural class of polymers"[106] and substantiates the high optimism for the future of dendrimers in this important field.

## Acknowledgments

The authors wish to thank all contributors to this fascinating field of research, as well as the funding agents that have supported this work over the years. In particular, DNT would like to acknowledge current funding by the US Army Research Laboratory (ARL) (Contract # W911NF-04-2-0030).

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# 14

# Drug Nanocrystals/Nanosuspensions for the Delivery of Poorly Soluble Drugs

*Rainer H. Müller and Jens-Uwe A. H. Junghanns*

## 1. Introduction

Since the last ten years, the number of poorly soluble drugs is steadily increasing. According to estimates, about 40% of the drugs in the pipelines have solubility problems.[1] The increased use of high throughput screening methods leads to the discovery of more drugs being poorly water soluble. In the literature, figures are quoted that about 60 percent of the drugs coming directly from synthesis are nowadays poorly soluble.[2] Poor solubility is not only a problem for the formulation development and clinical testing, it is also an obstacle at the very beginning when screening new compounds for pharmacological activity. From this, there is a definite need for smart technological formulation approaches to make such poorly soluble drugs bioavailable. Making such drugs bioavailable means that they show sufficiently high absorption after oral administration, or they can alternatively be injected intravenously.

There is quite a number of formulation approaches for poorly soluble drugs which can be specified as "specific approaches". These approaches are suitable for molecules having special properties with regard to their chemistry (e.g. solubility in certain organic media) or to the molecular size or conformation (e.g. molecules to be incorporated into the cyclodextrin ring structure). Of course it would be much smarter to have a "universal formulation approach" applicable to any molecule. Such a universal formulation approach to increase the oral

bioavailability is micronization, meaning the transfer of drug powders into the size range between typically 1–10 $\mu$m. However, nowadays many drugs are so poorly soluble that micronization is not sufficient. The increase in surface area, and thus consequently in dissolution velocity, is not sufficient to overcome the bioavailability problems of very poorly soluble drugs of the biopharmaceutical specification class II. A consequent next step was to move from micronization to nanonization. Since the beginning of the 90s, the company Nanosystems propagated the use of nanocrystals (instead of microcrystals) for oral bioavailability enhancement, and also to use nanocrystals suspended in water (nanosuspensions) for intravenous or pulmonary drug delivery.

The solution was simple; in general, simple solutions possess the smartness that they can be realized easier than complex systems and introduction to the market is faster. Nevertheless, it took about ten years before the first nanocrystals in a tablet appeared on the market, the product Rapamune® by the company Wyeth in 2000. Compared with liposomes developed in 1968[3] with the first products on the market around 1990 (e.g. Alveofact®, a lung surfactant), this was still relatively fast. What were the reasons that it took about one decade for nanocrystals to enter the market?

From our point of view, pharmaceutical companies prefer to use formulation technology already established with know how available in the company. In addition, if formulation technologies are established, a company also has the possibility for production of the final product. Therefore, all the traditional formulation approaches were exploited to solve a formulation problem. In addition, formulation approaches were preferred, being even simpler than nanocrystals. For example, production of drug-containing microemulsions administered in a capsule is, in many cases, even simpler. Another reason for the reluctance of pharmaceutical companies at the beginning was the lack of large scale production methods. These were not available at the very beginning of the development of the nanocrystal technology. Meanwhile, this has changed and the major pharmaceutical companies try to secure or have already secured their access to nanocrystal technology. Access to nanocrystal technology is possible either by licencing in or alternatively by the attempt to develop one's own production technologies for the nanocrystals, which do not depend on already existing intelectual property (IP). This chapter discusses the physicochemical properties of nanocrystals which make them interesting for drug delivery, reviews and discusses briefly the various production methods available and highlights the opportunities for improved drug delivery using different application routes.

## 2. Definitions

Drug nanocrystals are crystals with a size in the nanometer range, meaning that they are nanoparticles with a crystalline character. There are discussions about the

definition of a nanoparticle, referring to the size of a particle to be classified as a nanoparticle. Depending on the discipline, e.g. in colloid chemistry, particles are only considered as nanoparticles when they are in sizes below 100 nm or even below 20 nm. Based on the size unit, in the pharmaceutical area, nanoparticles should be defined as having a size between a few nanometers and 1000 nm (1 $\mu$m); thus, microparticles possess consequently a size 1–1000 micrometer.

A further characteristic is that drug nanocrystals are composed of 100% drug; there is no carrier material as in polymeric nanoparticles. Dispersion of drug nanocrystals in liquid media leads to "nanosuspensions", in contrast to "microsuspensions" or "macrosuspensions". In general, the dispersed particles need to be stabilized, e.g. by surfactants or polymeric stabilizers. Dispersion media can be water, aqueous solutions or non-aqueous media [e.g. liquid polyethylene glycol (PEG), oils]. Depending on the production technology, processing of drug microcrystals to drug nanoparticles can lead to either a crystalline or to an amorphous product, especially when applying precipitation. In the strict sense, such an amorphous drug nanoparticle should not be called nanocrystal. However, one often refers to "nanocrystals in the amorphous state".

## 3. Physicochemical Properties of Drug Nanocrystals

### 3.1. *Change of dissolution velocity*

The reason for micronization is to increase the surface area, thus consequently according to the Noyes-Whitney equation, increasing the dissolution velocity. Therefore, micronization can be succesfully employed if the dissolution velocity is the rate-limiting step for oral absorption (drugs of BSC II). Of course, by moving one dimension further to smaller particles, the surface area is further enlarged and consequently, the dissolution velocity is further enhanced. In most cases, a low dissolution velocity is correlated with a low saturation solubility.

### 3.2. *Saturation solubility*

The general textbook statement is that the saturation solubility $c_s$ is a constant depending on the compound, the dissolution medium and the temperature. This is valid for powders of daily life with a size in the micrometer range or above. However, below a critical size of 1–2 $\mu$m, the saturation solubility is also a function of the particle size. It increases with decreasing particle size below 1000 nm. Therefore, drug nanocrystals possess an increased saturation solubility. This has two advantages:

1. According to Noyes-Whitney, the dissolution velocity is further enhanced because $dc/dt$ is proportional to the concentration gradient $(c_s - c_x)/h$ ($c_x$ — bulk concentration, h — diffusional distance).

2. Due to the increased saturation solubility, the concentration gradient between gut lumen and blood is increased, consequently, the absorption by passive diffusion.

The interesting question very often asked is "How manyfold is the increased saturation solubility?". Data published in the literature or available to us from discussions range from 2–14 fold. What are the factors affecting the increase in saturation solubility? The factors can be identified when looking at the theoretical background. The Kelvin equation describes the increase in the vapor pressure of droplets in a gas medium as a function of their particle size, i.e. as a function of their curvature:

$$\ln\left(\frac{P}{P_0}\right) = \frac{-\gamma * V_L * \cos\theta}{r_K * RT}$$

**Fig. 1.** The Kelvin equation.

$P$ = vapor pressure
$P_0$ = equilibrium pressure of a flat liquid surface
$\gamma$ = surface tension
$V_L$ = molar volume
$\cos(\theta)$ = contact angle
$r_K$ = radius of droplet
$R$ = universal gas constant
$T$ = absolute temperature (K)

The vapor pressure increases with increasing curvature of the surface, that means decreasing particle size. Each liquid has its compound specific vapor pressure, thus the increase in vapor pressure will be influenced by the available compound-specific vapor pressure. The situation of a transfer of molecules from a liquid phase (droplet) to a qas phase is in principal identical to the transfer of molecules from a solid phase (nanocrystal) to a liquid phase (dispersion medium). The vapor pressure is equivalent to the dissolution pressure. In the state of saturation solubility, there is an equilibrium of molecules dissolving and molecules recrystallizing. This equilibrium can be shifted in case the dissolution pressure increases, thus increasing the saturation solubility. Identical to liquids with different vapor pressures under normal conditions (micrometer droplet size), each drug crystal has a specific dissolution pressure in micrometer size.

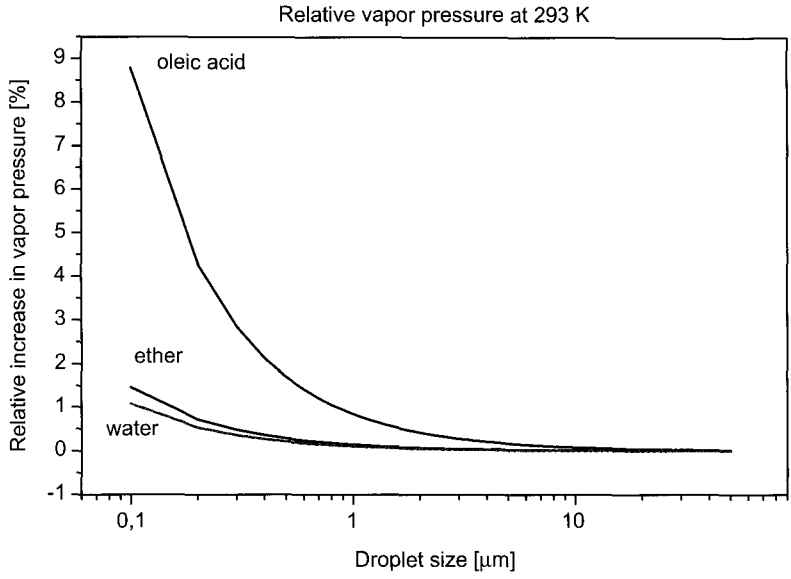


**Fig. 2.** Comparison of the relative increase in vapor pressure between water, ether and oleic acid (calculated using the Kelvin equation) as a function of the droplet size (with permission after[4]).

The important question is how the dissolution pressure changes, depending on the specific dissolution pressure of each compound and on the particle size. Model calculations were performed applying the Kelvin equation to compounds with different vapor pressures (droplets) as a function of droplet size (Fig. 2). Liquids with low medium and high vapor pressure were selected, such as oleic acid as an oil, water and ether. The important result for a drug formulation was:

1. The increase in vapor pressure is more pronounced for compounds having *a priori* a low vapor pressure. Applied to solid compounds, increase in dissolution pressure will be more pronounced for compounds having *a priori* a low dissolution pressure, i.e. the relative increase is highest for poorly soluble drugs.
2. The increase in vapor pressure is exponential, with a very pronounced increase occurring at droplet sizes below 100 nm.

Figure 3 shows a calculated increase for barium sulfate as solid model compound.

## 3.3. *Does size really matter?*

Transferring this to drug nanocrystals means that really smart crystals with highest increase in saturation solubility should have a size of e.g. 50 nm or 20–30 nm. From this, it can be concluded that the slogan "size matters" is correct regarding the increase in saturation solubility, and consequently, the increase in dissolution

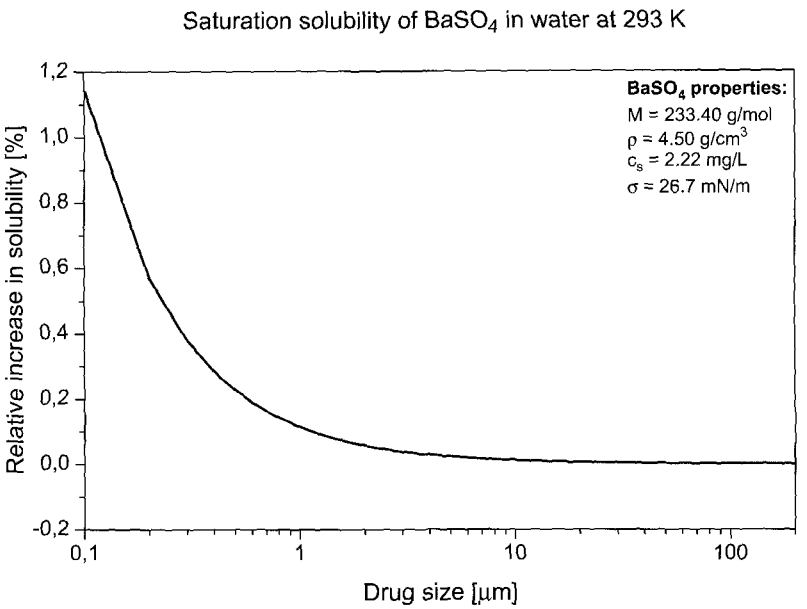


**Fig. 3.** Increase in saturation solubility of $BaSO_4$ in water as a function of the particle size calculated using the Kelvin equation (with permission after[4]).

velocity caused by a higher $c_s$. It needs to be kept in mind which blood profile is anticipated with a certain drug. In many cases, too fast a dissolution is not desired (creation of high plasma peaks, reduction of $t_{max}$). There is the request to combine drug nanocrystals with traditional controlled release technology (e.g. coated pellets) to avoid too fast a dissolution, too high plasma peaks, too early a $t_{max}$ and to reach prolonged blood levels. To summarize, the optimal drug nanocrystal size will depend on:

1. Required blood profile
2. Administration route

In the case of i.v. injected nanocrystals, the size should be as small as possible in case the pharmacokinetics of a solution should be mimicked. In the event that a targeting is the aim (e.g. to the brain by PathFinder technology,[5] the drug nanocrystals should possess a certain size to delay dissolution and to give them the chance to reach the blood-brain barrier (BBB) for internalization by the endothelial cells of the BBB.[6]

## 3.4. *Effect of amorphous particle state*

It is well known that amorphous drugs possess a higher saturation solubility, compared with crystalline drug material. A classical example from the literature

is chloramphenicol palmitate. The polymorphic modification I has a solubility of 0.13, the high energy modification II a solubility of 0.43 and the amorphous material of 1.6 mg/mL.[7,8] The same is valid for drug nanoparticles, amorphous drug nanoparticles possess a higher saturation solubility, compared with equally sized drug nanocrystals in the crystalline state. Therefore, to reach highest saturation solubility increase, a combination of nanometer size and amorphous state is ideal. However, prerequisite for exploitation in pharmaceutical products is that the amorphous state can be maintained for the shelf life of the product.

# 4. Production Methods

## 4.1. *Precipitation methods*

### 4.1.1. *Hydrosols*

The Hydrosol technology was developed by Sucker and the intellectual property owned by the company Sandoz, now known as Novartis.[9,10] It is basically a classical precipitation process known to pharmacists under the term "via humida paratum" (v.h.p.). This v.h.p. process was employed to prepare ointments containing finely dispersed, precipitated drugs. The drug is dissolved in a solvent, the solvent added to a non-solvent leading to the precipitation of finely dispersed drug nanocrystals. A problem associated with this technology is that the formed nanoparticles need to be stabilized to avoid growth in micrometer crystals. In addition, the drug needs to be soluble at least in one solvent. This creates problems for the newly synthesized or discovered drugs, being poorly soluble in water and simultaneously in organic media. Lyophilization is recommended to preserve the particle size.[1] To our knowledge, this technology has not been applied to a product to date.

### 4.1.2. *Amorphous drug nanoparticles (NanoMorph®)*

Depending on the precipitation methodology, drug nanoparticles can be generated which are in the amorphous state. A nice example are carotine nanoparticles in food industry.[11]

A solution of the carotinoid, together with a surfactant and a digestible oil, are admixed into an appropriate solvent at a specific temperature. The solution is mixed with a protective colloid. This tranforms the hydrophilic solvent components into the water phase and the hydrophobic phase of the carotinoid forms a monodisperse phase. X-ray analysis after subsequent lyophilization shows that approximately 90% of the carotinoid is in the amorphous state.[11]

Amorphous precipitation technology is used by the company Soliqs and the technology is advertised under the tradename NanoMorph®. The preservation of

the amorphous state could be achieved successfully for food products. To exploit the amorphous technology for pharmaceutical products, the stricter requirements for pharmaceuticals need to be met.

## 4.2. *Homogenization methods*

### 4.2.1. *Microfluidizer technology*

The previous Canadian company RTP (Montreal, now Skyepharma Canada Inc.) employed the microfluidizer to homogenize drug suspensions. The microfluidizer is a jet stream homogenizer of two fluid streams collied frontally with high velocity (up to 1000 m/sec)[12] under pressures up to 4000 bar. There is a turbulant flow, high shear forces, particles collied leading to particle diminution to the nanometer range.[13–15] The high pressure applied and the high streaming velocity of the lipid can also lead to cavitation additionally, contributing to size diminution. The patent describes examples requiring up to 50 passes through the microfluidizer to obtain a nanosuspension.[16] Sometimes, up to 100 cycles are required when applying the microfluidizer technology. This does not pose any problem on the small lab scale, but it is not production friendly for larger lab scale. The dispersion medium is water.

### 4.2.2. *Piston-gap homogenization in water (Dissocubes®)*

In 1994, Mueller *et al.*[17,18] developed a high pressure homogenization method based on piston-gap homogenizers for drug nanosuspension production. Dispersion medium of the suspensions was water. A piston in a large bore cylinder creates pressure up to 2000 bar. The suspension is pressed through a very narrow ring gap. The gap width is typically in the range of 3–15 micrometer at pressures between 1500–150 bar. There is a high streaming velocity in the gap according to the Bernouli equation.[19] Due to the reduction in diameter from the large bore cylinder (e.g. 3 cm) to the homogenization gap, the dynamic pressure (streaming velocity) increases and simultaneously decreases the static pressure on the liquid. The liquid starts boiling, and gas bubbles occur which subsequently implode, when the suspension leaves the gap and is again under normal pressure (cavitation). Gas bubble formation and implosion lead to shock waves which cause particle diminution. The patent describes cavitation as the reason for the achieved size diminution.[17,20] Piston-gap homogenizers which can be used for the production of nanosuspensions are e.g. from the companies APV Gaulin, Avestin or Niro Soavi. The technology was aquired by Skyepharma PLC at the end of the 90s and employed in its formulation development.[21–23]

### 4.2.3. *Nanopure technology*

For oral administration, the drug nanosuspensions themselves are, in most cases, not the final products. For patient's convenience, the drug nanocrystals should be incorporated in traditional dry dosage form, e.g. tablets, pellets and capsules. An elegant method to obtain a final formulation directly is the production of nanocrystals in non-aqueous homogenization media. Drug nanocrystals dispersed in liquid polyethylene glycol (PEG) or oils can be directly filled as drug suspensions into gelatine or HPMC capsules. The non-aqueous homogenization technology was established against the teaching that cavitation is the major diminution force in high pressure homogenization. Efficient particle diminution could also be obtained in non-aqueous media.[24–30]

To prepare tablets or pellets, the dispersion medium of the nanosuspension needs to be removed, i.e. in general, evaporated. Evaporation is faster and possible under milder conditions when mixtures of water with water miscible liquids are used, e.g. water-ethanol. To obtain isotonic nanosuspensions for intravenous injection, it is beneficial to homogenize in water-glycerol mixtures. The IP owned by Pharmasol covers, therefore, water-free dispersion media (e.g. PEG, oils) and also water mixtures.

## 4.3. *Combination Technologies*

### 4.3.1. *Microprecipitation™ and High Shear Forces (NANOEDGE™)*

The Nanoedge technology by the company Baxter covers a combination of precipitation and subsequent application of high energy shear forces, preferentially high pressure homogenization with piston-gap homogenizers.[31] As outlined in Sec. 4.1.1, the precipitated particles have a tendancy to grow. According to the patent by Kipp *et al.*, treatment of a precipitated suspension with energy (e.g. high shear forces) avoids particle growth in precipitated suspensions (= annealing process). The relative complex patent description can be summarized in a simplified way that the subsequent annealing stabilizes the obtained particle size by precipitation. As described in Sec. 4.1.2, precipitated particles can be amorphous or partially amorphous. This implies the risk that during the shelf life of a product, the amorphous particles can recrystalize, leading subsequently to a reduction in oral bioavailability or a change in pharmacokinetics after intravenous injection. The annealing process by Baxter converts amorphous or partially amorphous particles to completely crystalline material.[31]

### 4.3.2. *Nanopure® XP technology*

An important criteria for a technology is its scaling up ability and the possibility to produce on large scale, applying "normal" production conditions. The number of 50–100 passes through a homogenizer as partially required for the microfluidizer technology[16] is not production friendly. Piston-gap homogenizers (Sec. 4.2.2) proved to be more efficient, typically between 10–20 homogenization cycles are sufficient to obtain a nanosuspension. However, it would of course be desirable to apply even less homogenization cycles, reducing production time, potential product contamination by wearing of the machine and production costs. Pharmasol developed a new combination process, Nanopure XP (Xtended Performance)[32] leading to:

1. Identical particle sizes compared with high pressure homogenization in water (Sec. 4.2.2), but at half the cycle numbers or less.
2. Lower particle sizes at identical cycle numbers.

The process is again a combination technology, a pre-treatment step is followd by a high pressure homogenization step, typically performed with a piston-gap homogenizer.[34,35] The code for this homogenization technology is H42. Figure 4

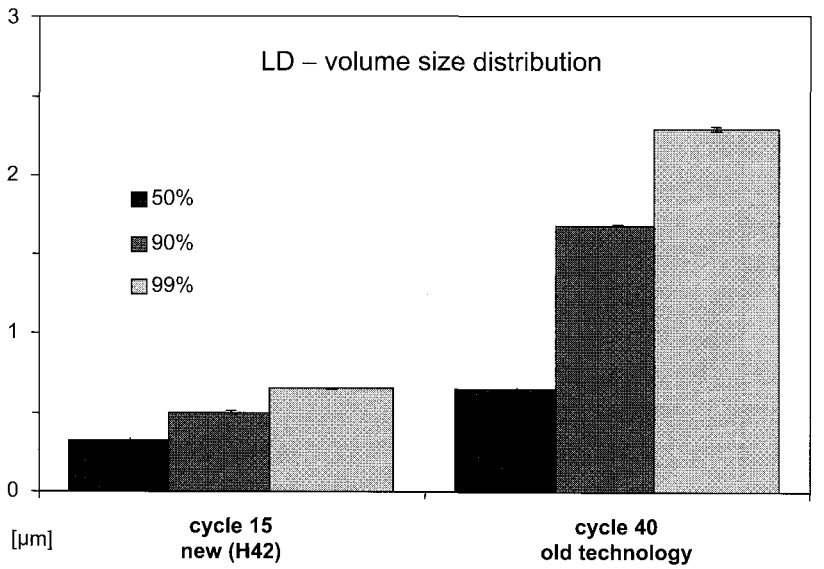


**Fig. 4.** Comparison of the old homogenization technology (homogenization in water, piston-gap homogenizer) on the right side to the new technology on the left side, presented are the laser diffractometry (LD) diameters 50%, 90% and 99% (volume distribution, Coulter LS230, Beckman-Coulter/Germany) (with permission after[33]).

demonstrates the efficiency of method processing a very hard drug material. Applying the novel H42 technology leads to distinctly smaller crystals after just 15 cycles, compared with the "old" technology of applying 40 cycles.

## 5. Application Routes and Final Formulations

### 5.1. *Oral administration*

Most attractive regarding regulatory and commercial aspects is the oral administration route. Compared with parenteral administration, the regulatory hurdles are much lower. In addition, the patient prefers an oral dosage form, that is why oral products possess the largest percentage of the pharmaceutical market. However, for the oral administration route, it is generally necessary to transfer the liquid nanosuspension into a solid dosage form.

Aqueous nanosuspensions can be used as a granulation fluid for producing tablets or as a wetting agent for pellet production. In addition, spray drying can be performed in order to obtain a product which can subsequently be processed to oral products. The first nanosuspension product in the market was Rapamune®, introduced in 2000 by the company Wyeth. Rapamune® is available on the market as oral solution, and alternatively as tablet. The tablet is more user-friendly. Comparing the oral bioavailabilities of solution and nanocrystal tablet, the bioavailability of the nanocrystals is 21% higher compared with the solution. The oral single dose of Rapamune® is 1 or 2 mg, the total tablet weight being 364 mg for 1 mg formulation and 372 mg for the 2 mg formulation, meaning that it contains a very low percentage of its total weight as nanocrystals. An important point is that the drug nanocrystals are released from the tablet as ultrafine suspension. In the event that crystal aggregation takes place to a pronounced extent, the dissolution velocity, and subsequently, the oral bioavailability of the BSC II drugs will be reduced. Therefore, there is an upper limit to load tablets with nanocrystals. In case the limit is exceeded and nanocrystals get in contact with each other within the excipient mixture of the tablet, the nanocrystals might fuse to larger crystals under the compression pressure during tablet production. For drugs with a low oral single dose such as Sirolimus in Rapamune®, incorporation into tablets causes little issues. A total nanoparticle load of less than 1% is well below the percentage being critical.[36]

The second product on the market was Emend®, introduced in 2001 by the Company Merck. The drug Aprepiptant is for the treatment of emesis (single dose is either 80 or 125 mg). Aprepiptant will only be absorbed in the upper gastrointestinal tract. Bearing this in mind, nanoparticles proved to be ideal in overcoming this narrow absorption window. The large increase in surface area due to nanonization leads to rapid *in vivo* dissolution, fast absorption and increased bioavailability.[37,38] The formulation of a tablet from micronized bulk powder made higher doses necessary,

leading to increased side effects.[39] The drug nanocrystals are contained within the hard gelatin capsules as pellets. Aprepiptant was formulated as capsules for it to be user friendly by healthcare providers and patients, and on the other hand, to make it applicable as pellets via a stomach tube. Currently, studies are being undertaken to evaluate the change in pharmacokinectics (if any) between the pellets and the capsules.

All nanocrystals in these first two products were produced using the pearl mill technology by Nanosystems/Elan. The prerequiste was the bioavailability of sufficient large scale production facilities for the respective product. In general, the candidates of first choice for nanosuspension technology are drugs with a relatively low dose. It is interesting that drugs such as Naproxen are formulated as nanosuspension (e.g. for fast action onset and reduced gastric irritancy),[40] however, it requires more sophisticated formulation technology to ensure the release of the drug nanocrystals as fine suspension when incorporated in a tablet in a relatively high concentration of a single dose of 250 mg. The tablet size (weight) has to be acceptable for the patient and that a dosing with two tablets should be avoided, for reasons of patient's compliance and marketing purposes.

Alternatively, to aqueous nanosuspensions, nanosuspensions in nonaqueous media can be produced by the Nanopure technology (Pharmasol). Nanocrystals dispersed in liquid PEG or oil can be directly filled into gelatine or HPMC capsules.[25] It saves the step of water removal and subsequent dispersion of the powder in a liquid capsule filling medium.

The Nanopure technology also allows production of nanocrystals in melted PEG (at 60°C). After solidification of the PEG nanosuspension, the drug nanocrystals are fixed (and kept seperated) in the solid PEG matrix. The solidified drug nanocrystal containing PEG can either be milled into powders and filled into the capsules, or alternatively, the hot liquid PEG nanosuspension can be directly filled into the capsules (Fig. 5, upper).

Instead of using aqueous nanosuspensions as fluids for the wet granulation process or extrusion of pellet mass, the nanosuspensions can be converted into a dry powder which is subsequently further processed into a tablet or a capsule. It also appears attractive to package such powders in sachets for redispersion in water or soft drinks prior to oral administration. Spray-drying is the only feasable cost-effective way to produce such powders. An attractive approach is the production of so-called "compounds" as described in the direct compress technology.[41] The term "compound" does not mean a chemical compound; in powder technology, "compounds" are defined as freely flowable granulate powders. In the direct compress technology, water-insoluble polymeric particles (e.g. Eudragit RSPO, ethylcellulose) are dispersed in the aqueous drug suspension, and lactose is dissolved. The mixture is a freely flowable compound yielded by spray-drying. The lactose

**Fig. 5.** Gelatin capsules filled directly with hot liquid PEG nanosuspension, solidification takes place in the capsules (upper) or filling of the capsules with milled solidified PEG nanosuspension (lower). (From Ref. 36 with permissions.)

is responsible for the good flowing properties. The water-insoluble polymeric particles also contribute to the formation of flowable granules, while at the same time allowing the compound to be compressed in a direct compaction process into tablets. The polymers form the matrix structure of the tablet. Depending on the percentage of the insoluble polymeric particles added, the resulting tablets may disintegrate fast or present a prolonged release system. A prolonged release of dissolving nanocrystal is desired in the case of high plasma that peaks at very early times (short $t_{max}$) and a targeted sustained blood level. Alternatively, the drug nanocrystal compound can be filled into hardgelatine capsules. Due to the presence of lactose and surfactant from the original nanosuspension, the compounds disperse relatively fast in liquids. Figure 6 shows the dispersion process of a compound after layering it on the surface of water in a beaker. As outlined above, efficient release and redispersion of the drug nanocrystals in a fine, nonaggregated state is a prerequisite for benefiting fully from the drug nanocrystal features.[42]

## 5.2. *Parenteral administration*

Intravenous administration is the second frequently investigated route. The company Baxter, with its technology NANOEDGE™, is presently focusing on intravenous nanosuspensions. They investigated Itraconazole nanosuspensions intensive.[44] It could be nicely shown that the side effects of the commercial product Sporanox® could be distinctly reduced by the administration of a nanosuspension. The nephrotoxicity of Sporanox® is not caused by the drug, but by the excipient

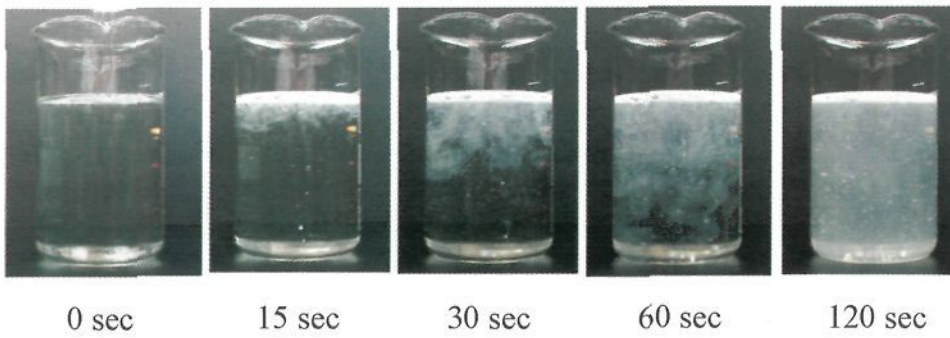


**Fig. 6.** Dispersion of a drug nanocrystal compound as a function of time after layering it on the surface of water in a beaker (with permission after[43]) (Compound: Aquacoat 40%, Lactose, 60%.)

used for solubilizing the drug, the hydroxypropyl-$\beta$-cyclodextrin.[45,46] The itraconzole nanosuspension was stabilized with Tween 80 surfactant being well tolerated intravenously.[44]

Administration of nanosuspsensions into body cavities is also of great interest, e.g. to increase the tolerability of the drug, to achieve a local treatment or to have a depot with slow release (e.g. into the blood). It could be shown that intraperitonal administration of a nanosuspension was well tolerated, whereas administration of a macrosuspension leads to irritancy [azodicarbonamide (ADA), unpublished data]. Intraperitonal administration can be used for local treatment or to obtain a depot with prolonged release into the blood. Interesting therapeutic targets include local inflammations, e.g. in joints. For instance, arthritic joint inflammations are caused by secretion products of activated macrophages. An interesting approach is therefore the administration of a corticoid nanosuspension directly into the joint capsule. The drug particles will be phagocytosed, the drug dissolves and reduces the hyperactivity of the macrophages. This concept is not new, being adopted by the company Boots in the 80s in an attempt to incorporate the corticoid prednisolone into polymeric nanoparticles made from PLA-GA-copolymer.[47] The particle load (polymer load) required to achieve a therapeutic drug level was being calculated. However, incubating macrophage cell cultures with the required particle concentration lead to cytotoxicity. The concept could not be realized, as it cannot occur with drug nanocrystals since no carrier polymer to required and present.

Producing parenteral products with drug nanocrystals has to meet higher regulatory hurdles and product quality standards distinctly. The produced drug nanosuspensions need to be terminally sterilized or alternatively produced in an aseptic process. In principal, sterilization is possible by autoclaving. However, the increase in temperature can reduce hydration of steric stabilizers, thus leading to some aggregation during the sterilization process. Gamma irradiation is *a priori* a

non-preferred process by the industry due to the necessary analytics (i.e. proof of absence of toxic irradiation products). In addition, it was also observed that irradiation can cause aggregation not by directly interacting with the drug nanocrystals, but with the stabilizing surfactant. Irradiation of Tarazepide nanosuspension leads to aggregation; simultaneously, a decrease in zeta potential also occurred during the irradiation process. A decrease in zeta potential, i.e. electrostatic repulsion, was considered as the cause for the aggregation process. It can be concluded that the production of drug nanosuspensions in an aseptic, controlled process has to be preferred, compared with the terminal sterilization by irradiation. The aseptic production process can be validated and documented relatively easy, therefore, being simpler to handle as an irradiation sterilization with accompanied analytics.

## 5.3. *Miscellaneous administration routes*

Oral and parenteral/intravenous routes are the ones in which developments are focusing, clearly due to the commercial background and the relation between the development costs for a market product versus its potential annual sales. However, drug delivery could also be improved when using drug nanocrystals for pulmonary and ophthalmic adminstration or dermal application.

Poorly soluble drugs could be inhaled as drug nanosuspension. The drug nanosuspension can be nebulized using commercially available nebulizers.[48,49] Disposition in the lungs can be controlled via the size distribution of the generated aerosol droplets. Compared with microcrystals, the drug is more evenly distributed in the droplets when using a nanosuspension. The number of crystals are higher, consequently, the possibility that one or more drug crystals are present in each droplet is higher.

It could be shown that nanoparticles possess a prolonged retention time in the eye, most likely due to their adhesive properties.[50–52] From this, poorly soluble drugs could be administered as a nanosuspension. However, the major obstacles are the commercial considerations. In many cases, the sales volume do not justify the costs for the development of a new market product. This is especially the case when a company has already a drug formulation which might be less efficient, but is already a product on the market. The price achievable with an improved product is not sufficiently high to cover the development costs of this new product. An additional major obstacle for the development of such improved products is the cost reduction policy of the healthcare systems worldwide. A longer treatment time with a less efficient product might still be less expensive for the healthcare system than a shorter treatment time with a more efficient, but distinctly more expensive product.

The same is valid for dermal products. Sales per product are lower compared with e.g. oral products, as the dermal market is smaller. Dermal nanosuspensions

are mainly of interest if conventional formulation technology fails or if it is distinctly less efficient. Dermal drug nanosuspensions lead to a supersaturated system because of their increased saturation solubility. The higher concentration gradient between topical formulation and skin can improve drug penetration into the skin. In addition, because of their small size, drug nanocrystals could target the hair follicle by protruding into the gap around the hairs. This was illustrated in solid lipid nanoparticles of a similar size.[53] Adhesive properties of drug nanocrystals are also an area of interest. Adherence to the skin reduces the "loss" of drug to the environment/third persons. This is especially so in the event that highly active compounds are applied, e.g. hormones. For this reason, the drug estradiole was incorporated into solid lipid nanoparticles to better localize it on the skin.[54]

## 6. Nanosuspensions as Intermediate Products

As described above, nanosuspensions can be produced such that nanocrystals appear in final products. Alternatively, drug nanosuspensions can be used as intermediate product, i.e. the drug nanocrystals do not appear in the final product. Recently, the SolEmuls® technology was developed to produce drug-loaded emulsions for intravenous injection, i.e. localizing poorly soluble drugs in the interfacial layer of lecithin emulsions.[55–57] The applicability of the technology has been proven for several drugs including amphotericin B,[58] itraconazole,[59,60] ketoconazole,[61] and carbamazepine,[62,63] among others. The drug Amphotericin B is on the market as a solution (Fungizone®), but also in liposomes (Ambisome®); the latter having the benefit of reduced nephrotoxicity.[64] Liposomes are relatively expensive (daily treatment costs approximately EUR 1000–2000[64,65]), therefore Amphotericin B was incorporated into parenteral emulsions. These emulsions can also reduce nephrotoxicity,[66] but for their production, it was necessary to use organic solvents. Egg lecithin and amphotericin B were dissolved in an organic solvent, the solvent evaporated and the obtained drug-lecithin mixture was used to produce an o/w emulsion. In these emulsions, amphotericin B was located in the interfacial lecithin layer as Amphotericin B is simultaneously poorly soluble in water and in oils.[67] There were also attempts to incorporate amphotericin B in the emulsion by simply adding Amphotericin B powder to the emulsion and subsequently shaking it. However, even shaking for 18 hours with 1800 rph was unable to completely dissolve the Amphotericin B. The reason was simply due to its low solubility in the water, and the dissolution velocity was also extremely low, i.e. the process of dissolution and redistribution into the lecithin layer takes too long for it to be used in pharmaceutical production. The problem was solved by the SolEmuls technology, i.e. simple co-homogenization of oil droplets and microcrystals. For a de novo production, a coarse pre-emulsion of lecithin stabilized

oil droplets in water is prepared, the drug powder is admixed under stirring and the obtained hybrid suspension subsequently homogenized at 600 bar (pressure being in the range to be used in pharmaceutical production lines). The high streaming velocities in the homogenization process lead to fast dissolution of the drug microcrystals and the re-distribution into the interfacial lecithin layer (Fig. 7).

Depending on the size of the drug crystals, 5–10 homogenization cycles are required. The number of homogenization cycles can be reduced when adding the drug not as microcrystals, but as nanocrystals in the form of a nanosuspension. A concentrated nanosuspension is prepared (e.g. 20–30% solid content) and added to the pre-emulsion. Ideally the nanosuspension is also stabilized by lecithin, i.e. the same emulsifier for the suspension and the emulsion. Alternatively, intravenously accepted stabilizers such as Tween 80 or Poloxamer 188 can be used. They are accepted intravenously without posing any regulatory issues. In addition, mixing the emulsion and nanosuspension at a ratio of 10:1 or higher will dilute the stabilizer concentration used in the nanosuspension by at least a factor of 10, meaning that in the final product, the nanosuspension surfactant concentration is typically 0.1 or 0.01%. The question might arise as to why an emulsion should be prepared using a nanosuspension as an intermediate product, when it can administer the nanosuspension itself intravenously?

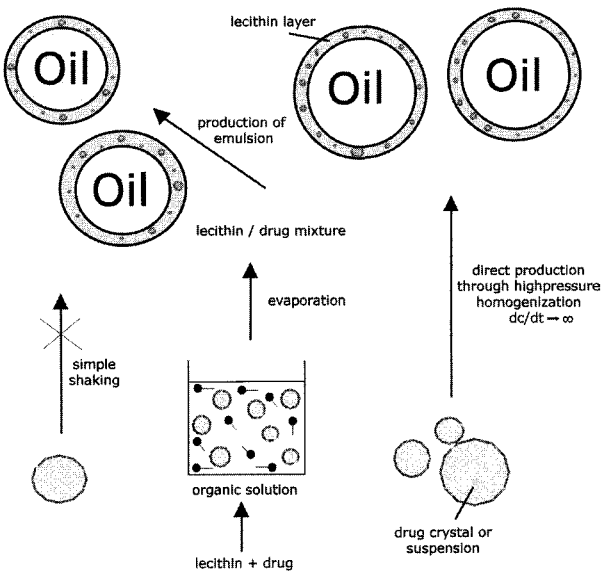


**Fig. 7.** Drug incorporation through various methods in comparison. Left: traditional attempt of shaking or alternatively use of organic solvent; Right: SolEmuls® process.

The reason is that drug-loaded parenteral emulsions are already products on the market (e.g. Diazepam-Lipuro, Etomidate-Lipuro, etc.), i.e. in a dosage form with which the regulatory authorities are already familiar with. Applying the SolEmuls technology and using lecithin-stabilized nanosuspension, the final product will only contain the excipients of an o/w emulsion for parenteral nutrition, without additional excipient plus the drug. It is an accepted known system with regard to the excipient status and its perfomance after intravenous injection. In contrast, drug nanosuspensions represent a new dosage form not yet present as intravenous formulations on the market. Registration of a completely new dosage form for a certain administration route is just more complicated and timely than registration of a product based on an established, known technology.

## 7. Perspectives

There was a "delayed" acceptance of the nanocrystal technology in the 90s. Pharmaceutical companies tried to solve their formulation problems with the traditional formulation approaches. However, the increasing number of drugs having a very low solubility, and not able to be formulated with these traditional formulation approaches, lead to a broad acceptance of the drug nanocrystal technology. This is clearly reflected in the increasing number of licensing agreements between companies holding nanocrystal IP and a number of medium and large pharmaceutical companies. The smartness of the technology is that it can be universally applied to practically any drug. Identical to micronization, it is a universal formulation principle, but limited to BSC drugs class II. The time between the beginning of intensive research in the drug nanocrystal technology and the first products on the market was relatively short, about one decade. The value of a formulation principle or technology can be clearly judged by looking at the number of products on the market, in the clinical phases, and/or the time of entry into the market. Based on these criteria, the drug nanocrystal technology is a successful emerging technology. Meanwhile, "Big Pharma" also realized the drug nanocrystal value. In combination with the further increasing number of poorly soluble drugs, a distinct increase in drug nanocrystal-based products on the market can be expected. In many cases, oral products will dominate because of the market share, higher sales volumes and less regulatory hurdles and quality requirements, compared with parenteral products.

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# 15

# Cells and Cell Ghosts as Drug Carriers

*José M. Lanao and M. Luisa Sayalero*

## 1. Introduction

Microparticle and nanoparticle polymeric systems currently occupy an important place in the field of drug delivery and targeting.[1] Nevertheless, there are biological drug carriers that offer an efficient alternative to such systems. Within the different systems of biological carriers, of great importance are cells and cell ghosts, which are both efficient and highly compatible systems from the biological point of view, capable of providing the sustained release and specific delivery to tissues, organs and cells of drugs, enzymatic systems and genetic material. Cell systems such as bacterial ghosts, erythrocyte ghosts, polymorphonuclear leukocytes, apoptotic cells, tumor cells, dendritic cells, and more recently, genetically engineered stem cells, are all examples of how cell systems of very diverse nature can be suitably manipulated and loaded with drugs and other substances, to permit specific drug delivery *in vivo* with important therapeutic applications.[2–8] Cell carriers for drug delivery are used in very different applications such as cancer therapy, cardiovascular disease, Parkinson's, AIDS, gene therapy, etc. Table 1 shows the classification of biological carriers for drug delivery based on the use of cells and cell ghosts.

## 2. Bacterial Ghosts

Bacterial ghosts are intact, non-living, non-denatured bacterial cell envelopes devoid of cytoplasmic contents. They are created by lysis of bacteria, but maintain

**Table 1** Kinds of cells and cell ghosts used for drug and gene delivery.

| Cell carrier | Target | Encapsulated substance |
|---|---|---|
| Bacterial ghost | Tissues, macrophages, cells | Drugs, vaccines, genetic material |
| Erythrocyte ghost | RES, macrophages | Drugs, enzymes, peptides |
| Engineered stem cells | Tumor cells, T cells, macrophages | Genetic material |
| Polymorphonuclear leucocytes | Tissues | Drugs |
| Apoptopic cells | Tumor cells | Drugs |
| Tumor cells | Tumor cells | Drugs |
| Denditric cells | T cells | Drugs |

their cellular morphology and native surface antigenic structures, including their bioadhesive properties.[3,9]

Bacterial ghosts allow the encapsulation of drugs and other substances, and their specific attachment to mammalian tissues and cells. This kind of cell carrier acts as a true drug delivery system, allowing the permanency of drugs in the systemic circulation to be increased together with tissue-specific targeting. They are thus a promising alternative to conventional drug delivery systems such as liposomes or nanoparticles.

The main advantages of bacterial ghosts as delivery systems are the fact that they are non-living, i.e. they can act as delivery systems of drugs, antigens or DNA; allow specific delivery to different tissues and cell types; and are well captured by phagocytic cells and antigen-presenting cells as dendritic cells. Among the drawback of bacterial ghosts is the possibility that they might revert to being virulent, the possibility of horizontal gene transfer, the stability of the recombinant phenotype, and pre-existing immunity against the carrier used.[10]

Usually, bacterial ghosts are produced by protein E-mediated lysis of Gram-negative bacteria.[11] The production of bacterial ghosts is based on the controlled expression of the bacteriophage PhiX174-derived lysis gene E. Expression of this gene from a plasmid in Gram-negative bacteria leads to the formation of a transmembrane lysis tunnel structure that penetrates the inner and outer membranes, and is formed by protein E with border values fluctuating between 40–200 nm in diameter. Protein E is a hydrophobic protein localized exclusively in the cell envelope.[12] E-mediated lysis has been achieved in many Gram-negative bacteria.[13]

Scanning electron micrographs of E-lysed cells reveal that bacterial ghosts contain only one E hole in a bacterial ghost, although in a few cases, there are two holes. The cytoplasm is expelled as a consequence of the high osmotic pressure inside the

cell. The collapse of membrane potential and the release of cytoplasmic components such as proteins, nucleic acids, etc occur simultaneously.[14] In the case of strains of *E. coli*, this effect occurs within a period of 10 min after the induction of expression.[15] The resulting empty bacterial cell envelope is considered a bacterial ghost. Bacterial ghosts show all the morphological, structural and immunogenic properties of a living cell.[9,15–17] Since bacterial ghosts are derived from Gram-negative bacteria that are able to adhere to structures such as fimbriae and lipopolysaccharide, they are used for specific binding to human tissue.[18]

Bacterial ghost drug-loading is accomplished by the suspension of lyophilised bacterial ghosts in a buffered medium containing the drug. The ghosts are then subjected to an incubation process varying from 5 to 30 min at 24°C. They are then washed to remove excess drug.[18,19]

In order to prevent rapid leakage of loaded water-soluble drugs or other substances, the bacterial ghosts are sealed by fusion of the cell membrane with membrane vesicles at the edges of the lysis pore. For the sealing step, the bacterial ghosts suspension is incubated in the fusion buffer at 28°C for 10 min.[18] Figure 1 shows a scheme of the production of bacterial ghosts by protein E-mediated bacterial lysis.

The *in vitro* release of drugs from loaded bacterial ghosts is performed from a suspension of drug-loaded bacterial ghosts that is dialysed through a membrane suitable for excluding the ghosts. Dialysis is performed at 28°C in PBS buffer.[19] The concentrations of drug released into the buffer at preset times are quantified using an appropriate analytical technique.

In studies addressing the adherence and capture of loaded bacterial ghosts by target cells such as macrophages, human colorectal adenocarcinoma cells (Caco-2) or dendritic cells, fluorescent markers such as fluorescein isothiocyanate (FITC) are used. These allow adherence to be assessed using fluorescence microscopy and flow cytometry techniques.[18,19] Macrophages internalize bacterial ghosts to a greater extent than Caco-2 cells.[18,19] Studies carried out using confocal laser scanning microscopy with *M. haemolytica* ghosts loaded with Doxorubicin have shown that the drug was associated with the ghosts membranes and the inner lumen.[19] Denditric cells that are professional phagocytic cells displaying the phagocytic capacity of antigens also have a good capacity for capturing bacterial ghosts, allowing the latter to be used as a vehicle for immunization and immunotherapy.[20]

## 2.1. *Application of bacterial ghosts as a delivery system*

Bacterial ghosts have important therapeutic applications. They can be loaded with drugs, proteins and other substances, and can be targeted selectively to macrophages, tumors or endothelial cells.[10,19]

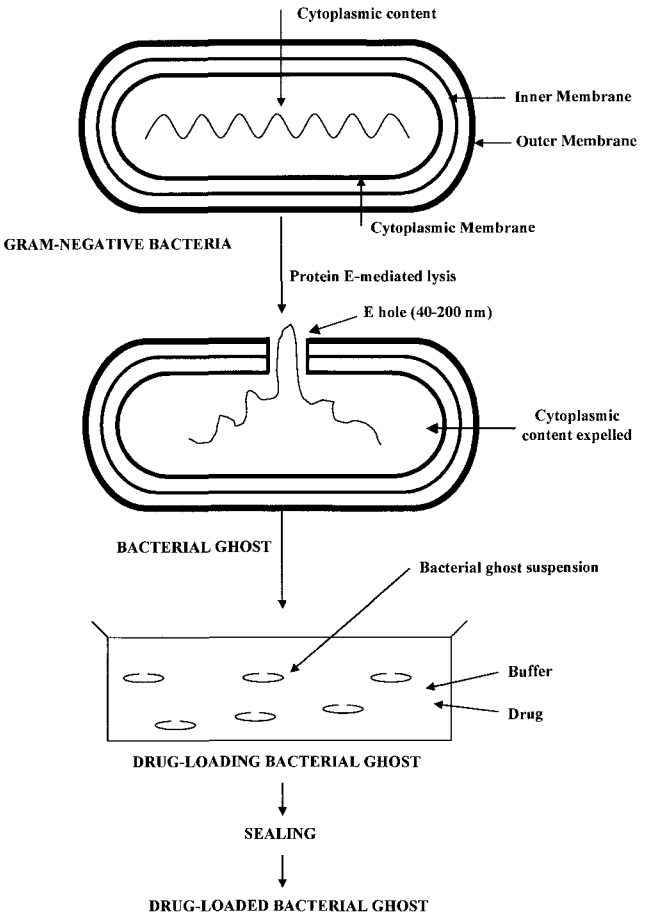


**Fig. 1.** Production and drug loading of bacterial ghosts.

Bacterial ghosts have been used as efficient drug delivery systems[10] in the field of anti-cancer drugs.[18] Bacterial ghosts obtained have been used as a delivery system of doxorubicin to human colorectal carcinoma cells. Cytotoxicity assays revealed that doxorubicin-loaded ghosts show better antiproliferative capacity in Caco-2 cells than when free doxorubicin is used at the same concentration.[18] Experiments have also been carried out using *E.coli* ghosts containing streptavidin, in order to increase the affinity of streptavidin for biotinylated compounds. Streptavidin-loaded ghosts permit specific targeting to mucosal surfaces of the gastrointestinal and respiratory tracts, and also to phagocytic cells.[3] Bacterial ghosts have been used as veterinary vaccines for the immunization of different animal species.[9]

*Pasteurella multocida* is a pathogen that causes morbidity and mortality in rabbits and its importance as a human pathogen has also been recognized. *P. multocida*

ghosts have been used to immunize rabbits and mice.[17] Similar results have been obtained in the immunization of cattle against pasteurellosis using *Pasteurella haemolytica* ghosts.[11]

*Actinobacillus pleuropneumoniae* is a highly contagious microorganism and is the cause of porcine pleuropneumonia, infecting 30–50% of pig populations. However, *Actinobacillus pleuropneumoniae* vaccines provide limited protection, since they decrease mortality but not morbidity in swine. Comparative studies have been carried out on immunization using a aerosol infection model for pigs vaccinated with loaded-ghosts or formalin inactivated *Actinobacillus pleuropneumoniae* bacterins. The results obtained showed that immunization with bacterial ghosts is more efficient in protecting pigs than bacteria.[21,22]

Bacterial ghosts can also be used as carriers of therapeutic DNA or RNA.[3,13] The use of nucleic acid vaccines currently offers a technique for the development of prophylactic or therapeutic vaccines, based on the use of DNA plasmids to induce immune responses by direct administration of DNA-encoding antigenic proteins into animals, and this is also suitable for the induction of cytotoxic T cells.[23,24] Bacterial ghosts loaded with DNA produce a high level of gene expression. They can be used to enhance the mucosal immune response to target antigens expressed in the bacterial ghost system. They can also be used for the specific targeting of DNA-encoded antibodies to primary antigens located in cells.[13] Ghosts of *Vibrium cholerae* have been tested as antigen carriers of *Chlamidia trachomatis* as potential vaccines for the control of genital infections produced by this bacteria. Recombinant *Vibrium cholerae* ghosts, previously cloned with a major outer membrane protein of *C. trachomatis*, afforded a high level of protective immunity against *Chlamydia* in a murine model.[25,26] *Mannheimia haemolytica* is a pathogen that causes ovine mastitis. *M. haemolytica* ghosts loaded with plasmid DNA stimulate the elicitation of efficient immune responses in mice, with no symptoms of acute or subacute toxicity during the experiment.[27]

## 3. Erythrocyte Ghosts

Erythrocytes constitute the largest population of blood cells and are produced in the bone marrow. They are mature blood cells that produce haemoglobin and carry out the exchange of oxygen and carbon dioxide between the lungs and the body tissues.

The term "erythrocyte ghost" attempts to define the resulting cell-like structure when erythrocytes are subjected to a reversible process of osmotic lysis.[28] For more than 30 years, many studies, both *in vivo* and *in vitro*, have been carried out to explore the use of erythrocyte ghosts as delivery systems of drugs and other substances.[2]

Erythrocyte ghosts are obtained from fresh erythrocytes coming from human blood or the blood of different animal species such as the rat, mouse, rabbit, etc, and are loaded with different types of substance, mainly drugs, peptides and enzymes, using different encapsulation methods. The most frequent methods for collecting erythrocyte ghosts are osmosis-based methods such as hypotonic dialysis.[2,29]

Autologous erythrocyte ghosts offer a drug delivery system that can act as a reservoir of the drug or substance encapsulated, providing the sustained release of the drug into the organism together with selective targeting of the drugs to the reticuloendothelial system (RES) of the liver, spleen and bone marrow.[2]

The main advantages of carrier erythrocytes as drug delivery systems are their high degree of biocompatibility, the possibility of encapsulating the drug in a small amount of cells, the sustained release of the encapsulated drug or substance into the body, the selective targeting to the RES, and the possibility of encapsulating substances of high molecular weight such as peptides. Among the drawbacks of these systems are the rapid leakage of some drugs out of the loaded erythrocytes and other problems related to their standardized preparation, storage and potential contamination.[2]

Erythrocyte ghosts can be obtained by diverse procedures such as hypotonic dilution, hypotonic pre-swelling, osmotic pulse, hypotonic hemolysis, hypotonic dialysis, electroporation, drug-induced endocytosis and chemical methods.[2,30] Of the different ways of obtaining carrier erythrocytes, hypotonic dialysis is undoubtedly the most frequently used encapsulation method. The reasons why it is so popular are its simplicity, its ease of application for a large number of drugs, enzymes and other substances, and because it is the method that best conserves the morphological and haematological properties of the erythrocyte ghosts obtained.

Hypotonic dialysis is based on the exposure of red cells to the action of a hypotonic buffer, inducing cell swelling and the formation of pores that permit the drug to enter erythrocytes by means of a passive mechanism. Figure 2 shows a scheme of the production of erythrocyte ghosts using a hypotonic dialysis method.

Morphological inspection of erythrocyte ghosts is usually performed using transmission (TEM) or scanning (SEM) electron microscopy.[2] Some physical parameters of red cell membranes can also be studied from the diffusion of haemoglobin.[28] The haemolytic methods employed in the production of erythrocyte ghosts normally affect the haemolytic volume, surface area and tension.[28] Figure 3 shows the morphological changes observed by SEM that occur in amikacin-loaded erythrocytes due to hypotonic dialysis.[31]

Haematological parameters determine the effects of the procedure used to collect erythrocyte ghosts on their haematological properties. Among others, parameters such as reduced glutathione (GSH), mean corpuscular volume (MCV) or red cell distribution width (RDW), may be evaluated using a haematology analyzer.

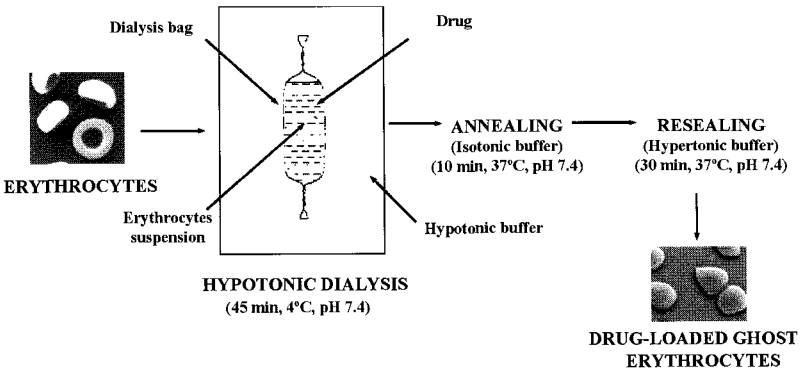


**Fig. 2.** Production and drug loading of erythrocyte ghosts using a hypotonic dialysis method.

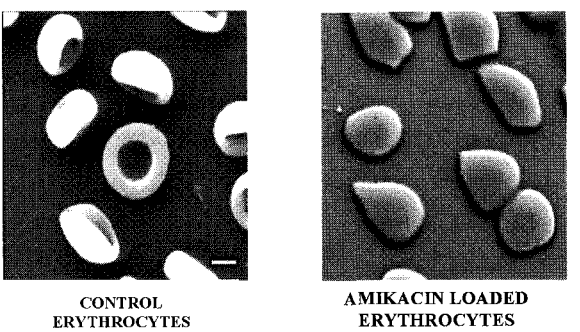


**Fig. 3.** SEM micrographs of amikacin carrier erythrocytes obtained by hypotonic dialysis[31] (Copyright 2005 from *Encapsulation and in vitro Evaluation of Amikacin-Loaded Erythrocytes* by C. Gutiérrez Millán. Reproduced by permission of Taylor & Francis Group, LLC, http://www.taylorandfrancis.com).

Erythrocyte ghosts obtained by hypotonic dialysis show a decrease in the mean corpuscular volume and an increase in size dispersion.[28,29] Erythrocyte ghosts show a greater degree of haemolysis than normal erythrocytes.[29]

## 3.1. *Applications of erythrocyte ghosts as a delivery system*

Erythrocyte ghosts can be used as potential drug delivery systems for enzymes, proteins and peptides, allowing sustained release into the systemic circulation and the delivery of these substances into the RES.[2]

*In vitro* release of drugs from loaded erythrocyte ghosts is usually tested using autologous plasma or an isoosmotic buffer at 37°C; alternatively, a dialysis bag may be used.[32] The *in vitro* release of drugs and substances from loaded erythrocytes is usually a first-order process, suggesting that the drug crosses the plasma membrane through a passive diffusion mechanism.[33] However, zero-order release

kinetics from loaded erythrocytes has also been described.[34] *In vitro* studies about the release kinetics of different drugs, enzymes and peptides from loaded erythrocytes have shown a slow release of the encapsulated substance.[2]

When loaded erythrocyte ghosts are administered *in vivo*, changes in the pharmacokinetics of the encapsulated drugs occur, involving a systemic drug clearance related to the biological half-life of the erythrocytes.[35] Increased serum half-lives and the areas under the curve of drugs encapsulated in loaded erythrocyte ghosts, in comparison with the free drug, have been observed in animals and humans.[36,37] At the same time, erythrocyte ghosts show a greater accumulation in tissues such as liver and spleen.[38,39]

Surface treatment of erythrocyte ghosts with substances such as glutaraldehyde, ascorbate, $Fe^{(+2)}$, diamide, band 3-cross-linking reagents, trypsin, phenylhydrazine and the N-hydroxysuccinimide ester of biotin (NHS-biotin), enhances the recognition of erythrocyte ghosts by macrophages *in vitro* and liver targeting *in vivo*.[40–43]

Red cells may be used as carriers for some drugs such as antineoplastics, anti-infective agents, antihypertensives, corticosteroids, etc.[2] Thus, carrier erythrocytes have been widely studied as delivery systems of antineoplastic drugs for targeting the RES located in organs such as liver and spleen.

Different antineoplastic drugs have been encapsulated in erythrocyte ghosts in both *in vitro* and *in vivo* experiments.[2] Increases have been obtained in average survival times in the treatment of mice bearing hepatomas, using methotrexate-loaded carrier erythrocytes.[44] Better recognition and capture of erythrocyte ghosts by macrophages have been obtained by using biotinylated erythrocytes containing methotrexate,[45] by alterations to the membrane using band-3 cross-linkers of erythrocyte ghosts containing etoposide,[46] or by treatment of erythrocytes containing doxorubicin with glutaraldehyde.[47]

Anti-infective agents such as gentamicin, metronidazole, primaquine or imizol have also been encapsulated in erythrocytes.[2] Human erythrocytes containing gentamicin have proven to act as an efficient slow-release system *in vivo*.[48,49]

Erythrocyte ghosts containing dexamethasone have been used *in vivo* in rabbits and humans. A sustained release of dexamethasone *in vivo* in animals and humans was observed using carrier erythrocytes. An increased anti-inflammatory effect of the drug using carrier erythrocytes was observed in rabbits.[50,51]

Moreover, new prodrugs of anti-opioid drugs such as naltrexone and naloxone have been encapsulated in erythrocytes to solve stability problems of the primary drug within the erythrocyte. The encapsulated prodrugs are transformed into the active compound, following their release from erythrocyte ghosts.[52]

In the fields of biochemistry and enzymatic therapeutics, the encapsulation of enzymes in erythrocytes has been studied in some depth. Enzymatic

deficiencies or the treatment of specific illnesses may be approached using carrier erythrocytes loaded with enzymes. The encapsulation of enzymes in erythrocytes solves some of the problems associated with enzyme therapy, such as the short half-life deriving from the action of plasma proteases, intolerant reactions, and the immunological disorders or allergic problems associated with the use of enzymes in therapeutics. *In vitro* or *in vivo* studies with enzyme carrier erythrocytes have been performed using L-asparaginase,[53] hexokinase,[54] alcohol dehydrogenase,[55] aldehyde dehydrogenase,[56] alcohol oxidase,[57] glutamate dehydrogenase,[58] uricase,[59] urokinase,[60] lactate 2-mono oxigenase,[61] arginase,[62] rhodanase,[63] recombinant phosphotriestearase,[64] delta-aminolevulinate dehydratase,[65] urease,[66] pegademase,[67] brinase[68] and alglucerase.[69] One of the best examples of the use in therapeutics of carrier erythrocytes containing enzymes, is that of L-asparaginase encapsulated in human erythrocytes. This has been successfully used in the treatment of acute lymphoblastic leukaemia in paediatrics.[70]

Erythrocyte ghosts may act as carrier systems for the delivery of peptides and proteins. One of the main therapeutic applications of carrier erythrocytes in this field is that of anti-HIV peptides. Nucleoside analogues successfully inhibit the replication of immunodeficiency virases. In view of the importance of the monocyte-macrophage system in infection by HIV-1, it would be of maximum therapeutic interest to have available, the specific delivery of these therapeutic peptides into macrophages, which act as an important reservoir for the virus. Carrier erythrocytes containing anti-HIV peptides such as azidothimidine (AZT) and didanosine (DDI), significantly reduced the pro-viral DNA content in comparison with the administration of free peptides in a murine AIDS model.[71] Similar results have been obtained with 2′,3′-dideoxycytidine 5′-triphosphate'(ddCTP),[72] 2′,3′-dideoxycytidine (ddCyd)[73] and AZT prodrugs[74] encapsulated in erythrocytes.

Anti-neoplastic peptides such as 2-fluoro-ara-AMP (fludarabine) and 5′-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), a pro-drug of 5-fluro-2′-deoxyuridine (FdUrd), have been encapsulated in human carrier erythrocytes, behaving as a slow-release delivery system.[75,76]

Macrophage uptake *in vitro* of antisense oligonucleotides may be increased by using carrier erythrocytes.[77,78] Other peptides, such as erythropoietin,[79] heparin,[80] dermaseptin S3,[81] interleukin-3[82] or vaccines,[83] have also been encapsulated in erythrocytes to increase their stability,[84] acting as a slow release system with a prolonged half-life,[80,84] or for their specific targeting to bacterial membranes.[85]

Erythrocyte ghost derivatives can also be used as drug delivery systems. Nanoerythrosomes are erythrocyte membrane derivatives formed by spheroid vesicles, obtained by consecutive extrusion under nitrogen pressure through a polycarbonate filter membrane of a erythrocyte ghost suspension to produce small vesicles having an average diameter of 100 nm. *In vitro* and *in vivo* studies, carried out with

nanoerythrosomes loaded with daunorubicin, have shown that when linked covalently to nanoerythrosomes, the drug produces slow release of daunorubicin to the organism over a prolonged period of time and also that, in comparison with the free drug, cytotoxicity is greater.[86] The advantage of nanoerythrosomes, as compared with erythrocyte ghosts as drug delivery system, is that the former are able to escape from the reticuloendothelial system faster.[86,87] *In vitro* studies have shown that the nanoerythrosome-daunorubicin complex is rapidly adsorbed and phagocytosed by macrophages.[88] Liver, spleen and lungs are the organs in which nanoerythrosomes show the greatest capacity of accumulation.[89]

Another derivative of erythrocyte ghosts are reverse biomembrane vesicles loaded with drugs.[90] Reverse biomembrane vesicles are produced by spontaneous vesiculation of the ghost erythrocyte membrane by endocytosis, using an appropriate vesiculating medium, producing small vesicles containing the drug within the parent ghost. *In vivo* studies carried out using reverse biomembrane vesicles from erythrocyte ghosts loaded with doxorubicin in rats have revealed increases in the half-life and bioavailability of the drug, the liver and spleen, being the main organs for the clearance of this drug delivery system.[90]

## 4. Stem Cells

In gene therapy, a therapeutic transgene is introduced into the patient with a view of supplementing the functions of an abnormal gene. To achieve the delivery of genetic material into the target cell, it is necessary to have a suitable carrier. One important aim in the field of gene therapy is the design and development of gene carriers that encapsulate and protect the nucleic acid, and selectively release the vector/nucleic acid complex to the target tissue, so that the genetic material will be released at the cellular level later. In practice, there are several ways to achieve this. The first is through the use of modified viruses containing the genetic material of interest. The use of viruses for gene delivery has some drawbacks since it is limited to specific cells susceptible to being infected by the virus, and also the administration itself of the virus, has some immunological problems among others.[91–93] The second alternative is to use living cells modified genetically, such as stem cells, to deliver transgenic material into the body.[8,94]

Stem cell therapy is a new form of treatment, in which cells that have died or lost their function are replaced by healthy adult stem cells. One advantage of this kind of cell is that it is possible to use samples from adult tissues or cells from the actual patient, for culture and subsequent implantation.

Within the framework of stem cell research, the use of stem cells as delivery systems is a novel and attractive technique in the field of gene therapy, in which the cells of the patients themselves are genetically engineered, in order to introduce a therapeutic transgene used to deliver the genetic material. A promising therapeutic

strategy is the use of stem cells such as lymphocytes or fibroblasts as drug delivery systems. Experimental studies using stem cells as such systems have been tested in different therapeutic applications, especially in the field of cancer therapy. Considering the affinity of stem cells for tumor tissue, engineered stem cells have been successfully used for direct drug delivery to cancer cells.[8,94] *In vitro* cultures have been made of human mesenchymal stem cells from bone marrow that are transduced with an adenovirus vector carrying the human interferon beta-gene, which exerts therapeutic action against cancer. Engineered stem cells administered *in vivo* allow the delivery of the genetic material to cancer cells. This new drug delivery system has proven to be efficient in the treatment of experimental neoplasms, such as lung cancer, in mice.[94] Figure 4 shows a scheme of the application of stem cells as carriers for gene delivery in experimental cancer therapy.

*In vivo* studies have also been carried out with neural stem cells engineered using adenoviral vectors to express interleukin-12, an oncolytic gene, whose efficiency has been demonstrated in the treatment of intracranial malignant gliomas in mice.[8,95]

The used of haematopoietic stem cells has allowed antiviral genes to be introduced in both T cells and macrophages for the treatment of AIDS.[96] The use of stem cells as vehicles for gene therapy has also been suggested for the treatment of ischaemic heart disease.[97]

Stem cells have also been employed in the field of antiepileptic therapy. Glial precursor cells, which release adenosine, have been derived from adenosine kinase embryonic stem cells. In these experiments, the fibroblasts were engineered to release adenosine by inactivating adenosine metabolising enzymes. After encapsulation within polyethersulfone hollow-fibre capsules, and the introduction into

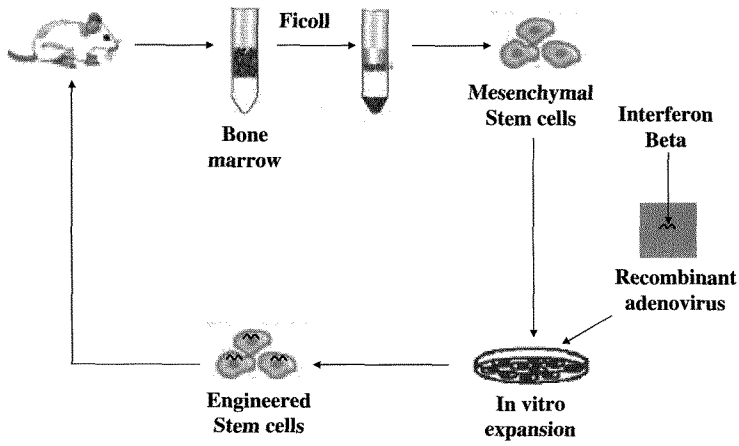


**Fig. 4.** Application of stem cells as carriers for gene delivery in experimental cancer.

the brain ventricles in a rat epilepsy model, the local release of adenosine allows drug-resistant focal epilepsy to be treated. These engineered cells were shown to suppress seizure activity.[98,99]

## 5. Polymorphonuclear Leucocytes

Polymorphonuclear leucocytes (PMN) can be used as carriers of antibiotics in view of their selective targeting to sites of infection. Simply incubating PMN in the presence of high concentrations of antibiotic for 1 hr at 37°C guarantees cell loading with the antibiotic. PMN loaded with the macrolide azithromycin have been found to be efficient in an *in vitro* model that permits the delivery of the antibiotic in a bioactive form to *Chlamydia* inclusions in polarized human endometrial epithelial (HEC-1B) cells infected with *Chlamydia trachomatis*. PMN carriers allow the accumulation of large amounts of antibiotic in endometrial epithelial cells and its retention over long periods of time.[4]

## 6. Apoptopic Cells

Programmed cellular death or apoptosis is a process that is controlled genetically in which the cells induce their own death in response to different types of stimulus such as the binding of death-inducing ligands to cell surface receptors.

A new strategy for drug delivery, called apoptopic induced drug delivery (AIDD), allows drug delivery to tumor cells upon the initiation of apoptosis by using a biological mechanism to achieve drug delivery.[5] This new system is based on the fact that apoptosis produces many changes in cell morphology that can be taken advantage of to achieve drug delivery.

Apoptosis is reflected in enhanced membrane permeability, which favors the release of the encapsulated drug from the apoptotic cells to the tissue. Phagocytosis of drug- loaded apoptotic carrier cells by tumor cells facilitates the localization of the drug within the tumor cell. One advantage of the apoptotic induced drug delivery system (AIDD) is that the drug carrier cells may be genetically engineered to modify their properties.

*In vitro* studies have been performed using S49 mouse lymphoma cells in which apoptosis is produced by exposure to dexamethasone. The cytotoxicity of RG-2 cells caused by temazolamide-loaded-S49 apoptotic cells was from 4 to 7 times higher than that of control temazolamide-loaded S49 cells.[5]

## 7. Tumor Cells

A novel strategy for drug delivery based on the use of cell systems is the drug-loaded tumor cell system (DLTC), developed for drug delivery and targeting in

lung metastasis.[6,100] The tumor cells as drug carriers permit drug targeting to the blood-borne cancerous cells and the lungs as potential metastatic organs. In practice, there is affinity between the plasma membrane of malignant tumor cells and the metastatic addressins expressed by the endothelial cells of the targeted organ.[6,101]

*In vivo* studies have been carried out with DLTC based on Doxorubicin-loaded B16-F10 murine melanoma cells. Doxorubicin accumulation in the mouse lung was several times higher than that seen after administering free Doxorubicin.[6]

## 8. Dendritic Cells

Dendritic cells (DC) are antigen-presenting cells. They ingest antigen by phagocytosis, degrade it, and present fragments of the antigen at their surface. Dendritic cells have huge potential for immunization against a broad variety of diseases, because they travel throughout the body in search of pathogens indicative of infection or disease. They are very important for the induction of T cell responses, which result in cell-mediated immunity.

Selective targeting of drugs incorporated in dendritic cells to T cells allows the response of these cells to be manipulated *in vivo*. It has been shown that when incorporated into dendritic cells, the drug O-galactosylceramide improves their anti-tumor activity.[7]

## 9. Conclusions

This chapter has focused on the use of cells and cell ghosts as delivery systems of drugs, enzymes or therapeutic genes. The use of carrier cells such as bacterial ghosts, erythrocyte ghosts and engineered stem cells, for drug delivery and targeting are reviewed among others. Their high biocompatibility, together with their capacity for selective delivery and targeting in cells and specific tissues mean that these types of carrier are promising alternatives to the use of nano- and microparticle systems, with applications in the fields of interest such as cancer therapy, cardiovascular therapy, AIDS, gene therapy, etc. As an alternative to the use of cell carriers, modified viruses can also be used as drug delivery systems, especially in the field of gene therapy. Despite their potential interest, clinical studies with these types of carrier are still very limited, although in the near future, increase in the use and therapeutic applications of cell delivery systems is expected.

## Acknowledgments

This chapter was supported in part by a project of the National Research and Development Plan (Project: SAF 2001-0740).

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# 16

# Cochleates as Nanoparticular Drug Carriers

*Leila Zarif*

## 1. Introduction

In spite of the availability of many non-traditional novel dosage forms, oral route remains the most attractive way for administration of therapeutical materials. However, many therapeutic agents, especially the increasing number of biological molecules cannot be taken up by intestine due to their intrinsic impermeability to tissue membranes and the enzymatic degradation through the wall of the GI tract. Carrier systems that facilitate intestine uptake of these molecules are of major interests in the drug delivery arena. Moreover, drug delivery systems that provide a route of administration that does not involve injection can improve patient compliance and expand the market for existing, injectable, drugs. The factors which are important for the oral efficiency of a vehicle system have been repeatedly summarized in the literature.[1,2] Small particle size, appropriate surface properties, mucoadhesive and targeting moieties, stability, as well as dose are the major factors imparting the efficiency of oral uptake.

Producing formulations of poorly soluble drugs with high bioavailability is an even higher challenge. Known technologies are nanocrystals and nanoparticles which use the approach of enhancing the bioavailability by a decrease in particle size, resulting in an increase of surface area and subsequently a faster dissolution. Other technologies such as solid dispersions, polymeric micelles and self-emulsifying systems were developed to increase the drug solubility.

Many lipid-based systems were developed to enhance oral bioavailability.[3,4] Examples are lipid-based emulsions & microemulsions[5–7]; Solid lipid nanoparticles (SLN), a high melting point lipids enclosed in a surfactant layer[8,9] adequate to enhance the oral bioavailability of poorly absorbed drugs; Lipid nanocapsules (LNC) for oral, injectable use[10] and improved bioavailability[11]; Lipid nanospheres prepared from egg lecithin and soybean, described for their low toxicity[12] and higher efficacy, compared with other delivery systems when incorporating amphotericin B,[13] due to their smaller particle size and lower uptake by reticuloendothelial system.[14,15] Recently, solid lipid microparticles, prepared by the solvent-in-water-emulsion-diffusion technique, were described for the encapsulation and oral delivery of insulin.[16]

In particular, lipid-based cochleate delivery system appears to provide answers to oral delivery challenges by (1) formulating different kind of molecules, especially hydrophobic ones[17,18] and (2) protecting the sensitive and biologically active molecules from harsh environmental conditions.

In this review, we will focus on cochleates nanoparticular drug carrier and will present the main features and the state of the art of this delivery technology.

## 2. Cochleates Nanoparticles in Oral Delivery

### 2.1. *Cochleate structure*

Cochleates were first described by Dimitrious Papahadjopoulos and his co-workers in 1975 as precipitates formed by the interaction of negatively charged phosphatidylserine and calcium.[19–21] He named these cylindrical structures "cochleate", meaning shell in the Greek language because of their rolled-up form, and explained the mechanism of cochleates formation by the fusion of negatively charged vesicles induced by the calcium cation[22] (Fig. 1).

These cigar-like structures have gained interest as antigen delivery system for vaccine applications.[23] More recently cochleates were studied as tools to deliver small molecule drugs.[17,18,24] A cochleate lipid formulation of amphotericin B has been developed as an oral composition to treat systemic fungal infections.[24–26] Other medical and non-medical applications are also under investigation.[27]

### 2.2. *Cochleate preparation*

#### 2.2.1. *Which phospholipid and which cation to use?*

Cochleates are a phospholipid-ion precipitates. Does that mean that cochleate is a structure obtained from precipitation of any phospholipid with any ion as presented in some litterature?,[28] i.e. a complex of negatively charged phospholipid with any cation or a complex made from a positively charged lipid with any anion?

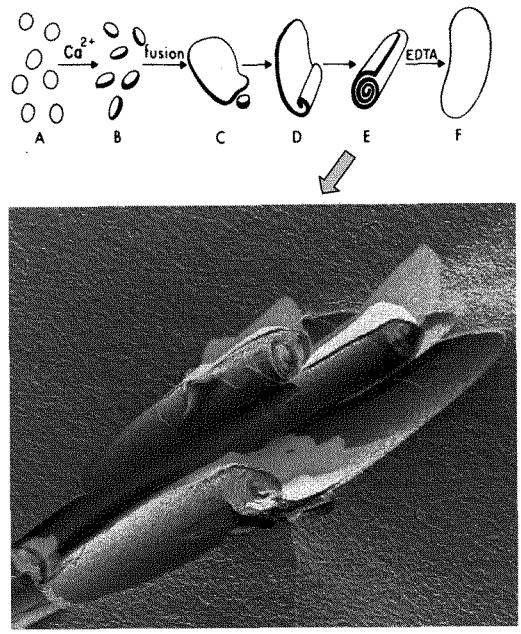


**Fig. 1.** Cochleate cylindrical structure and mechanism of formation (adapted from Refs. 19 and 69 with permission).

Papahadjopoulos has given in 1975 this appellation to a rolled phospholipid structure. So far, to our knowledge no physico-chemical evidence on the obtention of such cigar-like structure from positively charged phospholipid with an anion had been described ; on the contrary, extensive litterature is available on obtaining these cigar-like structure when negatively charged phospholipid such as phosphosphatidylserine (PS) had been precipitated with a cation such as calcium.[17,18,20–22,29,30] Other negatively charged phospholipids, such as phosphatidic acid (PA) or phosphatidyl glycerol derivatives, have been studied as well. Mixture of negatively charged phospholipids with other lipids can lead to cochleate formation. In this case, the cochleate formation depends on the negatively charged lipid/other lipid ratio and depends on the nature of the negatively charged lipid in the mixed lipid system. For example, PA derivatives form cochleate domains after the addition of calcium cation. However, when mixed with the corresponding diacylphosphatidylcholine (PC) and diacylphosphatidylethanolamine (PE), it was found that up to 20 mole% of PC or PE can be introduced into the cochleate phase of PA($Ca^{2+}$), above which a distinct PC rich or PE-rich phase appears.[31]

Other phospholipid derivatives such as galactosphingolipid hydroxy fatty acid cerebroside were reported to form cochleate cylinders by thermal mechanical treatment of glycol suspensions.[32] However, the addition of conjugated lipid, such as

poly(ethylene glycol)-lipid conjugates to PS vesicles, inhibited the calcium-induced fusion.[33]

In general, an additional desired feature of an oral drug delivery system is that the excipient permitting this transport to be classified is generally regarded as safe (GRAS). Soy phosphatidylserine fits this criteria. Furthermore, Soy PS has been used as a nutrient supplement since early 1980s. Clinical trials showed that PS may play a role in supporting mental functions in aging brains such as enhancing the memory, improving learning ability,[34–41] reducing the stress[42,43] and anxiety.[44]

Cochleates can be made from purified soy phosphatidylserine, which represents an affordable source of raw material.[45] A study comparing the purified soy phosphatidylserine (PSPS) to non-purified soy PS (NPSPS) has been disclosed in this patent, showing that PS should be present in an amount of at least 75% of the total lipid in order to allow the formation of cochleates. The other 25% phospholipids present can be selected either from the anionic group such as phosphatidic acid, phosphatidylglycerol, phosphatidyl inositol or phosphatidylcholine. PSPS cochleates can be loaded with different bioactive materials such as nutritional supplement, vitamins, antiviral, antifungal, small peptides. Proof of principle of the use of purified soy PS has been achieved using a polyene antifungal agent, amphotericin B. The preparation method for amphotericin B cochleates can be either via High pH-trapping or film method[18] or by hydrogel method;[29] the latter leading to nanocochleates formation.

The nature of the cation is an important factor in cochleate formation. In the precipitation process, divalent cations are preferred to monovalent cations. Monovalent cations such as $Na^+$ were described to prevent the cochleate formation.[46] Increases concentration of $Na^+$ ions was shown to interfere with the destabilization effect of $Ca^{2+}$. A critical Ca/PS ratio is necessary for the destabilization effect of divalent cations and the formation of cochleate phases.[46]

The formation of cochleate is easier from small unilamellar vesicles (SUV). However, multilamellar vesicles (MLV) can also lead to cochleate formation. In this case, the first mechanism is a destabilization of the outer bilayer of PS by $Ca^{2+}$ which causes its collapse, leading to a higher access of $Ca^{2+}$ to inner PS bilayers and so forth.

### 2.2.2. *Which molecules can be entrapped in cochleates nanoparticles*

Due to the intrinsic nature of the lipid-contained cochleates, these nanoparticles can encapsulate a variety of molecules of all shapes and sizes. Preference is given, however, to hydrophobic molecules, for which a need to enhance chemical stability or bioavailability is desired [Fig. 2(a)]. Amphiphatic molecules which can easily

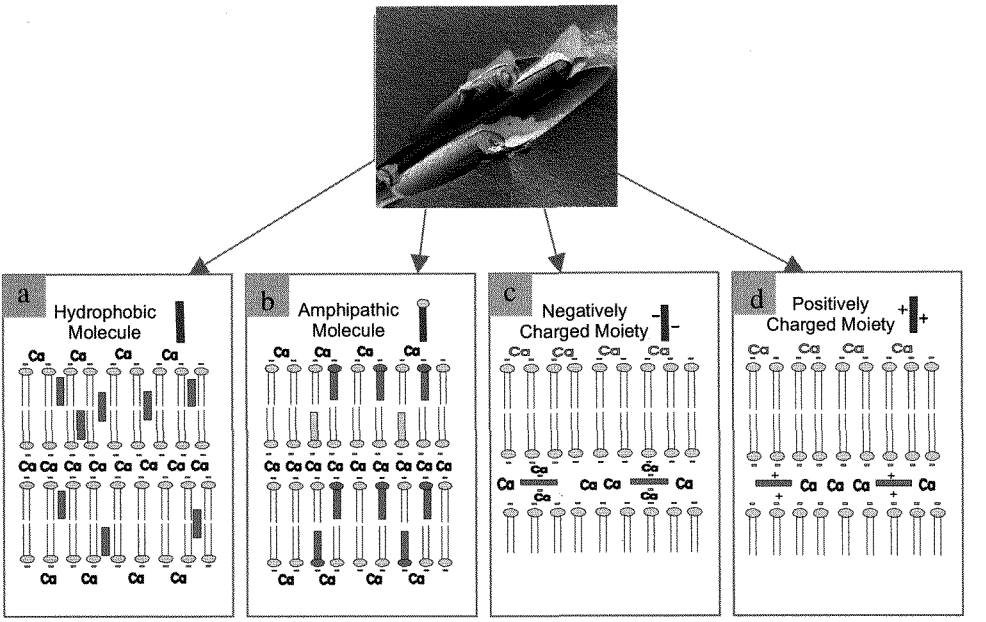


**Fig. 2.** Type of molecules which can be encapsulated into lipid based cochleate (adapted from Ref. 18 with permission).

insert in the membrane bilayers [Fig. 2(b)], negatively charged moiety [Fig. 2(c)] or positively charged moiety [Fig. 2(d)] could be encapsulated in the cochleate nanoparticle structure.

The nature of the drug influence the percentage of encapsulation. Hydrophobic drug shows a quantitative encapsulation, whereas less was seen for amphiphatic molecules. For instance, doxorubicin which presents hydrophobic regions is a water-soluble drug, has a partition between the bilayers and the external aqueous phase [Fig. 2(b)]. As calcium induces dehydration of the interbilayer domains, the amount of water in this region is low,[47] therefore, small hydrophilic molecules will not be suitable for cochleate system.

### 2.2.3. *Multiple ways of preparing cochleates*

Several processes were developed to obtain cochleates with a nanosize range, with the objective to allow oral delivery.[24,29,48–59] Particle size is process dependent. When a small nanosized particle is desired, the "hydrogel method" can be used, based on the use of an aqueous-aqueous emulsion system.[29] Briefly, this method consists of 2 steps: The preparation of small size liposomes either by high pH method[18,25] or by film method,[18] then the liposomes are mixed with a high viscosity polymer

such as dextran. The dextran/liposome phase is then injected into a second, non-miscible, polymer (i.e. PEG). The calcium was then added and diffused slowly from one phase to another, resulting in the formation of nanocochleates. The final step is the washing of the gel. These nanosized cochleates showed potential in the oral delivery of drugs.[18,29,48,59]

Electron microscopy and X-Ray crystallography of the nanoparticles show a unique multilayered structure consisting of continuous, solid lipid bilayer sheets, rolled up in a spiral with no internal aqueous space and the localization of AmB in the lipid bilayer.[25]

Other preparation techniques are known, e.g. the trapping method, useful for the encapsulation of hydrophilic and hydrophobic molecules,[17,18] which consist in the preparation of the liposomal suspension containing the drug either in the aqueous space of liposome (when hydrophilic) or intercalated in between the bilayers (when hydrophobic). A step of addition of calcium follows, and an aggregate of cochleates are formed. The cochleates made by the Trapping method present higher aggregation compared with other methods. This has been demonstrated using Electron microscopy after Freeze-fracture.[25]

Another method was developed for hydrophobic drugs,[61] known as "the solvent drip method" which consists of preparing a liposomal suspension separately based on soy PS and a hydrophobic or amphipathic cargo moiety solution. Solvent for hydrophobic drug can be selected from DMSO, DMF. The solution is then added to liposomal suspension. Since the solvent is miscible in water, a decrease of the solubility of the cargo moiety is observed, which associates at least in part with the lipid-hydrophobic liposomal bilayers. The cochleates are then obtained by addition of calcium and the excess solvent is being washed.

Usually, the cochleate formation can be characterized by optical microscopy when they are present in needle form in the micrometer size range. In this case, direct observation using a higher magnification can be used.[25] When nanocochleate are obtained, optical microscope can be used as an indirect method to assess the formation of cochleate, i.e. observation of the liposome formation after chelation of the calcium present, by addition of EDTA (ethylene diamine tetraacetate) to nanocochleate. A more sophisticated method is the electron microscopy after freeze-fracture[18,25] which allows the observation of the tighted packed bilayers. Recently, other methods were described using Laurdan (6-dodecanoyl-2-dimethylamino naphtalene) to monitor the cochleate phase formation.[62] In this case, the lipid vesicles are labeled with Laurdan and the addition of calcium to the laurdan labeled vesicles resulted in a shift in the emission peak maximum of Laurdan. Due to dipolar relaxation, excitation and emission, generalized polarization ($GP_{Ex}$ and $GP_{Em}$) indicates the transition from a LC to a rigid and dehydrated cochleate phase.

## 2.3. *Cochleates as oral delivery system for antifungal agent, amphotericin B*

Among the drug of choice using nanocochleate delivery system, amphotericin B (AmB) presented all aspects of a good candidate. Amphotericin B is a hydrophobic drug with poor oral bioavailability. This drug had been used for decades in injectable form to treat systemic fungal infections of candida, cryptococcus and aspergillosis species.[63–65]

Lipid formulations of Amphotericin B such as liposomes, lipid complexes, lipid emulsions and colloidal dispersions, were developed with the aim to achieve a higher therapeutic index.[26,66] These formulations indeed showed enhanced therapeutic index, even though none of these formulations showed ability to deliver AmB orally. Cocheate technology seems to offer the advantage over other delivery systems in providing the possibility for the oral delivery of AmB. Oral administration of amphotericin B cochleates (CAMB) to healthy mice achieved potentially therapeutic concentrations in key target tissues.[51]

Preclinical studies demonstrate a promising activity of CAMB in murine models of clinically relevant invasive fungal infections such as disseminated candidiasis,[25,48,67] disseminated aspergillosis[17,18,58,59] and central nervous system cryptococcosis.[68]

### 2.3.1. *In candidiasis animal model*

In *Candida albicans* infected murine animal model, AmB cochleates showed potential either after intraperitoneal (i.p.) or oral (p.o.) administration.[17,18,48,49,54,55,57,60,66–68]

After i.p. administration CAMB provided protection against *C. albicans* at doses as low as 0.1 mg/kg/day, kidney tissues burden showed that CAMB was more potent than Fungizone® at 1 mg/kg/day and was equivalent to AmBisome® at 10 mg/kg/day[18,25,60] (Fig. 3). CAMB was also effective after oral administration. Complete eradication of *C. albicans* from the lungs was noticed after p.o. administration at 2.5 mg/kg/day. These results were comparable to i.p. Fungizone® at 2.0 mg/kg/day.[48,54–56]

### 2.3.2. *In aspergillosis animal model*

Oral administration of CAMB was shown to be protective in a dose-dependent manner against systemic infection of *Aspergillus fumigatus* in animals immunosusppressed with cyclophosphamide.[58,59] In this mouse model, intragastric administration of CAMB at 40 mg/kg/day for 15 days resulted in 80% survival, while Fungizone at 4 mg/kg/day (i.p.) resulted in 20% survival; higher doses of Fungizone were lethal to animals.

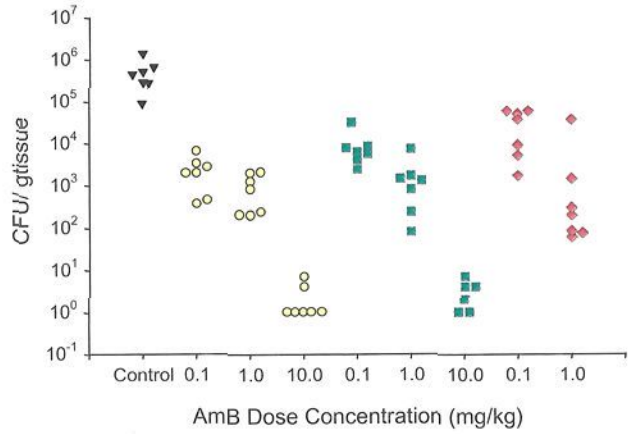


**Fig. 3.** Kidneys tissue burden of infected mice treated with either CAMB (•), Fungizone (♦) or AmBisome (■), compared with controls (▼) (from Ref. 18 with permission)

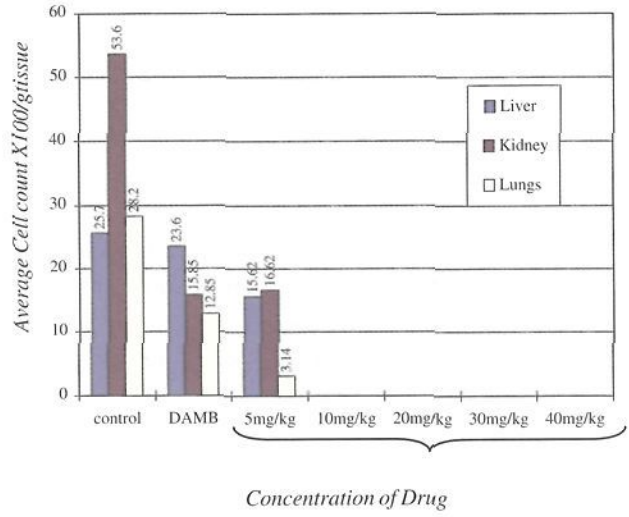


**Fig. 4.** Tissue burden for mice infected in a model of invasive *aspergillosis* after oral administration of CAMB (from Ref. 58 with permission).

The tissue fungal burden for target organs, kidneys, liver and lungs, demonstrated the benefic effect of CAMB (Fig. 4 ). CAMB showed a pronounced dose-dependent reduction in the fungal burden in all organs. The near eradication of *Aspergillus* was observed above a concentration of 20 mg/kg/day. CAMB at 30 mg/kg (PO) was as effective as CAMB at 20 mg/kg (PO) in reducing fungal tissue burden.[58]

### 2.3.3. *In cryptococcal meningitis animal model*

Oral amphotericin B cochleates were effective in a murine *cryptococcal meningitis* model with an 80% survival after 17 days, obtained after oral treatment with CAMB (10 mg/kg) to mice having intracerebral infection with *cryptococcus neoformans*.[68]

### 2.3.4. *Toxicity of amphotericin B cochleates*

*In vitro*, Amphotericin B cochleates (CAMB) showed a low toxicity on red blood cells when compared with Fungizone (DAMB). CAMB showed no hemoglobin release and therefore no hemolysis of red blood cells when incubated at 500 $\mu$g/ml. In contrast, DAMB was hemolytic at 10 $\mu$g/ml due to the presence of the detergent, sodium desoxycholate.[25]

*In vivo*, CAMB was non toxic to mice when administered orally at 50 mg/kg/day for 14 days. No nephrotoxicity was observed as demonstrated by the normal BUN level, and the histopathology of kidneys, lungs, liver, spleen and GI tract showed that animals dosed with CAMB were comparable to controls.[18]

### 2.3.5. *Pharmacokinetics of amphotericin B cochleates*

*Oral pharmacokinetics*3pt

Pharmacokinetic studies have shown that after oral administration of CAMB, AmB is distributed into the target tissues (e.g. brain, liver, lung, spleen and kidneys)[18,50,52] in healthy mice and AmB tissue level suggests a zero-order uptake process for all tissues.

When CAMB was administered po to C57BL/6 mice at 10 mg/kg ($n = 5$), and blood and tissues collected and AmB level measured by HPLC, blood shows a plateau-shaped profile with $T_{max}$ = 6 h and $C_{max}$ = 0.05 mg/ml. Non-compartmental (NCA) analysis showed blood $AUC_{0-\infty}$ = 1.20 $\mu$g*h/ml, $t_{1/2}$ = 12.8 h, $MRT_{0-\infty}$ = 21.1 h, Cl/F = 139.2 ml/min/kg, $V_z/F$ = 153.91 L/kg. AmB tissue exposure ($AUC_{0-\infty}$, $\mu$g*h/g) evaluated using NCA was greater for lungs (23.11), followed by liver (16.91), spleen (15.40) kidneys (14.97) and heart (3.34). Tissue elution $t_{1/2}$(h): kidneys 9.3, lungs 5.6, heart 5.3, liver 4.9 and spleen 4.3. For all tissues, $T_{max}$ = 12 h and $C_{max}$ ranged between 0.23 $\mu$g/ml for heart and 1.58 $\mu$g/ml for lungs.[52]

The delivery of AmB by cochleates after multiple oral doses (10) was assessed in the same mouse model and was compared with AmBisome. It was found that cochleate provides therapeutic levels in tissue and presents better delivery and transfer efficiency of AmB to the target tissue, as well as better tissue penetration.[53]

The ability of cochleate vehicles to deliver systemic AmB after single or multiple oral dosing suggest the potential of CAMB formulations to treat and prevent systemic fungal infections.

*Pharmacokinetics*

AmB given intraveneously (IV) to mice showed a two-phase pharmacokinetic profile.[69,70] Pharmacokinetic analysis in target tissues (liver, spleen, kidney and lungs) shows a multi-peak profile, large AUC and MRT.

After IV administration of 0.625 mg/kg, AMB presented a two-phase blood concentration time course [Fig. 5(A)]. This profile is characterized by a very fast distribution phase and an elimination phase with $t_{1/2} = 11.68$ hrs. The $AUC_{0-\infty}$ was 1.006 $\mu$g*h/ml, Cl = 10.36 ml/min/kg, $MRT_{0-\infty} = 15.41$ hrs and $V_{SS} = 9.587$ L/kg.

This pharmacokinetic profile indicates that CAMB is removed fast from blood. In addition, the large $V_{SS}$ also indicates a large distribution into the tissues. The results obtained in target tissues showed this extensive distribution and penetration [Fig. 5(B)].

Calculation of pharmacokinetic parameters showed that the main target tissues have a large AMB exposure reflected in the AUC and $C_{MAX}$ values (Table 1), as well as the tissue to blood AUC ratio.

The large AMB exposure in liver and spleen suggests involvement of the mononuclear phagocyte system (MPS) in the removal of CAMB. Cochleates are particulates that can be quickly cleared from the circulation by the macrophages of the reticular endothelial system (RES) related to the liver and the spleen. In addition, "physical retention" seems to play a role in the kinetic profile of the lungs due to its capillary nature.

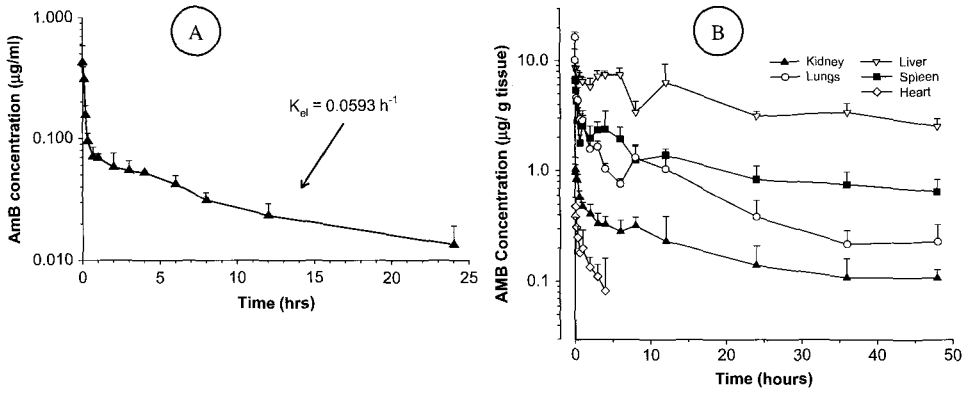


**Fig. 5.** (A) AMB profile in blood after a single dose (B) IV PK profile of AMB in target tissues, (from Ref. 69, with permission).

**Table 1** Pharmacokinetics parameters for CAMB in different target organs after IV administration to C57BL/6 mice ($n$ = 5 per time point) (From Ref. 69, with permission).

| Tissue[a] | $AUC_{0-\infty}$ ($\mu$g*h/g) | $T_{max}$ (min) | $C_{max}$ ($\mu$g/g) | $t_{1/2\lambda_z}$[b] (hrs) |
|---|---|---|---|---|
| Liver | 474.519 | 10 | 8.559 | 75.03 |
| Spleen | 116.388 | 2 | 6.633 | 66.71 |
| Lung | 39.707 | 2 | 16.408 | 22.34 |
| Kidney | 12.564 | 5 | 1.032 | 21.86 |
| Heart | 0.970 | 5 | 0.478 | 2.82 |
| Intestine | 9.173 | 20 | 0.609 | 13.88 |
| Stomach | 8.184 | 20 | 0.343 | 20.77 |

This phenomenon and the mobility of the macrophages seem to cause certain redistribution of cochleates that gives a multi-peak and plateau shape profiles in liver and spleen. Finally, AMB was also detected in bile and intestine contents, suggesting that bile excretion may be an additional elimination route.

## 2.4. *Other potential applications for cochleates*

### 2.4.1. *Cochleate for the delivery of antibiotics*

As cochleate has shown a high affinity to be engulfed by macrophages [Fig. 6(A)] probably due to a dual mechanism, the cochleate essential particulate feature[71] and possibly a PS receptor mediated internalization of the cochleate into macrophage.[72]

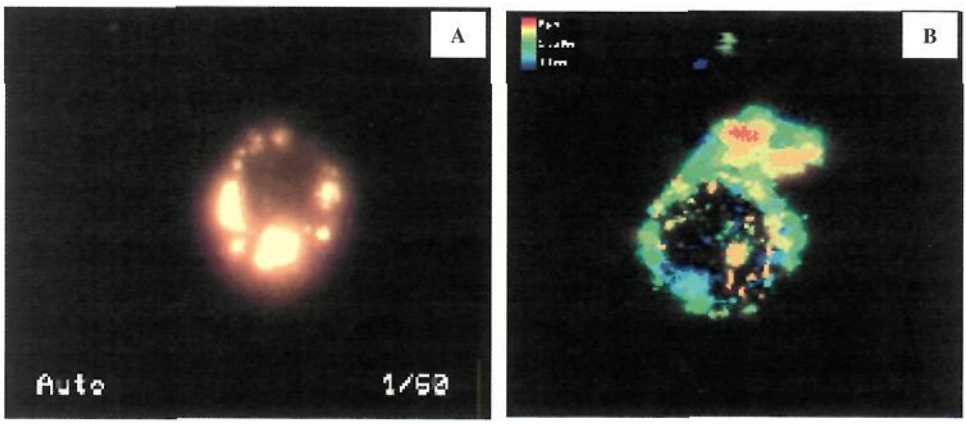


**Fig. 6.** Uptake of amphotericin B cochleates by J774 macrophages as seen by (A) fluorescence microscopy, (B) confocal microscopy (from Ref. 17, with permission).

This particulate system would have potential for the delivery of antibacterial agents such as aminoglycosides and vancomycin.[17] Illustration is given by the encapsulation of clofazimine, an anti-TB drug, and tobramycin, an aminoglycoside antibiotic used in treating bacterial infections, both given intraveneously thus far. The cochleate system may possibly offer a new oral way of delivery.

### 2.4.2. *Delivery of clofazimine*

Clofazimine cochleates were prepared by the Trapping method.[18] Clofazimine is a known hydrophobic anti-TB drug, the efficacy of Clofazimine cochleate was assessed by measuring the $IC_{50}$ in *Vero* Cells and in bone marrow derived macrophage (BM-M).[73] Clofazimine cochleates exhibit a greater decrease in toxicity *versus* free clofazimine and had a higher efficacy in killing intracellular *M. Tuberculosis* than free clofazimine:2 Log reduction (CE99) was achieved at 20.9 $\mu$g/ml for cochleates, while free clofazimine was toxic at this concentration. This shows that encapsulation of clofazimine in cochleates potentiates the antimicrobial efficacy of the drug, i.e. when higher concentration of drug can be used because of less toxicity, bactericidal levels of the drug could be attained.

### 2.4.3. *Delivery of tobramycin*

A recent research work has been published on the possible use of nanocochleates as an oral delivery system for Tobramycin.[74] Tobramycin is a well known aminoglycoside antibiotic used in treating bacterial infections, and is usually administered by intravenous (i.v.) infusion, intramuscular (i.m.) injection, or inhalation. This aminogycoside drug is known for its side effects such as mineral depletion (i.e. calcium, magnesium, potassium) after i.v. administration.[75,76]

In this work, the author described that tobramycin which is positively charged at low pH, will be encapsulated in the inter-bilayer space of cochleates. The fusion of unilamellar liposomes is no longer induced by a metal cation such as $Ca^{2+}$, but by the organic molecule to be encapsulated. The cochleate cylinders formation has been described by Papahadjoupolos as resulting partly from the intrinsic properties of the calcium cation. Indeed, phosphatidylserine shows considerable selectivity for calcium due to the propensity of calcium to lose part of its hydration shell, and to displace water upon complex formation.[19,77] In the cochleate solid crystalline structures formation, calcium plays a crucial role in bringing bilayers together closely through partial dehydration of the membrane surface and the cross-linking of opposing molecules of phosphatidylserine. In our opinion, in this recent work where formation of cochleate is claimed with no calcium present, additional

relevant physico-chemical evidence on cochleate formation and the localization of the drug in the interbilayer space will be needed.

### 2.4.4. *Cochleate for the delivery of anti-inflammatory drugs*

As a result of the deep embedding of the molecules in the cochleates structures, drug molecules are hidden from the outside environment. This should have two beneficial effects: one is to hide and protect the molecule from the degradation due to environment; the other is to protect, the environment when needed, from the active molecule when such molecule presents side effects.

This is the case of anti-inflammatory drugs, which associates cure to the disturbance of GI tract (stomach for instance). Cochleates were described to act beneficially in this area, reducing the stomach irritation when anti-inflammatory drugs such as aspirin is hidden in the cochleate structure, and administered to a carrageenan rat model for acute inflammation.[27,61]

## 2.5. *Other uses of cochleates*

Cochleates were also described as vehicles for nutrients[27] as an improved drug and contrast agent delivery system,[28] as well as intermediate in the preparation of special liposomes such as Large Unilamellar Vesicles (LUV) and proteoliposomes. In fact, the discovery of the cochleate structures was a result of the desire to prepare LUV by Pr papahadjoupoulos,[19,20] which were developed for the delivery of hydrophilic drugs. Proteoliposomes prepared from cochleates intermediates were described for vaccine applications in general,[78] and more recently, when containing lipopolysaccharide as a novel adjuvant.[79]

# 3. Conclusion

Cochleates lipid-based nanocarrier appears to have potential for the oral delivery of bioactive molecules. Future work should be directed towards more fundamental science, as many research aspects of the cochleate drug carrier system are still hardly known (e.g. localization of the drug in lipid bilayers, impact of multivalent cations on the cochleate formation, mechanism of action of cochleate after oral uptake). In addition, the development of friendly analytical assays to monitor the drug localization and loading percentage in cochleates will be desired. This nano drug carrier is currently under development by Biodelivery Sciences International.[27] Having the first drug-cochleate in the market place represents a big challenge. For instance, when oral amphotericin B cochleates are ultimately available for patients, thus will provide a new opening in the treatment of systemic fungal infections.

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# 17

# Aerosols as Drug Carriers

*N. Renée Labiris, Andrew P. Bosco and Myrna B. Dolovich*

## 1. Introduction

As the end organ for the treatment of local diseases or as the route of administration for systemic therapies, the lung is a very attractive target for drug delivery (Table 1). The lung provides direct access to the site of disease for the treatment of respiratory illness, without the inefficiencies and unwanted effects of systemic drug delivery. In addition, it provides an enormous surface area and a relatively low enzymatic environment for the absorption of drugs to treat systemic diseases (Table 1).

Inhaled medications have been available for many years for the treatment of lung diseases. Inhalational delivery has been widely accepted as being the optimal route of administration of first line therapy for asthmatic and chronic obstructive pulmonary diseases. Drug formulation plays an important role in producing an effective inhalable medication. In addition to being pharmacologically active, it is important that a drug be efficiently delivered into the lungs, to the appropriate site of action and remain in the lungs until the desired pharmacological effect occurs. A drug designed to treat a systemic disease, such as insulin for diabetes, must be deposited in the lung periphery to ensure maximum systemic bioavailability. For gene therapy, anti cancer or anti infective treatment, cellular uptake and prolonged residence in the lungs of the drug may be required to obtain the optimal therapeutic effect. Thus, a formulation that is retained in the lungs for the desired length of time and avoids the clearance mechanisms of the lung may be necessary.

The human lung contains airways and approximately 300 million alveoli with a surface area of 140 $m^2$, equivalent to that of a tennis court.[1] As a major port of

**Table 1** Advantages of pulmonary delivery of drugs to treat respiratory and systemic disease.

| Treatment of respiratory diseases | Treatment of systemic diseases |
|---|---|
| • Deliver high drug concentrations directly to the disease site<br>• Minimizes risk of systemic side effects<br>• Rapid clinical response<br>• Bypass the barriers to therapeutic efficacy, such as poor gastrointestinal absorption and first-pass metabolism in the liver<br>• Achieve a similar or superior therapeutic effect at a fraction of the systemic dose. For example, oral salbutamol 2–4 mg is therapeutically equivalent to 100–200 $\mu$g by MDI | • A non-invasive Needle-free delivery system.<br>• Suitable for a wide range of substances from small molecules to very large proteins<br>• Enormous absorptive surface area (140 $m^2$) and a highly permeable membrane (0.2 to 0.7 $\mu$m thickness) in the alveolar region.<br>• Large molecules with very low absorption rates can be absorbed in significant quantities; the slow mucociliary clearance in the lung periphery results in prolonged residency in the lung.<br>• A less harsh, low enzymatic environment<br>• Avoids first-pass metabolism.<br>• Reproducible absorption kinetics.<br>• Pulmonary delivery is independent of dietary complications, extracellular enzymes and inter-patient metabolic differences that affect gastrointestinal absorption. |

entry, the lung has evolved to prevent the invasion of unwanted airborne particles from entering into the body. Airway geometry, humidity, mucociliary clearance and alveolar macrophages play a vital role in maintaining the sterility of the lung, and consequently, they can be barriers to the therapeutic effectiveness of inhaled medications.

The size of the drug particle can play an important role in avoiding the physiological barriers of the lung and targeting to the appropriate lung region (Fig. 1). Nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm.[2] Studies have demonstrated that they are taken up by macrophages, cancer cells, and epithelial cells.[3–6] Their small size ensures the particles containing the active pharmacological ingredient will reach the alveolar regions. However, the use of an aerosol delivery system that generates nano-sized particles for inhalation, places these particles at risk of being exhaled, leaving very few drug particles to be deposited in the periphery of the lung. Residence time is not long enough for the particles to be deposited by sedimentation or diffusion.[7]

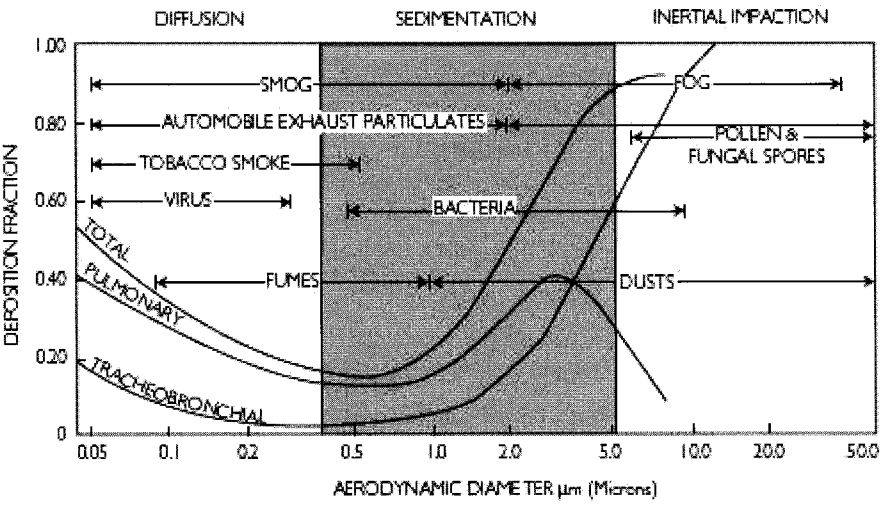


**Fig. 1.** Relationship between particle size and lung deposition.[105]

## 2. Pulmonary Drug Delivery Devices

The origin of inhaled therapies can be traced back 4000 years ago to India, where people smoked the leaves of the *Atropa belladonna* plant to suppress cough. In the 19th and early 20th centuries, asthmatics smoked asthma cigarettes that contained stramonium powder mixed with tobacco to treat the symptoms of their disease. Modern inhalation devices can be divided into three different categories (Fig. 2), the refinement categories (Fig. 2), the refinement of the nebulizer and the of compact portable devices, the pressurized metered dose inhaler (pMDI), and the dry powder inhaler (DPI). The advantages and disadvantages of each are summarized in Table 2.

### 2.1. *Nebulizers*

Nebulizers have been used for many years to treat asthma and other respiratory diseases. There are 2 basic types of nebulizers, jet and ultrasonic nebulizers. The jet nebulizer functions by the Bernoulli principle by which compressed gas (air or oxygen) passes through a narrow orifice, creating an area of low pressure at the outlet of the adjacent liquid feed tube. This results in the drug solution being drawn up from the fluid reservoir and shatter into droplets in the gas stream. The ultrasonic nebulizer uses a piezoelectric crystal, vibrating at a high frequency (usually 1 to 3 MHz), to generate a fountain of liquid in the nebulizer chamber; the higher the frequency, the smaller the droplets produced. Nebulizers can aerosolize

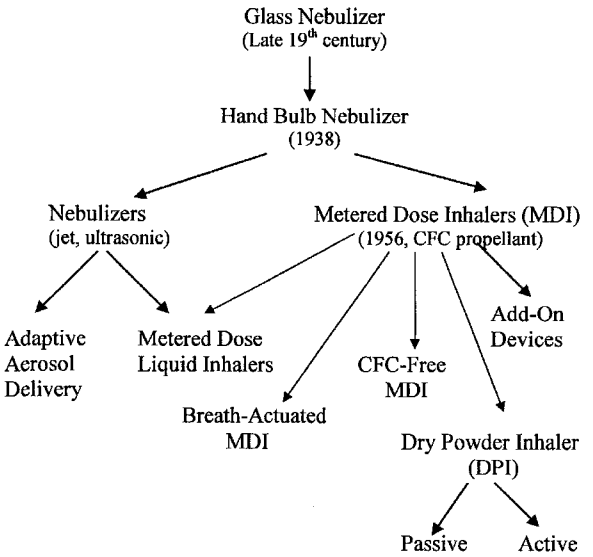


**Fig. 2.** Evolution of pulmonary delivery devices.

most drug solutions and provide large doses with very little patient coordination or skill. However, treatments using these nebulizers can be time consuming and inefficient, with large amounts of drug wastage e.g. 50% loss with continuously operated nebulizers.[8] Most of the prescribed drug never reaches the lung with nebulization. The majority of the drug is either retained within the nebulizer (referred to as residual or dead volume) or released into the environment during expiration. On average, only 10% of the dose placed in a continuous output jet nebulizer is actually deposited in the lungs.[8] Advances in technology have led to the development of novel nebulizers that reduce drug wastage and improve delivery efficiency. Breath-enhanced jet nebulizers such as the Pari LC Star, (PARI, Germany) increase aerosol output by directing auxiliary air, entrained during inspiration, through the nebulizer, causing more of the generated aerosol to be swept out of the nebulizer and available for inhalation. Drug wastage during exhalation is reduced to the amount of aerosol produced by the jet airflow rate that exceeds the storage volume of the nebulizer. Adaptive aerosol delivery (Halolite, Medic-Aid, Bognor Regis, UK) monitors a patient's breathing pattern in the first 3 breaths and then targets the aerosol delivery into the first 50% of each inhalation. This ensures that the aerosol is delivered to the patient during inspiration only, thereby eliminating drug loss during expiration that occurs with continuous output nebulizers.[9] A number of metered dose liquid inhalers, including AERx (Aradigm, Hayward, CA), AeroDose (AeroGen, Sunnyvale, CA) and Respimat (Boehringer Ingelheim, Ingelheim Rhein, Germany), have been developed to produce a fine aerosol in the respirable

**Table 2** Advantages and disadvantages of inhalation devices.

| Inhalation device | Advantages | Disadvantages |
|---|---|---|
| Nebulizers (jet, ultrasonic) | • no specific inhalation technique or coordination required<br>• aerosolizes most drug solutions<br>• delivers large doses<br>• suitable for infants and people too sick or physically unable to use other devices | • time consuming<br>• bulky<br>• non-portable<br>• contents easily contaminated<br>• relatively expensive<br>• poor delivery efficiency<br>• drug wastage<br>• wide performance variation between models and operating conditions |
| pressurized Metered Dose Inhalers (pMDI) | • compact<br>• portable<br>• multi-dose (~200 doses)<br>• inexpensive<br>• sealed environment (no degradation of drug)<br>• reproducible dosing | • inhalation technique and patient coordination required<br>• high oral deposition<br>• maximum dose of 5 mg<br>• limited range of drugs available |
| Dry Powder Inhalers (DPI) | • compact<br>• portable<br>• breath actuated<br>• easy to use<br>• no hand-mouth coordination required | • respirable dose dependent on IFR*<br>• humidity may cause powders to aggregate and capsules to soften<br>• dose lost if patient inadvertently exhales into the DPI<br>• most DPIs contain lactose |

*IFR = Inspiratory Flow Rate

range by forcing the drug solution through an array of nozzles, using vibrating mesh or electronic micropump platforms with 30 to 75% of the emitted dose being deposited in the lungs.[10,11]

## 2.2. *Metered-dose inhalers*

The pressurized metered-dose inhaler (pMDI) was a revolutionary invention that overcame the problems of the hand-bulb nebulizer, and it is the most widely used aerosol delivery device today. The pMDI emits a drug aerosol driven by

propellants, such as chlorofluorocarbons (CFC) and more recently, hydrofluoroalkanes (HFAs) through a nozzle at high velocity (>30 m/sec). pMDIs deliver only a small fraction of the drug dose to the lung. Typically, only 10 to 20% of the emitted dose is deposited in the lung.[12] The high velocity and large particle size of the spray causes approximately 50% to 80% of the drug aerosol to impact in the oropharygeal region.[13] Hand-mouth discoordination is another obstacle in the optimal use of the pMDI. Crompton and colleagues[14] found 51% of patients experienced problems coordinating the actuation of the device with inhalation, 24% of patients halted inspiration upon firing the aerosol into the mouth, and 12% inspired through the nose instead of the mouth when the aerosol was actuated into the mouth.

The delivery efficiency of a pMDI depends on a patient's breathing pattern, inspiratory flow rate and hand-mouth coordination. The studies by Bennett[15] and Dolovich[16] demonstrated that for any particle size between 1 to 5 $\mu$m mass median aerodynamic diameter (MMAD), deposition was more dependent on inspiratory flow rate than any other variable. Fast inhalations (>60 L/min) result in a reduced peripheral deposition because the aerosol is more readily deposited by inertial impaction in the conducting airway and oropharyngeal regions. When aerosols are inhaled slowly, deposition by gravitational sedimentation in peripheral lung regions are enhanced.[17] Peripheral deposition has also been shown to increase with an increase in tidal volume and a decrease in respiratory frequency. As the inhaled volume is increased, aerosols are able to penetrate more distally into the lungs.[18] A period of breath holding on completion of inhalation enhances deposition of particles in the periphery, thus preventing the particles from being exhaled during the expiratory phase. Thus, the optimal conditions for inhaling pMDI aerosols are from a starting volume equivalent to the functional residual capacity, the actuation of the device at the start of inhalation, inspiratory flow rate of <60 L/min, followed by a 10 second breath-hold at the end of inspiration.[17,19]

Spacer tubes, valved holding chambers and mouthpiece extensions have been developed to eliminate coordination requirements and reduce the amount of drug deposited in the oropharynx, by decreasing the particle size distribution and slowing the aerosol's velocity. Spacer geometry and materials of manufacture influence the quality and quantity of aerosol available. The aerosols from a pMDI and the holding chamber are finer than that with the pMDI alone, with an approximate 25% decrease in the mass median aerodynamic diameter (MMAD), compared with the original aerosol.[20,21] This finer aerosol is more uniformly distributed in the normal lung, with increased delivery to the peripheral airway. However, in patients with airway obstructions, the addition of a holding chamber to the pMDI may not change the distribution of the aerosol.[22]

### 2.3. *Dry powder inhalers*

Dry powder inhalers (DPIs) were designed to eliminate the coordination difficulties associated with the pMDI. There are a wide range of DPI devices on the market from single-dose devices loaded by the patient (e.g. Aerolizer from Novartis, Rotahaler from GSK, Ware UK) to multi unit dose devices provided in a blister pack (e.g. Diskhaler, GSK, Ware UK), multiple unit doses sealed in blisters on a strip that moves through the inhaler (e.g. Diskus, GSK, Ware UK) or reservoir-type (bulk powder) systems (e.g. Turbuhaler, AstraZeneca, Lund Sweden).

Lung deposition varies among the different DPIs. Approximately 12% to 40% of the emitted dose is delivered to the lungs with 20 to 25% of the drug being retained within the device.[10,23,24] Poor drug deposition with DPIs can be attributed to inefficient deaggregation of the fine drug particles from coarser carrier lactose particles or drug pellets. Slow inspiratory flow rate, high humidity and rapid, large changes in temperature are known to affect drug deaggregation and hence the efficiency of pulmonary drug delivery with DPIs.[25,26] With most DPIs, drug delivery to the lungs is augmented by fast inhalation. Borgstrom and colleagues[27] demonstrated that increasing inspiratory flow from 35 L/min to 60 L/min through the Turbuhaler[7], increased the total lung dose of terbutaline from 14.8% of nominal dose to 27.7%. This is in contrast to the MDI which requires slow inhalation and breath holding to enhance lung deposition of the drug. Each DPI has a different air flow resistance that governs the required inspiratory effort.[28,29] The higher the resistance of the device, the more difficult it is to generate an inspiratory flow great enough to achieve the maximum dose from the inhaler.[30–32] However, deposition in the lung tends to increase when using high resistance inhalers.[32–36]

Active DPIs are being investigated to reduce the importance of a patient's inspiratory effort. By adding either a battery driven propeller that aids in the dispersion of the powder (Spiros, Elan Pharmaceuticals, San Diego, CA), or using compressed air to aerosolize the powder and converting it into a standing cloud in a holding chamber, the generation of a respirable aerosol becomes independent of a patient's inspiratory effort (Inhance Pulmonary Delivery System, Nektar Therapeutic, San Carlos, CA).

## 3. Aerosol Particle Size

Aerosol particle size is one of the most important variables in defining the dose deposited and the distribution of drug aerosol in the lung (Fig. 3). Fine aerosols are distributed on peripheral airways, but deposit less drug per unit surface area than larger particle aerosols which deposit more drug per unit surface area, but on

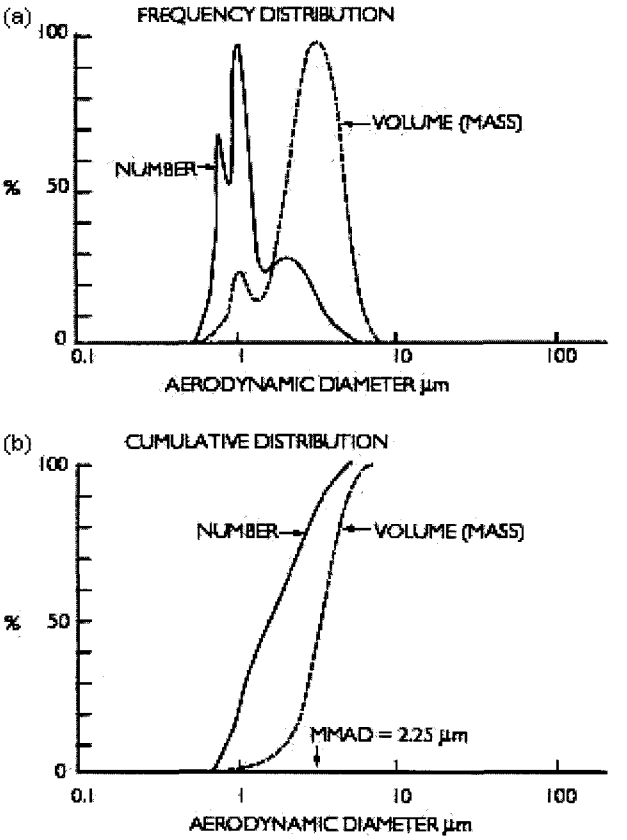


**Fig. 3.** Frequency (a) and cumulative (b) distribution curves for Beclovent MDI used with an Aerochamber, in terms of number of particles and volume (mass) of particles vs. particle aerodynamic diameter. The volume distribution curves are displaced to the right of the number distribution curves. The smaller number of large particles within the aerosol carry the greater mass of the drug; this is reflected in the larger, second peak of the volume distribution curve, which corresponds to the smaller second peak of the number distribution curve. MMAD is read from the cumulative distribution curve at the 50% point and if the distribution is log-normal, the GSD can be calculated as the ration of the diameter at the 84.1% point to the MMAD. Particle distribution was measured using the Anderson Cascade Impactor.[105]

the larger, more central airways.[37] Most therapeutic aerosols are nearly always heterodisperse, consisting of a wide range of particle sizes. These aerosols are described by the log-normal distribution, with the log of the particle diameters plotted against particle number, surface area or volume (mass) on a linear or probability scale and expressed as absolute values or cumulative %. Since delivered dose is very important when studying medical aerosols, particle number may be misleading as smaller particles contain less drug than larger ones. Particle size is defined from this distribution by several parameters. Mass median diameter of an aerosol refers to the

particle diameter that has 50% of the aerosol mass residing above and 50% of its mass below it. The aerodynamic diameter relates the particle to the diameter of a sphere of unit density that has the same settling velocity as the particle of interest, regardless of its shape or density. MMAD is read from the cumulative distribution curve at the 50% point (Fig. 3). Geometric standard deviation (GSD) is a measure of the variability of the particle diameters within the aerosol, and is calculated from the ratio of the particle diameter at the 84.1% point on the cumulative distribution curve to the MMAD. For a log-normal distribution, the GSD is the same for the number, surface area or mass distributions. A GSD of 1 indicates a monodispersed aerosol, while a GSD of >1.2 indicates a heterodispersed aerosol.

Particles can be deposited by inertial impaction, gravitational sedimentation or diffusion (Brownian motion), depending on their size. While deposition occurs throughout the airways, inertial impaction usually occurs in the first 10 generations of the lung, where air velocity is high and airflow is turbulent.[38] Most particles above 10 $\mu$m are deposited in the oropharyngeal region with a large amount impacting on the larynx, particularly when the drug is inhaled from devices requiring a high inspiratory flow rate (DPIs) or when the drug is dispensed from a device at a high forward velocity (MDIs).[39,40] The large particles are subsequently swallowed and contributed minimally, if at all, to the therapeutic response. In the tracheobronchial region, inertial impaction also plays a significant role in the deposition of particles, particularly at bends and airway bifurcations. Deposition by gravitational sedimentation predominates in the last 5 to 6 generation of airways (smaller bronchi and bronchioles), where air velocity is low.[38] In the alveolar region, air velocity is negligible and thus the contribution to deposition by inertial impaction is also negligible. Particles in this region have a longer residence time and are deposited by both sedimentation and diffusion. Particles not deposited during inhalation are exhaled. Deposition due to sedimentation affects particles down to 0.5 $\mu$m in diameter, whereas below 0.5 $\mu$m, the main mechanism for deposition is by diffusion.

Targeting the aerosol to conducting or peripheral airways can be accomplished by altering the particle size of the aerosol. It is difficult to predict the actual site of deposition, since airway calibre and anatomy differ among people. However, in general, aerosols with a MMAD of 5 to 10 $\mu$m are mainly deposited in the large conducting airways and the oropharyngeal region.[41] Particles 1 to 5 $\mu$m in diameter are deposited in the small airways and alveoli with greater than 50% of the 3 $\mu$m diameter particles being deposited in the alveolar region. In the case of pulmonary drug delivery for systemic absorption, aerosols with a small particle size would be required to ensure peripheral penetration of the drug.[42] Particles <3 $\mu$m have approximately 80% chance of reaching the lower airways, with 50 to 60% being deposited in the alveoli.[43,44] Nanoparticles <100 nm are deposited mainly in the alveolar region.

## 4. Targeting Drug Delivery in the Lung

The therapeutic effect of aerosolized therapies is dependent on the dose deposited and its distribution within the lung. If a drug aerosol is delivered at a suboptimal dose or to a part of the lung, devoid of the targeted disease or receptors, the effectiveness of therapy may be compromised. For example, the receptors for the $\beta_2$ agonist, salbutamol and the muscarinic$_3$ ($M_3$) agonist, ipratropium bromide, are not uniformly distributed throughout the lung. Autoradiographic studies have shown $\beta_2$ adrenergic receptors are present in high density in the airway epithelium from the large bronchi to the terminal bronchioles. Airway smooth muscle has a lower $\beta$-receptor density, greater in the bronchioles than bronchi.[45] However, greater than 90% of all $\beta$ receptors are located in the alveolar wall, a region where no smooth muscle exists and whose functional significance is unknown. Another autoradiographic study has shown a high density of $M_3$ receptors in submucosal glands and airway ganglia, and a moderate density in smooth muscles throughout the airways, nerves in intrapulmonary bronchi and in alveolar walls.[46] The location of these receptors in the lung suggests that ipratropium bromide needs to be delivered to the conducting airways, while salbutamol requires a more peripheral delivery to the medium and small airways to produce a therapeutic effect.

Since particle size affects the lung deposition of an aerosol, it can also influence the clinical effectiveness of a drug. Rees *et al.* reported the varying clinical effect of 250 $\mu$g of aerosolized terbutaline from a pMDI, given in three different particle sizes of <5 $\mu$m, 5 to 10 $\mu$m, and 10 to 15 $\mu$m.[47] In asthmatics, the greatest increase in forced expiratory volume in one second ($FEV_1$) was found with the smallest particle size (<5 $\mu$m), suggesting that the smaller particle aerosol was considerably more effective than larger particle size aerosols in producing bronchodilation, since it has the best penetration and retention in the lungs in the presence of airway narrowing. Using three monodisperse salbutamol aerosols (MMAD of 1.5 $\mu$m, 2.8 $\mu$m, 5 $\mu$m), Zanen and colleagues demonstrated in patients with mild to moderate asthma that the 2.8 $\mu$m particle size aerosol produced a superior bronchodilation, compared with the other two aerosols.[48] In patients with severe airflow obstruction ($FEV_1$ < 40%), Zanen *et al.* demonstrated that the optimal particle size for $\beta_2$ agonist or anticholinergic aerosols is approximately 3 $\mu$m.[49] They examined the effect on lung function of equal doses of three different sizes of monodisperse aerosols, 1.5 $\mu$m, 2.8 $\mu$m and 5 $\mu$m, of salbutamol and ipratropium bromide. Their findings suggest that small particles penetrate more deeply into the lung and more effectively dilate the small airways than larger particles, which are filtered out in the upper airways. The 1.5 $\mu$m aerosol induced significantly less bronchodilation than the 2.8 $\mu$m aerosol, suggesting that this fine aerosol may be deposited too peripherally to be effective, since smooth muscle is not present in the alveolar region.

The optimal site of deposition in the respiratory tract for aerosolized antibiotics depends on the infection being treated. Pneumonias represent a mixture of purulent tracheobronchitis and alveolar infection. Successful therapy would theoretically require the antibiotic to be evenly distributed throughout the lungs. However, those confined to the alveolar region would most likely benefit from a greater peripheral deposition. *Pneumocystis carinii* pneumonia, the most common life-threatening infection among patients infected with HIV, is found predominately within the alveolar spaces, with relapses occurring in the apical region of the lung after treatment with inhaled pentamidine given as a 1 $\mu$m MMAD aerosol.[50] The mechanism suggested for this atypical relapse is the poorer apical deposition of the aerosol. Regional changes in intrapleural pressure result in the lower lung regions receiving relatively more of the inspired volume than the upper lung, when sitting in an upright position or standing. This influence on deposition has been shown to occur in an experimental lung model, analyzing sites of aerosol deposition in a normal lung. The experiment showed a 2:1 ratio in the overall deposition for a 4 $\mu$m aerodynamic diameter aerosol between the lower and upper lobes when in the upright position.[51]

Chronic lung infection with *Pseudomonas aeruginosa,* in patients with cystic fibrosis or non-CF bronchiectasis, resides in the airway lumen with limited invasion of the lung parenchyma.[52,53] Infection starts in the smaller airways, the bronchioles, and moves into the larger airways. The optimal site of deposition for inhaled antimicrobial therapy would, therefore, be a uniform distribution on the conducting airways. Mucus plugs in the bronchi and bronchioles may prevent deposition of even small particle aerosols in regions distal to the airway obstruction, possibly the regions of highest infection, and thereby limiting the therapeutic effectiveness of the aerosolized antibiotic.[54–56]

Until recently, aerosol drug delivery has been limited to topical therapy for the lung and nose. The major contributing factor to this restriction was the inefficiencies of available inhalation devices that deposit only 10% to 15% of the emitted dose in the lungs. While appropriate lung doses of steroids and bronchodilators can be achieved with these devices, for systemic therapies, large amounts of the drug are necessary to achieve therapeutic drug levels systemically. Recent advances in aerosol and formulation technologies have led to the development of delivery systems that are more efficient and that which produce small particle aerosols, allowing higher drug doses to be deposited in the alveolar region of the lungs, where they are available for systemic absorption.

Most macromolecules cannot be administered orally because proteins are digested before they are absorbed into the bloodstream. In addition, their large size prevents them from naturally passing through the skin or nasal membrane; therefore, they cannot be administered intranasally or transdermally without the

use of penetration enhancers. Thus, the easiest route of administration for proteins has been through intravenous or intramuscular/subcutaneous injection. It has been known for many years that proteins can be absorbed from the lung as demonstrated with insulin in 1925.[57] Macromolecules < 40 kiloDaltons (kDa) (<5–6 nm in diameter) appear rapidly in the blood following inhalation into the airways. Insulin which has a molecular weight (mw) of 5.7 kDa and a diameter of 2.2 nm peaks in the blood 15 to 60 min after inhalation.[58–62] Macromolecules >40 kDa (>5–6 nm in diameter) are slowly absorbed over many hours; inhaled albumin (68 kDa) and alpha$_1$-antitrypsin (45–51 kDa) have a $T_{max}$ of 20 hrs and between 12 to 48 hrs respectively.[63]

The lung is the only organ through which the entire cardiac output passes. Before the inhaled drug can be absorbed into the blood from the lung periphery, it has several barriers to overcome such as lung surfactant, surface lining fluid, epithelium, interstitium and basement membrane, and the endothelium. Drug absorption in the lung periphery is regulated by a thin alveolar-vascular permeable barrier. An enormous alveolar surface area with epithelium, consisting of a thin single cellular layer (0.2 to 0.7 $\mu$m thickness), promotes efficient gas exchange through passive transport, but also provides a mechanism for efficient drug delivery into the bloodstream.[64] Although the mechanism of absorption is unknown, it has been hypothesized that macromolecules either pass through the cells via absorptive transcytosis (adsorptive or receptor mediated), paracellular transport between bijunctions or trijunctions or through large transitory pores in the epithelium caused by cell injury or apoptosis.[65] Thus, the high bioavailability of macromolecules deposited in the lung (10 to 200 times greater than nasal and gastrointestinal values) may be due to its enormous surface area, very thin diffusion layer, slow surface clearance and anti-protease defense system.

## 5. Clearance of Particles from the Lung

Like all major points of contact with the external environment, the lung has evolved to prevent the invasion of unwanted airborne particles from entering into the body. Airway geometry, humidity and clearance mechanisms contribute to this filtration process. The challenge in developing therapeutic aerosols is to produce an aerosol that eludes the lung's various lines of defense.

### 5.1. *Airway geometry and humidity*

Progressive branching and narrowing of the airways encourages impaction of particles. The larger the particle size, the greater the velocity of incoming air, while the greater the bend angle of bifurcations and the smaller the airway radius, the

greater the probability of deposition by impaction.[66] Drug particles are known to be hygroscopic and grow in size in high humidity environments, such as the lung which has a relative humidity of approximately 99.5%. The addition and removal of water can significantly affect the particle size and thus deposition of a hygroscopic aerosol.[67] A hygroscopic aerosol that is delivered at relatively low temperature and humidity into one of high humidity and temperature would be expected to increase in size when inhaled into the lung. The rate of growth is a function of the initial diameter of the particle, with the potential for the diameter of fine particles less than 1 $\mu$m to increase 5-fold, compared with 2 to 3-fold for particles greater than 2 $\mu$m.[68] The increase in particle size above the initial size should affect the amount of drug deposited, and particularly, the distribution of the aerosolized drug within the lung. Ferron and colleagues have predicted that for initial sizes between 0.7 $\mu$m and 10 $\mu$m, total deposition of hygroscopic aerosols increases by a factor of 2.[69] For particles with an initial size of 1 $\mu$m, Xu and Yu were able to predict changes in the distribution pattern due to particle growth.[70] The calculations showed a shift from deposition due to sedimentation to primarily impaction on more central airways.[69]

## 5.2. *Lung clearance mechanisms*

Once deposited in the lungs, inhaled drugs are either cleared from the lungs, absorbed into the circulatory or lymphatic systems, or metabolized. Drug particles deposited in the conducting airways are primarily removed through mucociliary clearance, and to a lesser extent, are absorbed through the airway epithelium into the blood or lymphatic system. Ciliated epithelium extends from the trachea to the terminal bronchioles. The airway epithelial goblet cells and submucosal glands secrete mucus forming a two-layer mucus blanket over the ciliated epithelium: a low-viscosity periciliary or sol layer covered by a high-viscosity gel layer. Insoluble particles are trapped in the gel layer and moved towards the pharynx (and ultimately to the gastrointestinal tract) by the upward movement of mucus generated by the metachronous beating of cilia. In the normal lung, the rate of mucus movement varies with the airway region and is determined by the number of ciliated cells and their beat frequency. Movement is faster in the trachea than in the small airways, and is affected by factors influencing ciliary functioning and the quantity and quality of the mucus.[40,71] For normal mucociliary clearance to occur, airway epithelial cells must be intact, ciliary structure and activity normal, the depth and chemical composition of the sol layer optimal, and the rheology of the mucus within the physiological range. Mucociliary clearance is impaired in lung diseases such as immotile cilia syndrome, bronchiectasis, cystic fibrosis and asthma.[72] In immotile cilia syndrome and bronchiectasis, the ciliary function can be

either impaired or nonexistent. In cystic fibrosis, the ciliary structure and function are normal, however, the copious amounts of thick, tenacious mucus present in the airways impairs their ability to clear the mucus effectively.[73] In these diseases, clearance of aerosolized drugs deposited in the conducting airways is generally decreased and secretions are cleared from the lung by cough.[74–76]

In addition to mucociliary clearance, soluble particles can also be removed by absorptive mechanisms in the conducting airways.[77] Lipophilic molecules pass easily through the airway epithelium via passive transport. Hydrophilic molecules cross via extracellular pathways such as tight junctions or by active transport via endocytosis and exocytosis.[78] From the submucosal region, particles are absorbed either into systemic circulation, bronchial circulation or lymphatic systems.

Drugs deposited in the alveolar region may be phagocytosed and cleared by alveolar macrophages or absorbed into the pulmonary circulation. Alveolar macrophages are the predominant phagocytic cell for the lung defense against inhaled microorganisms, particles and other toxic agents. There are approximately 5 to 7 alveolar macrophages per alveolus in the lungs of healthy, non-smokers.[79] Macrophages phagocytose insoluble particles deposited in the alveolar region are either cleared by the lymphatic system or moved into the ciliated airways along currents in alveolar fluid and then cleared via the mucociliary escalator.[65] This process can take weeks or months to complete.[7] As discussed above, soluble drug particles deposited in the alveolar region can be absorbed into the systemic circulation. The pulmonary epithelium appears to be more resistant to soluble particle transport than to the endothelium or the interstitium.[42]

The lung-blood barrier may behave as a molecular sieve, allowing the passage of small solutes but restricting the passage of macromolecules. Conhaim and colleagues proposed that the lung barrier was best fitted to a three pore size model, including a small number (2%) of large-sized pores (400 nm pore radius), 30% of medium-sized pores (40 nm radius) and 68% of small-sized pores (1.3 nm).[80]

The rate of protein absorption from the alveoli is size dependent. Effros and Mason demonstrated an inverse relationship between alveolar permeability and molecular weight.[42] In rats, after intratracheal instillation of DDAVP (1-desamino-8-D-arginine vasopressin) (mw = 1.1 kDa), peak serum DDAVP levels occurred at 1 hr compared with 16 to 24 hrs after the intratracheal instillation of albumin (mw = 67 kDa).[43] However, some proteins are cleared from the lung more rapidly than expected for their size. After intratracheal instillation or aerosolization of human growth hormone (mw = 22 kDa), peak serum levels were observed between 0.5 to 4 hrs, indicating a rapid, saturable clearance from the lung that is suggestive of receptor-mediated endocytosis.[65] Vasoactive intestinal polypeptide (VIP) is believed to be completely degraded during the passage across the pulmonary epithelium and into the bloodstream.[81]

Nanoparticles can pass rapidly into the systemic circulation. The distribution of radioactivity, after the inhalation of a $^{99m}$Technetium (Tc)-labeled ultrafine carbon particles (5 to 10 nm), was detected in the blood one min post-inhalation and peaked between 10 and 20 min. This blood radioactivity level was sustained up to 60 min. 8% of the initial lung radioactivity was measured in the liver 5 min post-administration and remained stable over time. The rapidity of the appearance of radioactivity systemically makes the translocation from the lung unlikely due to phagocytosis, by macrophages or endocytosis by epithelial and endothelial cells, but by passive diffusion.[82]

## 6. Nanoparticle Formulations for Inhalation

Delivery of nano-sized aerosols to the lung may result in very little drug being deposited in the lung. The majority of particles <500 nm inhaled will not have enough residence time in the lung to deposit, and therefore will be exhaled (Fig. 1). However, if the nanoparticles were delivered in larger carrier particles, they could be sufficiently deposited in the lung. The carrier particle would dissolve after contact with the lung surface fluid, releasing the nanoparticle at the target tissue or cells.

Sham and colleagues demonstrated that nanoparticles (173 to 242 nm) could be delivered into the lung in larger respirable lactose carrier particles produced by spray-drying.[83] The dry powder containing the nanoparticles had a MMAD of 3.0 $\mu$m. pMDI formulations are typically micronized drugs in the 2 to 3 $\mu$m range suspended in a hydrofluoroalkane (HFA) propellant. Solution pMDI such as QVAR produce smaller drug particles on propellant evaporation, resulting in better deposition and distribution than a micronized formulation.[84] However, for insoluble drug particles in the propellant, the efficiency of pMDI is limited. A study by Dickinson *et al.* proposed the use of nanoparticles suspended in propellant as a method of increasing the delivery efficiency of insoluble drugs in pMDIs.[85] They produced hydrophilic nanoparticles using a reverse phase microemulsion technique that captures nanoparticles by snap freezing, followed by freeze-drying. The nanoparticles of pure drug (salbutamol) and the drug in a non-polymer matrix (lecithin-based), with and without lactose, were dispersed in HFA-227 and in aerosol performance assessed by cascade impaction. The size of the salbutamol nanoparticles ranged from 34 to 216 nm. Dispersion of the nanoparticles in a HFA-227:hexane (95:5 v/v) blend resulted in a homogeneous fine suspension that showed no signs of sedimentation or creaming over several months. Rapid release of salbutamol from the nanoparticle was observed (approximately 4 min) as expected from the large surface area of the particles and the high water solubility of the drug. A high fine particle fraction (ex-device, % < 5.8 $\mu$m) of 58.3% to 65.5% and a low MMAD

(1.2 to 1.5 $\mu$m) were observed with the nanoparticle formulations. This data suggests that a high fraction of the nanoparticles would be distributed in the alveolar region of the lung and represents the best aerosol that can be produced using a pMDI.

Budesonide is a potent corticosteroid used as an inhaled anti inflammatory agent to treat asthma. It is available as a dry powder inhaler and as a suspension for inhalation with a nebulizer. A new formulation for nebulization has been developed that contains nanocrystals of budesonide that give the suspension solution-like qualities.[86] The particles are 75 to 300 nm in diameter, compared with 4400 nm for the marketed budesonide suspension (Pulmicort Respules, AstraZeneca). In a randomized crossover study, 16 healthy volunteers were given the nanocrystal budesonide formulation (0.5 mg and 1.0 mg doses), Pulmicort respules and placebo via nebulization using a Pari LC jet nebulizer. Nebulization times were shorter for the nanocrystal formulation, compared with Pulmicort respules (~7.1 min vs. 8.7 min). Similar AUCs were observed with the formulations, suggesting similar pulmonary absorption. However, a higher $C_{max}$ (1212 pg/mL vs. 662 pg/mL) and shorter $T_{max}$ (8.4 min vs. 14.4 min) for nanocrystal budesonide compared with the same dose of Pulmicort, suggests a more rapid drug delivery or absorption with the nanocrystal formulation.

## 6.1. *Diagnostic imaging*

Radiolabeled nanoparticles have been used for many years in pulmonary ventilation studies.[87] Ultrafine $^{99m}$Tc labeled carbon particles (Technegas) is a relatively new advance in ventilation scintigraphy.[88] Technegas (Vita Medical Ltd., Sydney Australia) consists of nanoparticles of carbon with a diameter of approximately 5 nm, that behaves more like a 0.2 $\mu$m particle.[89] Technegas is generated by the electrostatic heating of a graphite crucible to 2500°C in which a saline solution of $^{99m}$Tc-pertechnetate had been placed and dried. The aerosol is dispersed in a lead-lined chamber in an atmosphere of 100% argon gas that is then inhaled by the patient. It is deposited in alveoli by inhalation and distributes similarly as the inert gas radioisotopes. Once they are inhaled, the particles adhere to the alveolar structures without appreciable movement for at least 40 min.[88]

Pulmonary delivery of nanoparticles is also being investigated for lymphoscintigraphy to assess the spread of or the staging of lung cancer. Lung cancer usually exhibits metastasis proliferation, spreading through the lymphatic system and the blood circulation. Lymphatic drainage is responsible for the alveolar clearance of the deposited particulates and drugs up to a certain particle diameter (500 nm).[90] Thus, radiolabeled nanoparticles could be used to visualize the lymph nodes to determine the presence of tumors.

The lymphatic uptake of solid lipid nanoparticles has also been studied as an imaging method to stage lung cancer. The lipid nanoparticles were radiolabeled with the lipophilic tracer, D,L-hexamethylpropylene amine oxime (HMPAO), tagged with 99 m-Tc. The lipid nanoparticles were prepared by the melted homogenization method and had a mean diameter of 200 nm.[90] The radiolabeled nanoparticles were aerosolized using an ultrasonic nebulizer and delivered to rats until 200,000 cpm was achieved over the lung. After inhalation, the total activity in the lung was observed, followed by a fast clearance rate ($t_{1/2}$ = 10 min) that decreases activity in the lung to 25% of the total dose. A significant uptake (16.7%) was detected in the regional lymph nodes during the first 45 to 60 min, suggesting that aerosol delivery to the lungs of solid lipid nanoparticles could be used as an effective colloidal carrier for lymphoscintigraphy.

Drainage into the lymph nodes following the lung instillation of nanoparticles of insoluble iodinated CT x-ray contrast agents was studied in beagle dogs.[91] Nanoparticles of the contrast agent were prepared by microfluidization. A particle size of 150 to 200 nm was achieved. The nanoparticles were suspended in 2 different surfactant solutions. 1.5 mL of the suspension was instilled using a fiber optic bronchoscope at specific sites in the small airways and alveoli. The nanoparticles were transported from the lung to the draining lymph nodes, 6 to 9 days post instillation as visible on the CT radiographs. No adverse clinical signs were observed in the dogs. However, microscopic lung lesions were observed at the instillation sites for both formulations and vehicle. The lesions consisted of inflammatory infiltrates, mainly macrophages, in intra-alveolar, interstitial and perivascular locations. A few small sites had fibrosis and granulomatous nodules with the destruction of the lung parenchyma. The presence of foamy macrophages was observed in the lymph nodes. The microscopic findings suggest that instillation of these nanoparticles of contrast agent may be harmful to the lung. The authors suggested that administering the nanoparticles as an aerosol, rather than by instillation, would prevent high concentrations in focal areas believed to be responsible for these lesions.

## 6.2. *Vaccine delivery*

Mucosal vaccine administration is an attractive method of inducing an immune response, since many pathogens invade the body through mucosal surfaces in the nose, lung and gut. As it is the first contact point, the mucosa has developed barriers to protect the body. The mucosa associated lymphoid tissue (MALT) is one of these barriers. It contributes 80% of the immunocytes and secretes more immunoglobulins than any other organs in the body.[92] Antigens are delivered locally in the respiratory tract to nasal-associated and bronchus-associated lymphoid tissues (NALT and BALT, respectively) and a mucosal immunity is induced. Using nanoparticles,

systemic immunity may also be induced. Several studies have investigated the use of nanoparticles as carriers for the nasal delivery of vaccines. Using tetanus toxoid as a model antigen, Vila and colleagues have studied the use of chitosan nanoparticles as well as polyethyleneglycol and polylactic acid (PEG-PLA) nanoparticles as nasal vaccine carriers.[93,94] They compared PEG-PLA nanoparticles with PLA alone.[94] Tetanus toxoid was entrapped in the hydrophobic PLA core and protected from interacting with enzymes such as lysozymes, by a hydrophilic PEG coating. Upon incubation with lysozymes *in vitro*, PLA particles aggregate and do not reach the epithelium, whereas PEG-PLA nanoparticles remain stable and size unmodified. The nanoparticles were produced using a double emulsion technique. PEG-PLA tetanus toxoid nanoparticles had a similar diameter to the PLA particles (196 nm vs. 188 nm), but had a lower loading efficiency of 33.4% compared with 48.1% with PLA. The IgG antibody response induced by PEG-PLA was superior at weeks 2 to 24, after intranasal instillation of 30 $\mu$g of tetanus toxoid (10 $\mu$l per nostril) on days 1, 8 and 15 in male BALB/c mice. In a similar study, the same group compared radiolabeled PEG-PLA, PEG-PLA with gelatin stabilizer to radiolabeled PLA encapsulated tetanus toxoid. They reported that 1 hr after intranasal administration, PEG-PLA nanoparticles produced a radioactivity level 10-fold higher in the blood than PLA which remained constant for 24 hrs. The radioactivity detected in the lymph nodes, lungs, liver and spleen was 3 to 6 fold higher for PEG-PLA than PLA nanoparticles 24 hrs post instillation. The results of this work suggest that the PEG-PLA nanoparticles are partially taken up by the M cells of the NALT, as well as being transported to the submucosa and drained into the lymphatic system and blood stream.[95] Recent work by the same group has investigated the potential use of chitosan nanoparticles for nasal administration of vaccines.[93] Chitosan is a hydrophilic natural polysaccharide that is biodegradable and has mucoadhesive properties. The nanoparticles are formed spontaneously by adding the counter anion sodium TPP into the chitosan solution, without the use of energy sources or organic solvents required for the production of PEG-PLA nanoparticles. Again, using tetanus toxoid as the model antigen, the investigators studied the effect of chitosan dose (200 $\mu$g and 70 $\mu$g) and molecular weight (23, 38 or 70 kDa) on the efficacy of the nanoparticles. The nanoparticles produced were 300 to 350 nm and had a positive surface charge (+40 mV). The loading efficiency of tetanus toxoid was 50 to 60%, irrespective of the molecular weight of chitosan. *In vitro*, the formulations exhibited a rapid release over the initial 2 hrs followed by a slow release for 16 days, with the greater initial release at lower molecular weights of chitosan. 30 or 10 $\mu$g of antigen (associated with 200 and 70 $\mu$g of chitosan) was given intranasally to BALB/c mice on days 1, 8 and 15. The IgG levels induced by the nanoparticles were significantly higher than those elicited by free tetanus toxoid. The response lasted for the 24 weeks studied with the IgG titres increasing over time. Anti-tetanus IgA titers were detected in the saliva, bronchoalveolar and intestinal lavage fluids 24 weeks post

administration. The results were independent of the administered dose and were significantly higher for the nanoparticle than the free tetanus toxoid.

Jung and colleagues evaluated tetanus toxoid-loaded polymer nanoparticles as potential nasal vaccine carriers in mice.[96] The nanoparticles were produced with various diameters (100 nm, 500 nm) using a novel polyester, sulfobutylated poly (vinyl alcohol)-graft-poly(lactide-co-glycolide), SB(43)-PVAL-g-PLGA. The surface charge was −43 to 59 mV. Mice were immunized with tetanus toxoid nanoparticles or free toxoid in solution at weeks 1, 2 and 3, either by oral, intranasal or intraperitoneal administration. Four weeks after the first intranasal immunization, IgG and IgA titers were significantly higher than baseline. Oral immunization with the nanoparticles produced a weak IgG antibody response. Only 10% of the oral dose was administered to the nose (2.89 vs. 28.9 $\mu$g), however, intranasal immunization appeared to be more effective in inducting an immune response. Particle size had an effect on the titer levels. Particles $>1\,\mu$m did not induce an immune response, but no difference was observed between the 500 nm and 100 nm nanoparticles which both induced significantly levels of IgG and IgA.

These studies suggest that nasal delivery of vaccines using biodegradable nanoparticles are a promising method of inducing mucosal and systemic immunity.

## 6.3. *Anti Tuberculosis therapy*

Intracellular bacterial infections caused by pathogens such as *Mycobacterium tuberculosis* are difficult to eradicate because they are generally inaccessible to free antibiotics. By loading antibiotics into nanoparticles, it is expected that delivery to the infected cells would improve since nanoparticles have been shown to localize preferentially in organs with high phagocytic activity and in circulating macrophages as well.[97] The encapsulation of antibiotics has several advantages: (1) It modifies their pharmacokinetic characteristics by prolonging the antibiotics half-life and increasing the area under the concentration time curve (AUC), while decreasing its apparent volume of distribution. (2) It improves the targeting of the drug to the phagocytic cells. (3) It reduces toxicity of the antibiotics, such as the hepatotoxicity of anti tuberculosis drugs and the nephrotoxicity of aminoglycosides. Antibiotics encapsulated in nanoparticles have been shown to be superior at treating intracellular infections when administered intravenously. However, the pulmonary delivery of these nanoparticles have only been investigated recently.

Although effective therapy for tuberculosis is available, treatment failure and drug resistance is typically the result of patient's noncompliance. To improve compliance, investigators have been studying ways to reduce the dosing frequency of the drugs. Poly (lactide-co-glycolide) (PLG) nanoparticles as an aerosolized sustained release formulation for anti tuberculosis drugs, isoniazid, rifampicin and pyrazinamide, has been investigated since pulmonary tuberculosis is the most

common form of the infection.[98] The majority of the nanoparticles were 186 to 290 nm in diameter. Drug encapsulation efficiency was 56.9% to 68%. Aerosolized nanoparticles had a MMAD of 1.88 $\mu$m, with 96% of the particles in the respirable range (<6 $\mu$m). A single nebulization to guinea pigs resulted in sustained plasma drug concentrations for 6 to 8 days and in the lung for 11 days. The half-life and mean residence time of the drugs was significantly prolonged, compared with the oral free drugs. Nebulizing the nanoparticles every 10 days to guinea pigs infected with *Mycobacterium tuberculosis* resulted in no detectable bacilli in the lung after 5 doses of treatment, compared with 46 daily doses of orally administered drug to achieve the equivalent efficacy.

The use of lectin-based PLG nanoparticles as an aerosolized sustained release formulation of isoniazid, rifampicin and pyrazinamide has also been studied in guinea pigs.[99] Mucoadhesive drug delivery systems such as chitosan have been previously investigated as a method of prolonging residence at a site of absorption. The main drawback of mucoadhesive systems is that its residence time is limited by the turnover time of the mucous gel layer, which is only a few hrs. Attaching the polymeric nanoparticles to cytoadhesive ligands such as lectins could prolong the duration of adhesion, thereby prolonging residence time. Lectins bind to epithelial surfaces via specific receptors. Wheat germ agglutinin (WGA) is the least immunogenic lectin and has known receptors on the alveolar epithelium as well as the intestinal wall. WGA lectin-PLG nanoparticles were prepared by a two-step carbodiimide procedure. Their size ranged from 350 to 400 nm with drug encapsulation efficiency between 54% and 66%. The nanoparticles were delivered via nebulization to guinea pigs. 88% of the aerosol was in the respirable range (<6 $\mu$m) with a MMAD of 2.8 $\mu$m (GSD of 2.1). Three doses of nanoparticles were administered every 15 days for 45 days. The WGA-PLG nanoparticles resulted in a prolonged $T_{max}$, increased AUC and mean residence time after inhaled delivery. All three drugs were present in the lungs, liver and spleen at concentrations above the minimum inhibitory concentration 15 days post dosing, compared with orally-administered free drug. Chemotherapeutic studies in guinea pigs infected with *Mycobacterium tuberculosis* showed that 3 doses administered every 15 days for 45 days yielded undetectable mycobacterial colony forming units, which was only achievable with 45 doses of the oral free drugs. The study results suggest that WGA-based PLG nanoparticles could be potential drug carriers for anti tuberculosis through aerosol delivery, reducing the drug dosing frequency.

## 6.4. *Gene therapy*

Pulmonary gene delivery and DNA vaccinations are attractive therapies for a variety of lung diseases such as cystic fibrosis, asthma, chronic obstructive pulmonary

disease, lung cancer and infections caused by *Mycobacterium tuberculosis*, influenza or SARS-associated coronavirus. Gene delivery requires carriers to transfer DNA into the nuclei of cells. There are two approaches for delivery: viral and non viral carriers. Viral delivery systems, although very efficient at transfection, are problematic due to their inherent immunogenicity. Non viral are safer but their transfection efficiency is low. Recently, biodegradable polymer-based nanoparticles have been investigated as a non viral pulmonary gene delivery system, taking advantage of their prolonged residence time in the lung and ability to be taken up by macrophages and dendritic cells, and to escape degradation by lysosomes.

Asthma is characterized by elevated eosinophilic inflammation in the airway and increased airway hyperresponsiveness. Chronic inflammation can lead to structural damage and airway remodeling. IFN-$\gamma$ is a cytokine that promotes T-helper type 1 (Th1) responses which down regulates the Th2 immune responses present in asthma. Recombinant IFN-$\gamma$ has been shown to reverse inflammation in murine models of asthma. However, its short half-life and severe adverse effects at high doses have prevented its therapeutic use.[100] An intra-nasal IFN-$\gamma$ gene therapy had been developed as an attempt to circumvent the drawbacks to its use. Kumar and colleagues studied the effects of a chitosan-IFN-$\gamma$ plasmid DNA nanoparticle in a BALB/c mouse model of allergic asthma (using ovalbumin-sensitization).[101] Mice treated with the chitosan nanoparticles exhibited a significantly lower airway hyperresponsiveness (to methacholine challenge), reduced number of eosinophils and a significant decrease in epithelial denudation, mucus cell hyperplasia and cellular infiltration. Production of IFN-$\gamma$ was increased post-treatment while IL-5 and IL-4 and ovalbumin-specific IgE were reduced. Chitosan IFN-$\gamma$ nanoparticles induced IFN-$\gamma$ gene expression predominately in epithelial cells and worked within 3 to 6 hrs after intranasal administration.

Poly (D,L-lactide-*co*-glycolide (PLGA)–polyethyleneimine (PEI) nanoparticles are also being investigated for pulmonary gene delivery. PLGA had been extensively evaluated for its sustained-release profile and ability to be taken up by macrophages. PEI is a cationic polymer. Its high positive charge density suggests that it would be a promising candidate as a non viral vector.[102] PLGA nanoparticles with PEI on their surface had a mean particle diameter between 207 and 231 nm, surface charge $> 30$ mV and a DNA loading efficiency of $>99\%$. Internalization of the nanoparticles in the human airway submucosal epithelial cell line, Calu-3, was observed and DNA detected 6 hrs after administration. However, *in vivo* efficiency of this system still needs to be studied.

Respiratory syncytial virus (RSV) infection is a major cause of respiratory tract infections and is associated with approximately 17000 deaths annually on a worldwide basis, with no anti viral therapy or vaccine available.[103] RSV NS1 protein appears to antagonize the host type 1 interferon–mediated response. Zhang and

colleagues hypothesized that blocking the NS gene expression might inhibit RSV replication and thus provide effective antiviral therapy.[104] Small interfering RNA (siRNA) targeting the NS1 gene (siNS1) were encapsulated in chitosan nanoparticles. BALB/c mice were intranasally treated with siNS1 chitosan-nanoparticles before or after RSV infection. A significant decrease in virus titers in the lung was observed, in addition to a decrease in inflammation and airway hyperresponsiveness, compared with controls. The effect of siNS1 lasted at least 4 days. The data show that siNS1 nanoparticles may be a promising anti viral therapy against RSV infection.

## 7. Conclusion

Innovations in the biotechnology and pharmaceutical industries have led to novel approaches for delivering drugs more efficiently and to specific targets in the lung and the body. One of the growth areas is the development of nanoparticles as carriers of active pharmaceutical agents for diagnosis and treatment.

Aerosol delivery systems, discussed at the beginning of this chapter, are the current technologies for delivering therapies to treat respiratory diseases and some systemic diseases. The accepted philosophy, and one based on sound *in vitro* and *in vivo* clinical data, is that the optimal size of aerosol needed to target the distal lung is of the order of 3 $\mu$m. This size is 10–100 times greater than the nanoparticles being considered in the design of agents including antibiotics, vaccines and gene therapies for inhaled delivery. Novel techniques and formulations are being studied to produce successful vehicles for delivering these types of products *in vivo*. Positive outcomes using animal models to test these new aerosol formulations have been reported. However, clinical studies still need to be conducted to determine their efficacy in humans.

As with any new technology, there will be benefits and risks associated with its use. The use of nanotechnology to provide improved targeting of drugs via the inhaled route is an exciting development that has the potential to yield novel treatments for many diseases in the near future.

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# 18

# Magnetic Nanoparticles as Drug Carriers

*Urs O. Häfeli and Mathieu Chastellain*

Magnetic nanoparticles possess many characteristics that make them promising as drug carriers and for use in biomedical applications. They can be attracted or magnetically guided by strong magnetic fields, thus acting as drug carriers. They can also be used for hyperthermia applications, due to the heat they produce in an alternating magnetic field. The resulting temperature increase can be used to modify or inhibit specific cell activities locally, or even to release drugs in a precisely controlled, temperature-increase activated manner. Magnetic nanoparticles can also serve as contrast agents for diagnostic applications such as magnetic resonance imaging.

## 1. Introduction

Magnetic nanoparticles occur frequently in nature. They are found not only in the mineral world but also in living organisms. Well known examples are magnetotactic bacteria, which are believed to navigate the waters they live in, by using internal magnetic crystals aligned in chains that function as a compass. Higher forms of life, such as humans, also employ iron as an essential metal. In order to ensure a constant supply of iron, the body stores it within the well-defined protein shell ferritin as a 5 to 7 nm hydrous ferric oxide nanoparticle.[1,2]

The use of magnetic powders in medical applications was already conceptualized by ancient Greek and Roman scientists.[3] However, magnetic nanoparticles have only been used since the mid 1970s in the area of biological and medical sciences.[4] A wide range of *in vivo* as well as *in vitro* applications have been or are

currently being developed.[5–14] These applications include magnetic drug delivery, magnetic fluid hyperthermia, magnetic cell separation and extraction when an external magnetic field is applied, and contrast enhancement for diagnostic imaging procedures, as the magnetic nanoparticles' own magnetic field influences their surrounding. From a practical point of view, magnetic nanoparticles are thus versatile tools that enhance yields for many *in vitro* processes such as cell purifications. In addition, in general, no invasive procedures are required when they are used for *in vivo* therapies.

## 2. Definitions

The development of nanoparticles for biomedical applications requires contributions from the basic to the medical sciences. Such interdisciplinary interactions can sometimes lead to communication problems. For example, the term magnetic nanoparticle has a different meaning for a physician and a biochemist, and might have no meaning at all for a physicist. For this reason, definitions satisfying all partners involved in the present research field are required. With the following simplified definitions, we attempt to provide a universal starting point.

### 2.1. *Properties of magnetic materials*

The magnetic properties of materials are mainly related to electrons, with all materials showing some kind of magnetic behavior. Materials can be classified according to their response to external magnetic solicitations. Magnetic susceptibility is defined by the initial slope of the magnetic curve, presenting the magnetization "M" (response) as a function of an applied magnetic field "H" (solicitation) (Fig. 1).

The observed behavior of different materials can be explained in terms of their magnetic structure at the atomic level, and can be summarized as diamagnetism, paramagnetism, and ferromagnetism. Diamagnetic materials consist of atoms with no net magnetic moment. Nevertheless, they tend to oppose any external magnetic field change due to induced dipoles in the material. For this reason, they are characterized by a slight negative magnetic susceptibility. Paramagnetic materials are made of atoms showing a net magnetic moment. The random orientation of these moments is responsible for a slight positive magnetic susceptibility and no magnetization remains when the external magnetic field is switched off (see Fig. 1). Ferromagnetic materials react strongly to external magnetic fields, unlike dia- and para-magnetic materials. They can be viewed as paramagnetic materials with an organized domain structure (see Fig. 1). Within a domain, all atomic magnetic moments are parallel. When submitted to an external magnetic field, the different domains, initially in a random orientation, tend to align according to the

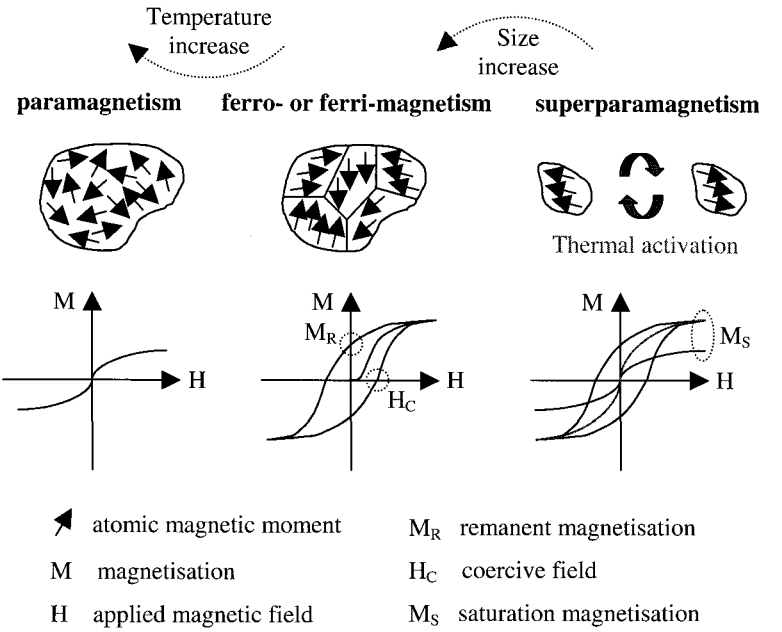


**Fig. 1.** Atomic magnetic moment structure (upper drawings) and corresponding magnetization curves (lower graphs). Paramagnetic materials show random atomic moment orientation which is responsible for their weak response to magnetic solicitations and no remanence. A typical ferro- or ferri-magnetic material shows a characteristic domain structure with associated hysteresis magnetization curve. Superparamagnetic materials present a thermally induced oscillating magnetic moment and a strong magnetic response to external magnetic fields (red curve). Their saturation magnetization is comparable to ferro or ferri-magnetic materials (black curve), but without remanence as in the case of paramagnetic materials (blue curve). For a given particle composition, all three behaviors might be encountered, depending on temperature or particle size (upper arrows).

external field. This alignment requires domain wall motions and results in hysteresis of the magnetization curve. After the external field is switched off, a remaining or remanent magnetization "$M_R$" can be observed. Again, in order to achieve a random domain orientation, more energy must be provided by means of an external magnetic field applied in the opposite direction. A coercive field "$H_C$" is defined as the value of the external field necessary to misalign the domains to a random state. More detailed information is available in the literature.[16–20]

Magnetic materials can be composed of different atoms and ions with various magnetic moments. The most well known example is magnetite ($Fe_3O_4$), which consists of $Fe^{2+}$ and $Fe^{3+}$ ions. The crystallographic structure of such materials determines whether or not antiferromagnetic or ferrimagnetic properties are present. For magnetic drug targeting, only ferro- and ferri-magnetic materials are of interest, as they react strongly to external magnetic fields due to their non-zero atomic or lattice

unit magnetic moment and the domain structure. Temperature also plays a role in the magnitude of magnetic response, as high thermal energy can disturb the atomic moment orientation within the domains, leading to paramagnetic behavior.

When ferro- or ferri-magnetic materials are divided, the obtained nanoparticles can become small enough to show single domain structure with a non-zero magnetic moment. Depending on the particle size, the thermal energy might be high enough to have the particles magnetic moment switch between energetically favorable (or easy) directions. These directions are defined by the particle structure, especially the crystallography, the shape and the surface. As a result of the moment oscillation, the net particle magnetization is zero and no remanent magnetization is observed, but the particles still strongly react to external magnetic solicitations (see Fig. 1). This behavior, called superparamagnetism, is generally encountered for particles that are a few nanometers in size. Superparamagnetism can be influenced by magnetic interparticle interactions, which lead to collective behavior of several particles acting as one bigger particle. The observation time is also important and must be longer than the particle relaxation time, necessary to switch from one to the other easy direction.

## 2.2. *Nanoparticles*

No single definition exists to describe a nanoparticle. Most of the time, an arbitrary size range is used ("nanometer sized", from $10^{-9}$ to $10^{-6}$ m). In view of the recent developments in nanotechnology, some people now use the drastic behavior changes arising below a critical size (such as the superparamagnetic state described earlier) to define nanoparticles. When reducing nanoparticle size, not only does the surface over volume ratio increase gradually, but a complete modification of the material properties may also occur. This is of primary importance in the biomedical field, where a change in size can lead to toxic effects. Many unanswered questions remain in this field and legal aspects related to nanoparticles are currently under discussion.[21]

For use in biomedical applications, ferro-, ferri- or superparamagnetic particles must be coated to ensure colloidal stability, increased circulation time in the body, functional surfaces, and appropriate diagnostic properties. In this regard, the term "magnetic nanoparticle" not only refers to an inorganic core responsible for magnetic properties, but also to a composite structure with one or several cores coated or embedded in a matrix. Coatings are reviewed elsewhere in this book.

In addition to a compatible coating, magnetic nanoparticles used in clinical applications must form stable aqueous suspensions. Suspensions are complex dynamic systems. Their equilibrium is influenced by the forces present, including Van der Waals, electrostatic, steric, and magnetic forces, as well as by Brownian

motion. On this account, it is crucial to realize that solvent modifications can drastically influence the behavior of the system. The term "ferrofluid" is correctly used only in the case of a colloidal stable suspension of single domain nanoparticles.[22]

## 3. Magnetic Nanoparticles

In general, a single particle type cannot be used for all applications. Instead, the composition, size and production route of synthesized magnetic nanoparticles is determined by the target application. Although superparamagnetic, ferro- and ferrimagnetic particles can all be used for magnetic drug carrier applications, superparamagnetic particles are favored for biomedical applications, due to the fact that they behave non-magnetically when they are not under the influence of an external magnetic field, thus preventing undesired magnetic agglomeration. To further assist in preventing agglomerations, to optimize bio-interactions with the host environment and to maximize biocompatibility, the choice of appropriate surface chemistries and functionalizations is also important. Many magnetic nanoparticles are available with different surface chemistries, and details about the properties of these chemistries are given elsewhere in this book.

The following subsections provide an overview of magnetic nanoparticles as drug carriers, classified according to magnetic composition. The final subsection deals with the general biocompatibility issues of magnetic nanoparticles.

### 3.1. *Iron oxide based magnetic nanoparticles*

In biomedical applications, the most commonly used magnetic nanoparticles are superparamagnetic magnetite ($Fe_3O_4$) and maghemite ($\gamma$-$Fe_2O_3$). This is due to their ease of synthesis using chemical or physical approaches,[23–33] as well as their general bio-compatibility (and FDA approval). Massart's aqueous coprecipitation method,[34] which leads to particles easily dispersible in water, is the most cited method of magnetic nanoparticle preparation. The particle size can be tuned in the 3 to 30 nm size range[35] and the particles usually show an ellipsoidal shape. The stoichiometry ranges from magnetite to maghemite, the two crystallographic structures being very similar.[35–38] The size distribution is about 10 to 20% [see Fig. 2(a)]. Time consuming size sorting procedures allow for further narrowing of the size distribution to about 5% in the best case. A thorough characterization of such particles was carried out by the group of Jolivet *et al.*[39–47]

Iron oxide nanoparticles have been synthesized intensively during the past decades, but until recently, phase and size control have been problematic. A newly developed two-step approach has allowed for much better control over the particle

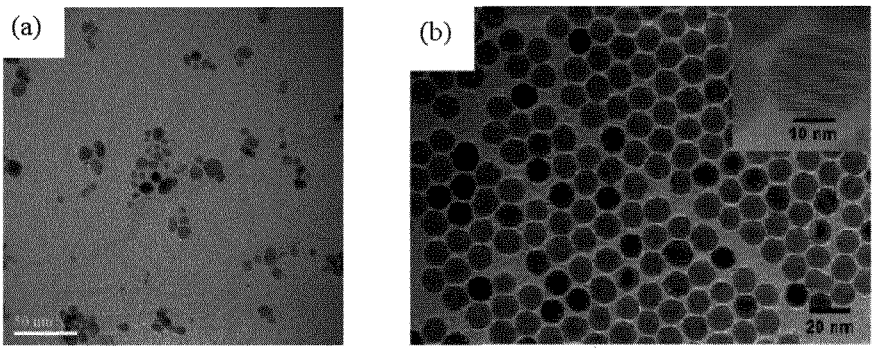


**Fig. 2.** Typical TEM bright field pictures of maghemite nanoparticles. (a) Classical co-precipitation synthesis[48] and (b) Decomposition at high temperature of organic precursors.[49] Despite its much improved size and shape distribution, the second particle type suffers from two major drawbacks for biomedical applications: Biocompatibility and the ease of dispersion in water based solvents.

structure. In this approach, metal particles are first obtained and then oxidized in a controlled way.[50] Size distributions of better than 5% can be achieved in the 4 to 16 nm range, as shown by Alivisatos *et al.*[51] and Hyeon *et al.*[49] [see Fig. 2(b)]. These particles are, however, often not appropriate for biomedical applications as they do not disperse easily in water.[52]

Many other magnetite and maghemite nanoparticle synthesis approaches can be found in the literature, but none are significantly different from the ones presented above. Slightly modified nanoparticles can also be obtained by partly replacing the iron in the magnetite or maghemite structure with cobalt or nickel. This in turn changes the magnetic properties of the particles. More details are given in a recent and extensive review by Tartaj *et al.*[53]

## 3.2. *Cobalt based magnetic nanoparticles*

From a magnetic point of view, particles showing a stronger reaction to magnetic fields are desirable. Cobalt achieves this aim, but its toxicity is a major drawback. One way of preventing or minimizing this toxicity caused by cobalt ion leakage is the inorganic encapsulation of cobalt with, for example, silica.

## 3.3. *Iron based magnetic particles*

Pure iron nanoparticles can be synthesized, but their sensitivity to oxidation is a major drawback for biomedical applications. Thus, a coating, as described for cobalt particles, should be used. Iron has also been coated or alloyed with platinum, cobalt and carbon.

## 3.4. *Encapsulated magnetic nanoparticles*

Depending on the application, magnetic nanoparticles may be combined into larger conglomerates to increase the overall magnetic moment (see Fig. 3). Great care should be paid to interparticle magnetic interactions. The superparamagnetic behavior of a system might, for example, be lost due to such interactions. Also, the magnetic core concentration must be kept constant among the magnetic conglomerates to yield homogeneous magnetic moments, and thus a consistent response to an applied magnetic field.

Either a single or a two-step approach can be used to synthesize magnetic particles.[54] In the one-step approach, a "linker" is present while synthesizing the magnetic nanoparticles. In the two-step approach, the linker is added subsequently. The linker can be organic or inorganic and is chosen for its chemical and biocompatible characteristics. For biomedical applications, dextran, starch, polyethylene glycol (PEG), polyvinyl alcohol (PVA), silica and gold are among the most common compounds.[54]

## 3.5. *Biocompatibility issues of magnetic nanoparticles*

One of the first papers to discuss the biocompatibility issues of magnetic particles was published in the early 1970's by Nakamura *et al.*[55] The authors prepared fine carbonyl-iron particles and infused them into different animal species *in vivo*. They concluded that to achieve optimal results, the magnetic particles should be coated with a biocompatible material and be as round as possible.

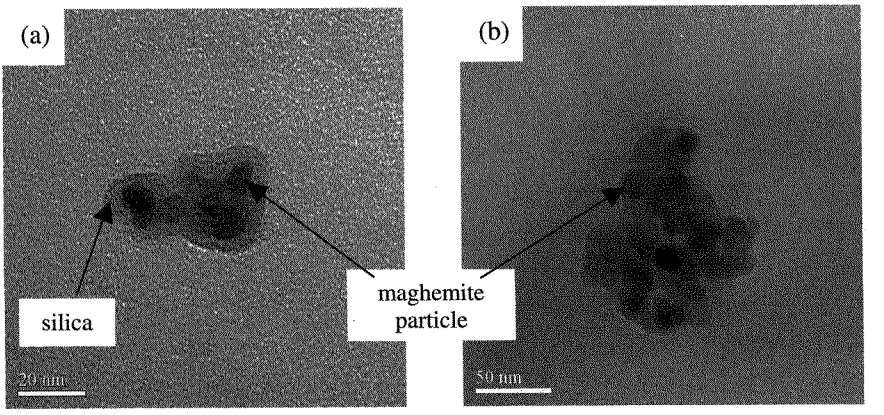


**Fig. 3.** Bright field TEM pictures of different types of magnetic particles[48]: (a) silica-magnetite composite and (b) dextran-magnetite composite. The silica layer can be observed easily, whereas dextran does not produce enough contrast to be seen clearly.

Further research showed that pure magnetic metal particles, such as iron, cobalt and nickel particles, should not be used directly *in vivo* because they oxidize easily and release +2 or +3 charged metal ions that can exert unwanted as well as toxic effects. Iron ions, for example, are problematic in that they produce and catalyze oxygen radical formation.[56] Cobalt and nickel ions have been found to induce adverse tissue reactions, and to promote infection and metal sensitivity.[57]

In contrast to pure magnetic particles, iron oxides and superparamagnetic iron oxide nanoparticles (SPION) coated and stabilized with hydrophilic polymers have been found to be quite thermodynamically stable under physiological conditions, not exerting obvious toxic effects. In fact, they are similar in size and core composition to the natural non-toxic magnetic nanoparticles found in magnetotactic bacteria[58] and in human tissue.[59] Pharmacokinetic studies of small magnetite nanoparticles destined for magnet resonance imaging[60,61] have shown that the magnetite nanoparticles are taken up by the cells of the reticuloendothelial system (RES) and are transported intracellularly to lysosomes, where they slowly oxidize at low pH and are then recycled by the body.[62] Within 20–40 days, up to 60% of the iron is recovered in the red blood cells, as determined using radiolabeled $^{59}$Fe.

Recent discussions have centered on the fate and toxic effects of (magnetic) nanoparticles in humans after inhalation. Rodent models have shown the potential problematic effects of such particles to include the induction of asthma, inflammation, and potentially even cancer.[63] Some of these effects might be due to the fact that particles smaller than 100 nm are not exhaled, but are almost completely retained in the alveoli.[64] For this reason, acute effects can rapidly turn into chronic effects. Another possible cause for concern is the report that small particles have been found behind the blood brain barrier[65] (see also Chap. 24). Further research needs to clarify if these particles directly crossed the blood brain barrier or via the nose. Care must be taken to relate the effects seen in animal models to the human situation, especially since effects seen in rodents do not seem to develop in humans.[63] Clarification of the short and long term risks of nanoparticle use is the aim of several programs being initiated in 2005 by the European, American and Canadian governments.

## 4. Application of Magnetic Nanoparticles as Drug Carriers

The following section presents an overview of the use of magnetic nanoparticles sized 1 $\mu$m or less for the delivery of drugs. Magnetic microspheres of larger than 1 $\mu$m size are also mentioned in a few places, but for a recent and thorough review, as well as for the history of magnetic drug delivery, the reader is advised to consult a more extensive treaty on this matter such as the recent volume of the MML series.[66] In this section, magnetic nanoparticles will be grouped according to their mechanism of action including magnetic hyperthermia; the delivery of magnetic nanoparticles that (slowly) release drugs (tumor treatment, thrombolysis, delivery

of antiinfective, antiarthritic, antifungal, and antiscar agents, and local anesthesia or neuroblocking agents) or act without drug release (radiotherapy or embolization); and the improved delivery of peptides (gene transfer). Results from *in vitro*, *in vivo* and clinical work will be discussed.

## 4.1. *Magnetic hyperthermia*

Magnetic nanoparticles in an alternating current (AC) magnetic field produce heat by Néel and Brownian relaxation.[67] Heat production above a person's normal body temperature is called hyperthermia and can be medically used for the eradication of cancer cells. Temperatures above 56°C lead to thermoablation. Once magnetic nanoparticles have successfully reached certain organs or tissues, especially tumors, magnetic hyperthermia can be induced. Normal tissue nearby, not containing the magnetic nanoparticles, remains at body temperature and is thus spared.

One of the first to examine this effect was Gilchrist who published a seminal paper in 1956 on the selective inductive heating of lymph nodes, after injection of maghemite particles sized between 20 and 100 nm diameter directly into the lymph nodes near surgically removed canine tumors.[68] Using 5 mg of $Fe_2O_3$ per gram of lymph node and a magnetic field strength of 240 Oe, a maximum temperature rise of 14°C was reached within 3 minutes. To prevent a reoccurrence of the cancer, hyperthermia is normally combined with a second treatment modality such as chemotherapy or irradiation.

Twenty years later, Rand *et al.* showed that ferrosilicone can induce heat after being infused into a tumor's blood supply and placed under the influence of a strong magnetic alternating field.[69] Rand's ferromagnetic silicone microspheres were based on Turner *et al.*'s research in 1973, in which magnetic particles of unknown but probably larger size in a silicone fluid were infused into and then clogged (embolized) the capillary bed of several targeted organs.[70] With this technique, the researchers successfully embolized the blood supplies of different tumors. No side effects were reported in the 7 patients who had brain tumors, pheochromocytomas, a tongue tumor and a hypernephroma. Rand's so-called "magnetic field induced hyperthermia" was then further developed by Sako *et al.*,[71,72] who showed that heating was reproducible and proportional to the amount of iron used.

The use of single domain, dextran-coated magnetite nanoparticles for tumor hyperthermia was developed by Jordan[73] and Chan,[74] and is currently undergoing *in vivo* and clinical testing. Jordan reported the optimal nanoparticle core diameter to be in the 10 nm range,[73,75] although type of coating and coating stability also seemed to be important.[76] Using these nanoparticles, 5 mg of material per gram of tumor was sufficient to increase the tumor temperature by 10°C to cell toxic levels. Jordan is currently conducting a clinical phase II trial combining magnetic hyperthermia and radiation therapy.[77,78] He recently presented the first clinical results from 8 patients

at the 5th International Conference on the Scientific and Clinical Applications of Magnetic Carriers in Lyon, France. The patients were treated for cervix (2), rectal, and prostate (2) carcinoma, as well as for a chondrosarcoma, rhabdomyosarcoma, and liver metastasis. During the 1-hour sessions, after local injection of the magnetic particles, the tumor temperature increased to 43–50°C under the influence of a magnetic field of 3 to 9.5 kA/m and a frequency of 100 kHz. While no additional nanoparticle injections were necessary, the hyperthermia treatment was repeated from 2–11 times. The magnetic fluid hyperthermia was well tolerated. Two patients showed complete remission 9 and 14 months after treatment, while the other six patients showed local control with no recurrent growth of the tumors. These results are very promising.

Another group in Germany led by Hilger is working on circumventing the drawback of having to directly inject the particles into a tumor, by using antibody-bound magnetic nanoparticles which are able to target breast cancer, followed by magnetic field hyperthermia.[79,80] Although their particles are taken up extensively by tumor cells and show a specific heating power of up to 170 W/g,[81] there is still more work needed to increase the number of particles in the tumor and to reach a homogeneous tumor distribution.[82]

Magnetic hyperthermia is also possible with large microspheres that contain magnetic nanoparticles.[83,84] As an example, Moroz *et al.* incorporated 100 nm maghemite particles into 32 $\mu$m biocompatible plastic particles and then embolized the arterial blood supply of liver tumors with them. In an animal study with 10 rabbits, the VX2 tumor volumes decreased significantly within 2 weeks.[85–87]

The development of maghemite nanoparticles with very high AC losses is ongoing. Hergt *et al.* are in the process of characterizing the largest, but still superparamagnetic particles,[88] optimizing the coatings such as carboxydextran or polyethylene glycol,[89] and investigating the exact mechanism of heat production in an AC magnetic field.[88]

Magnetic hyperthermia is an exciting cancer treatment possibility and is profiting from ongoing research into its mechanism of action and from improved magnetic materials. The proof of principle has advanced to the clinical stage with the construction and clinical testing of Jordan's magnetic field therapy system.[77] The targeted (cancer) cell uptake of sufficient amounts of magnetic nanoparticles from a patient's blood supply could make magnetic hyperthermia the method of choice for many different kinds of tumors.

## 4.2. *Magnetic chemotherapy*

Magnetic drug delivery is able to concentrate drugs in a tumor if the tumor is accessible through the arterial system and has a good supply of blood. Magnetic

drug delivery thus promises to deliver highly effective anticancer drugs with fewer side effects, and with shorter and less toxic treatments.

Most drug release from magnetic particles occurs passively, by desorption from and diffusion out of the particle matrix. The main driving forces are pH, osmolarity and concentration differences between particles and the blood/tissue. Widder and Senyei were the first to successfully illustrate this concept with the chemotherapeutic drug doxorubicin encapsulated into albumin-coated magnetite particles sized around 1–2 $\mu$m.[90] Targeting a distinct area of a rat's tail, they were able to deliver 200 times more of the drug than intravenous application of the same amount of free drug could achieve.[91] Taking it a step further, they treated Yoshida rats with sarcomas in their tails and attained complete remission in 77% of the rats.[92,93]

The magnetic albumin microspheres were never tested clinically, likely because the magnetophoretic mobility (overall magnetic responsiveness to a magnetic field) was considered too low for deeper applications. This changed with the introduction of iron-carbon particles originally developed in Russia[94] and then brought to clinical trial by the company FeRx. FeRx's irregularly-shaped carbon-coated iron particles, of 0.5 to 5 $\mu$m in diameter and with a very high magnetic susceptibility, were loaded with doxorubicin and showed promising results and very low therapy-related toxicity in the treatment of inoperable liver cancer.[95,96] Unfortunately, FeRx ceased to exist in 2004 when a preliminary analysis of their ongoing clinical trial failed to convince investors of the method's superiority over other treatment methods.

Not only doxorubicin, but also many other chemotherapeutic drugs can be and have been adsorbed to magnetic nanoparticles made from many different matrix materials. Examples of chemotherapeutic magnetic nanoparticles tested *in vivo* include polyalkylcyanoacrylate nanoparticles of 220 nm diameter filled with (adsorbed) dactinomycin,[97] chitosan nanoparticles of 530 nm diameter loaded with oxantrazole,[98] solid lipid nanoparticles of 450–570 nm diameter loaded with methotrexate,[99] and ferro carbon of 100 nm diameter loaded with carminomycine.[94] Each of these nanoparticles has been tested in animal experiments with positive results. Specifically, after intravenous injection, the drug concentration tripled in the target organ when a magnet was placed above it, compared with a control without an applied magnet.

It seems that the intravenous injection of magnetic nanoparticles, even very close to the target region, is not optimal. This was well documented in a clinical cancer therapy trial performed by Lübbe *et al.* in 14 patients.[100,101] They used magnetic nanoparticles of 100 nm in diameter loaded with 4'-epidoxorubicin for the treatment of advanced solid cancer. The phase I study clearly showed accumulation of magnetic nanoparticles in the target area without toxic effects. MRI measurements, however, indicated that more than 50% of the magnetic nanoparticles were deposited in the liver. This was likely due to the particles' low magnetic

susceptibility and small size, which limited their ability to be held at the target organ. Intraarterial injection into the blood supply that leads to the target region might be much more effective for magnetic drug targeting for this reason.

The above examples of magnetic drug targeting with magnetic nanospheres are only a subset of all the magnetic drug delivery attempts. A more complete compilation is given by Hafeli.[66] In addition, all the important factors in magnetically controlled targeted chemotherapy are extensively described in a review by Gupta and Hung.[102]

## 4.3. *Other magnetic treatment approaches*

Under the influence of a magnetic field, magnetic particles align in chains and eventually agglomerate. Depending on particle size and shape, this can lead to embolization (clogging) of the blood vessels and especially of the small capillaries of 7 to 10 $\mu$m in diameter. This accumulation of particles can be used on its own to starve the target tissue of oxygen, produce hypoxia and induce necrosis. The magnetic particles used for this approach are generally larger, such as the "iron sponge" of 10–30 $\mu$m used by Sako *et al.*,[103,104] but can also consist of nanoparticles in a more lipophilic solvent such as the ferrosilicone employed by Turner *et al.*[70] Turner added a catalyst to their ferrosilicone suspension, which resulted in vulcanization of the viscous slurry 14 min after injection into 7 patients with diverse solid tumors.[70]

Magnetic particles can also be used for tumor treatment without releasing any drugs. For this purpose, magnetic particles can incorporate radioisotopes either in the matrix or bound to their surface, and then deliver tumor cell-toxic radiation doses wherever they accumulate.[105] External magnetic fields or internal magnetizable wires[106,107] can be used to accumulate radioactive magnetic particles and hold them at the target site. The particles irradiate the area within the specific treatment range of the isotope. Initial experiments in mice showed that intraperitoneally injected radioactive poly(lactic acid) based magnetic microspheres (10–20 $\mu$m in diameter) could be concentrated near a subcutaneous tumor in the belly area above which a small magnet had been attached.[108] The dose-dependent irradiation from the $\beta$-emitter $^{90}$Y-containing magnetic particles resulted in the complete disappearance of more than half of the tumors.

Iron carbon-based smaller radioactive particles of around 1 $\mu$m have been radiolabeled with different diagnostic ($^{99m}$Tc, $^{111}$In) and therapeutic ($^{188}$Re, $^{90}$Y) radioisotopes.[109,110] Targeting studies to distinct liver regions in swine by our group (unpublished results) showed that more than 90% of the injected radioactive magnetic particles were accumulated underneath a strong NdFeB-magnet. The radioactive particles stayed in the target region for at least 3 days, even after the removal of the magnet.

Magnetic nanoparticles of much smaller diameters are being used clinically for diagnostic purposes, mainly as contrast agents.[111] The accumulation of these magnetic particles is, however, based on non-specific properties such as the tissue-specific pore size (fenestration) or enhanced permeability and retention effect (EPR) seen in tumor tissues, but not on magnetic targeting. Recent examples of non-specific targeting are the internalization into cells of the positively charged at peptide bound to therapeutic agents, such as radioactively complexed $^{99m}$Tc and $^{188}$Re,[112] and superparamagnetic iron oxides known as tat-CLIO (tat-cross linked iron oxides).[113]

Magnetic drug delivery is also able to deliver other types of drugs such as highly potent antiinfective, blood clot-dissolving, anti-inflammatory, anti-arthritic, photodynamic therapy and paralysis-inducing drugs, among many others.[66] A good example of these applications was reported in 1988.[114] In this study, Torchilin *et al.* surgically induced a thrombus in both carotid arteries of a dog, fixed a permanent magnet near one of them, and 1 hr later, intravenously injected 1 $\mu$m-sized dextran microspheres with covalently bound streptokinase. The side without the magnet completely occluded within 4 hrs, while the magnet side returned to initial blood flow conditions after about 30 min and appeared completely open at histological examination. Torchilin noted that these results were achieved using doses 10 times lower than those used when streptokinase is directly injected. Similar results were obtained in the same year by another group using 30–60 nm PEGylated magnetite particles containing urokinase.[115] In both cases, the thrombolytic activity remained at background levels outside the targeted region.

## 4.4. *Magnetic gene transfer*

The newest application of magnetic nanoparticles is for targeted and enhanced gene delivery in potential applications such as wound repair[116] and the treatment of cancer,[117,118] eye disease,[119] and cystic fibrosis.[120] Magnetically enhanced gene transfer may be able to overcome the current lack of selectivity of the existing vectors and low efficiency of gene transfer. The mechanisms by which magnetic nanoparticles can improve on transfection rates are by magnetically forced contact[121,122] and by increasing the plasmid concentration magnetically.[123]

Magnetofection was first described in a Japanese patent by Harata *et al.* who used magnetic liposomes to transfect cells.[124] Bergemann *et al.* described the first experiments of transfecting cytokine-induced killer cells (CIK-cells) with plasmid DNA carrying distinct interleukin genes. However, their magnetic nanoparticles were only used as plasmid carriers, not as the driving force, which was provided by electroporation.[12] Using a magnet as the driving force was then described a couple of years later by Plank *et al.*[122,125] For successful *in vitro* and *in vivo* transfections,

only very small amounts of plasmid were necessary, and the transfection occurred in a matter of just a couple of minutes. This speedy and efficient transfection at low vector doses is the main advantage of magnetofection. Furthermore, remotely controlled vector targeting *in vivo* seems possible.

## 5. Conclusions

The technological advancements in the material and engineering sciences, and especially in the nanotechnology revolution, with its increasing molecular approach to the synthesis, derivatization, combination, self-assembly, and manipulation of materials, will guide improvements in all aspects of magnetic nano- and microparticles. These advancements include the synthesis of higher magnetic nanophases; the increasing availability of stronger magnets and engineered magnetic fields; the ability to prepare more uniform particles; and the rendering of these particles biocompatible and ultimately biodegradable without toxicity.

For real therapeutic breakthrough, however, a few challenges in the field of magnetic drug delivery still need to be addressed. One of the challenges is the difficulty involved in generating the focused field profiles needed to target magnetic nanoparticles deep within the body, due to the speed with which the magnetic fields drop off as their distance from the source increases (intensity = $1/r^3$). Another challenge centers on attaining homogeneous particle distributions given that blood flow in the target region can vary from very fast (100–200 cm/s) to very slow (0.05 cm/s). A final challenge is the optimization of magnetic nanoparticles in terms of magnetization and size uniformity. All these problematic areas are currently being addressed by multidisciplinary groups worldwide, as evident from a special issue of the *J Magn Magn Mater* **293**:1–736, containing 107 original peer-reviewed papers that were submitted at the 5th International Conference on the Scientific and Clinical Applications of Magnetic Carriers in 2004 (www.magneticmicrosphere.com).

The potential of magnetic drug delivery is great. In addition to their magnetic responsiveness, magnetic nanoparticles carry an innate signal that can be used for magnetic resonance imaging.[126] Furthermore, other imaging modalities such as radioisotope or fluorescence imaging can be used after derivatizing the particle surface. There is no limitation to the kind of drugs that can be encapsulated or bound to magnetic nanoparticles.[66] Also, current pharmaceutical techniques allow for the development of drug release profiles for a large group of drugs and diseases. Magnetic targeting devices such as the recently FDA approved Niobe system (Stereotaxis Inc., St. Louis, Missouri, U.S.A.)[127] improve on the precise manipulation of magnetic forces. Anatomical and physiological conditions in a patient are, however, complicated, and successful therapies will have to be specifically adapted to each disease and ideally applied under imaging control.

The treatment of cancer is an especially good target for magnetic drug delivery. However, any disease that could benefit from precise control over the delivery of highly effective, but potentially toxic substances would also be a good candidate. For example, antiangionic drugs could be delivered to the back of the eye to prevent blindness in patients with age-related macular degeneration. Attempts to develop such a drug delivery system are ongoing.[128,129]

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# 19

# DQAsomes as Mitochondria-Specific Drug and DNA Carriers

*Volkmar Weissig*

## 1. Introduction

DQAsomes (i.e. ***dequa***linium based lipo***some***-like vesicles; pronounced *dequasomes*) have been proposed in 1998 as the first mitochondria-specific colloidal drug and DNA delivery system.[1] These unique mitochondria-targeted drug carriers have been designed based on the intrinsic mitochondriotropism of amphiphilic cations with a delocalized charge center, i.e. on cations that accumulate at and inside mitochondria of living cells, in response to the mitochondrial membrane potential. Prerequisite for creating this system was the distinct self-assembly behavior of dicationic quinolinium derivatives, which are mitochondriotropic cations resembling "bola"-form electrolytes, i.e. they are symmetrical molecules with two charge centers separated by a hydrophobic chain at a relatively large distance. Such "bola"-form like amphiphiles form upon sonication of aqueous suspensions cationic vesicles ("bolasomes") are termed "DQAsomes" when prepared from dequalinium salts.[1]

The need for mitochondria-specific delivery systems arises from the central role mitochondria play in a multitude of metabolic pathways. Mitochondria are vital for the cell's energy metabolism and for the regulation of programmed cell death. In addition, mitochondria are critically involved in the modulation of intracellular calcium concentration and the mitochondrial respiratory chain is the major source of damaging reactive oxygen species. Consequently, mitochondrial dysfunction either

causes or at least contributes to a large number of human diseases. Malfunctioning mitochondria are found in several adult-onset diseases including diabetes, cardiomyopathy, infertility, migraine, blindness, deafness, kidney and liver diseases and stroke. The accumulation of somatic mutations in the mitochondrial genome has been suggested to be involved in aging, age-related neurodegenerative diseases, neuromuscular diseases, as well as in cancer. Consequently, mitochondria are increasingly recognized as a prime target for pharmacological intervention.[2–5]

The development of methods for selectively delivering biologically active molecules to the site of mitochondria, along with the identification of new mitochondrial molecular drug targets, will potentially launch new therapeutic approaches for the treatment of mitochondria-related diseases, based on either the selective protection, repair or eradication of cells.[6–9]

## 2. The Self Assembly Behavior of Bis Quinolinium Derivatives

### 2.1. *Monte Carlo computer simulations*

Dequalinium salts (Fig. 1A) are dicationic mitochondriotropic compounds resembling bola form electrolytes, i.e. they are symmetrical molecules with two charge centers separated at a relatively large distance. Such symmetric bola-like structures are known from archaeal lipids, which usually consist of two glycerol backbones connected by two hydrophobic chains.[10] The self-assembly behavior of bipolar lipids from Archaea has been extensively studied (reviewed by Gambacorta *et al.*[11]). It has been shown that these symmetric bipolar archaeal lipids can self-associate into mechanically very stable monolayer membranes.

The most striking structural difference between dequalinium and archaeal lipids lies in the number of bridging hydrophobic chains between the polar head groups. Contrary to the common arachaeal lipids, in the dequalinium molecule, there is only one alkyl chain that connects the two cationic hydrophilic head groups.

Monte Carlo simulations were applied to a system of bola form amphiphiles in a coarse-grained model, in which the amphiphilic molecules consist of connected segments with each segment of the chain representing several atoms of a real amphiphilic molecule.[12] All segments of the coarse grained model are therefore either head-like (hydrophilic) or tail-like (hydrophobic) as shown in Fig. 1B.

The formation of molecular aggregates was studied by employing a sequence of lattice simulations in an NVT ensemble (constant number of particles, $N$, constant volume, $V$, constant temperature, $T$), starting from an isotropic three-dimensional distribution of model molecules. The unoccupied lattice sites were considered water-like, i.e. hydrophilic. All computer simulations were done at reduced temperatures $T^* = k_BT/\varepsilon$ and interactions were modeled based on nearest neighbor repulsions $\varepsilon$ (in units of $k_BT$) between hydrophobic tail segments and hydrophilic

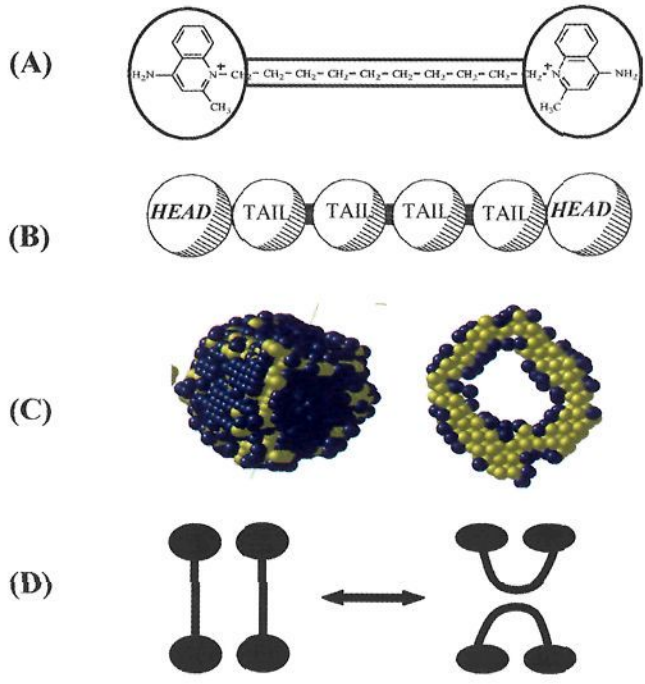


**Fig. 1.** (A) Chemical structure of dequalinium. (B) Dequalinium after coarse graining.[12] (C) Snapshot from Monte Carlo Computer Simulation: Left, whole vesicle left; Right, cross section.[12] (D) Possible conformation of single-chain bola amphiphiles: Left, stretched (bola); Right, bended (horse shoe).

heads. At $T^* = 0.925$ and at 10 vol% amphiphiles (i.e. 10% of all lattice sites were occupied with amphiphilic molecules), self-assembled vesicular structures could be found, as shown in the snapshot in Fig. 1C. Monte Carlo Simulations were also used to predict the conformational state of dequalinium within a self-assembled structure. While the stretched conformation (Fig. 1D, left) would give rise to the formation of a monolayer, assuming the horseshoe conformation (Fig. 1D, right), it would result in the formation of a bilayer. It was found that both conformations could theoretically co-exist, although the balance between them appeared to be temperature dependent.

## 2.2. *Physico-chemical characterization*

The self-assembly behavior, as predicted by Monte Carlo Computer Simulation, was confirmed by electron microscopy (Fig. 2) and photon correlation spectroscopy (Fig. 3).[1] It was found that dequalinium forms upon sonication spheric appearing aggregates with a diameter between approximately 70 and 700 nm. Freeze fracture images (Fig. 2, panel C) show both convex and concave fracture faces. These images

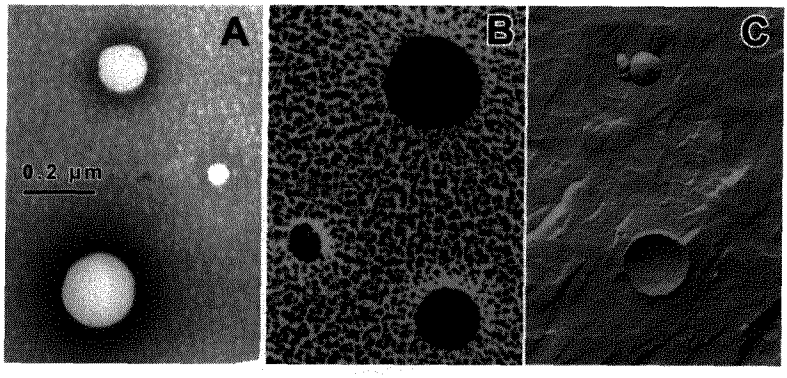


**Fig. 2.** Electron photomicrograph of DQAsomes. Panel A, negatively stained; Panel B, rotary shadowed; Panel C, freeze fractured.[1]

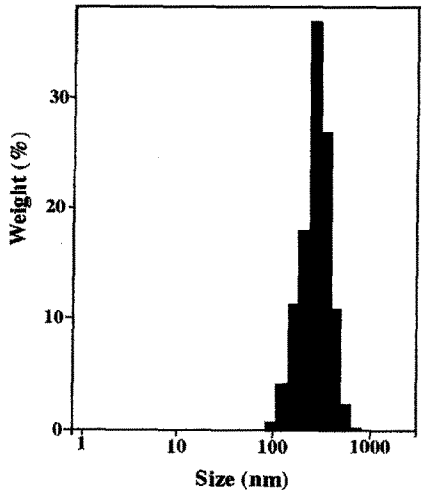


**Fig. 3.** Size distribution of DQAsomes.[13]

strongly indicate the liposome-like aggregation of dequalinium. Negatively stained samples (Fig. 2, panel A) demonstrate that the vesicle is impervious to the stain and appears as a clear area surrounded by stain with no substructure visible. Rotary shadowed vesicles (Fig. 2, panel B) appeared very electron dense without showing any substructure.

## 2.3. *Structure activity relationship studies*

To define relationships between the structure of dequalinium-like bola amphiphiles and their ability to form bolasomes, the self-assembly behavior of nine dequalinium derivatives with varying hydrophilic head groups and different hydrophobic tail segments was evaluated.[14] It was found that the methyl group in ortho position to

the quaternary nitrogen at the quinolinium ring system seems to play an essential role in the self-assembly behavior of these bola amphiphiles; this seems surprising, considering the bulky and hydrophobic nature of this group. While the removal of this methyl group significantly impairs the stability of formed vesicles, replacing the methyl group by an aliphatic ring system (Fig. 4) confers unexpected superior vesicle forming properties to this bola amphiphile. Vesicles made from this cyclohexyl derivative of dequalinium are contrasted with vesicles made from dequalinium, with a very narrow size distribution of $169 \pm 50$ nm which hardly changes at all, even after storage at room temperature for over 5 months. In contrast to vesicles made from dequalinium, bolasomes made from the cyclohexyl derivative are also stable upon dilution of the original vesicle preparation. While dequalinium-based bolasomes, slowly disintegrate over a period of several hours upon dilution, bolasomes made from the cyclohexyl compound do not show any change in size distribution following dilution. It appears that bulky aliphatic residues, attached to the quinolinium heterocycle, favor self-association of the planar ring system. It has therefore been speculated that the bulky group sterically prevents the free rotation of the hydrophilic head of the amphiphile around the $CH_2$ – axis (Fig. 5), thus contributing to improved intermolecular interactions between the amphiphilic monomers.[14]

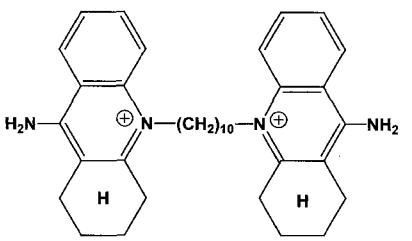


**Fig. 4.** Structure of the cyclohexyl derivative of dequalinium.

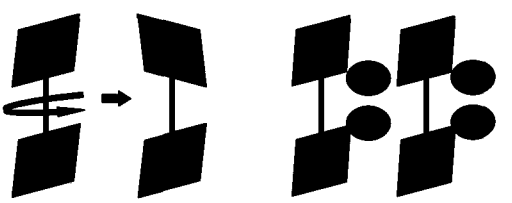

**Fig. 5.** Schematic illustration of the stabilizing effect of the cyclohexyl ring system (black circles).

## 3. DQAsomes as Mitochondrial Transfection Vector

The number of diseases found to be associated with defects of the mitochondrial genome has grown significantly since 1988. Despite major advances in understanding mtDNA, defects at the genetic and biochemical level, there is no satisfactory treatment available for a vast majority of patients. Objective limitations of conventional biochemical treatment, for patients with defects of mtDNA, warrant the exploration of gene therapeutic approaches. Two different strategies for mitochondrial gene therapy are imaginable.[15] The first involves expressing a wild-type copy of the defective gene in the nucleus, with cytoplasmic synthesis and subsequent targeting of the gene product to the mitochondria ("allotopic expression"). Besides the different codon usage in mitochondria, however, there are possibly four major difficulties in adapting this nuclear-cytosolic approach for mitochondrial gene therapy to mammalian cells, as recently reviewed by D'Souza.[16] Firstly, the majority of mtDNA defects involve tRNAs, and to date, no natural mechanism has been reported for the mitochondrial uptake of cytosolic tRNAs in mammalian cells. Secondly, it is generally agreed that the 13 proteins encoded for by mtDNA are very hydrophobic peptides, which would not be readily imported by the mitochondrial protein import machinery. However, since the 13 mitochondrial coded proteins are not equally hydrophobic, the allotopic expression of at least some of the peptides appears as possible.[17] Thirdly, it has been hypothesized that some of the proteins encoded by the mitochondrion may potentially be toxic if synthesized in the cytosol.[18] Fourthly, according to a hypothesis termed co-location for redox regulation[19], the co-location of mtDNA and its products may be essential for the rapid control of gene expression by the redox state in the mitochondrial matrix. Considering all the problems associated with the nuclear-cytosolic approach, the development of methods for the direct transfection of mitochondria as an alternative approach towards mitochondrial gene therapy is warranted.

Based on the intrinsic mitochondriotropism of dequalinium salts and the ability of dequalinium-based vesicles, i.e. DQAsomes, to bind and condense pDNA, a strategy for the direct transfection of mitochondria within living mammalian cells has been proposed.[20,21] This new strategy involves the transport of a DNA-mitochondrial leader sequence peptide conjugate to mitochondria using cationic mitochondriotropic vesicles (DQAsomes), the liberation of this conjugate from the cationic vector upon contact with the mitochondrial outer membrane, followed by DNA uptake via the mitochondrial protein import machinery. In a series of papers,[22–27] it was then shown that DQAsomes indeed fulfill all pre-requisites for

a mitochondria-specific DNA delivery system:

- DQAsomes bind pDNA forming so-called DQAplexes and protect the DNA from nuclease digestion.
- The cytotoxicity of DQAsomes and of DQAsome/pDNA complexes is comparable to non-viral transfection vectors, which are already being used in clinical trials.
- DQAsomes mediate the cellular uptake of bound pDNA, most probably via non-specific endocytosis.
- DQAsomes are endosomolytically active, thereby presumably contributing to an early endosomal release of the DQAsome/pDNA complex.
- DQAplexes do not release pDNA upon contact with anionic phospholipids from the inner cytoplasmic membrane.
- DQAplexes release pDNA upon contact with mitochondria-like membranes, as well as upon contact with whole isolated mitochondria.
- Tested under identical experimental conditions, DQAsomes were shown to transport pDNA as well as oligonucleotides to the site of mitochondria, while lipofectin was demonstrated to deliver pDNA and oligonucleotides towards the nucleus.
- Plasmid DNA dissociates from DQAplexes upon contact with mitochondria within living mammalian cells.

Perhaps the most surprising finding among the above listed results is the selective DNA release from DQAplexes upon contact with different membranes. Why do anionic phospholipids such as phosphatidylserine displace pDNA from lipofectin (as shown by Xu and Szoka[28]), but not from DQAplexes, and why do DQAplexes in turn become destabilized upon contact with mitochondrial membranes? When looking at data obtained from studies with living mammalian cells,[23] it appears reasonable to assume that dequalinium molecules could be pulled into the mitochondrial matrix in response to the high mitochondrial membrane potential (as demonstrated in 1987),[29] which in turn might lead to the destabilization of the DQAsome/pDNA complex. However, the first detailed study, which demonstrated the selective DNA release from DQAplexes, was performed using membrane-mimicking liposomes (Fig. 6). As a model for the intracellular release of DNA from DQAsomes, the capacity of anionic liposomes to displace the DNA from its cationic carrier was studied. The association of DNA with the cationic carrier was assessed by employing SYBR™ Green I. The fluorescence signal of this dye is greatly enhanced when bound to DNA. Non-binding results in loss of fluorescence. It can be clearly seen that in the vicinity of a 1/1 charge ratio, DQAsomes do not release any DNA in the presence of cytoplasmic membrane mimicking liposomes

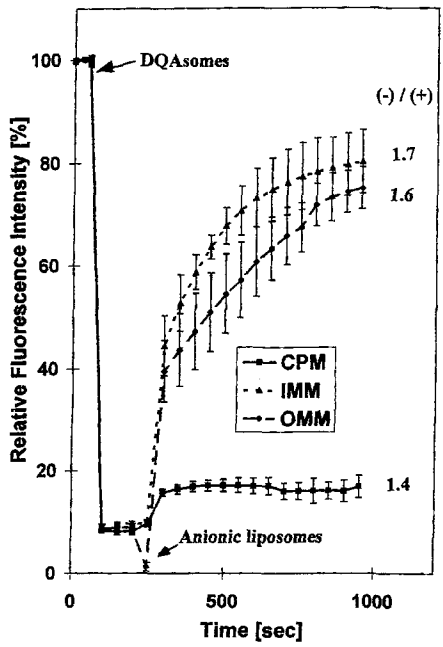


**Fig. 6.** Effect of anionic liposomes on DNA release from DQAsome/pDNA complexes. DNA was preincubated with SYBR until stabilization of the signal, followed by adding (indicated by arrow) the minimal amount of DQAsomes necessary to decrease the signal to background level. Anionic liposomes were then injected (arrow) at an anionic to cationic charge ratio (−)/(+) as shown. The displacement of DNA from its carrier is indicated by the increase of the fluorescence signal. CPM, cytoplasma membrane like liposomes; IMM, inner mitochondrial membrane like liposomes; OMM, outer mitochondrial membrane like liposomes.[22]

(CPM), not even at a 1.4 fold excess of anionic charge. However, with a similar charge excess of anionic liposomes to cationic DQAsomes, 1.6 and 1.7 respectively, inner and outer mitochondrial membrane mimicking liposomes (IMM and OMM, respectively) are able to displace up to 75% of the DNA from its DQAsomal carrier. In agreement with these data, it was found that for the complete liberation of DNA from DNA/DQAsome complex, a fourfold excess of dicetylphosphate and an eightfold excess of phosphatidylserine, respectively, are necessary.

The finding that CPM liposomes, at an anionic to cationic charge ratio of 0.82, displace up to 75% of the DNA from lipofectin, which was used as a control, do not liberate any DNA from DQAsomes even at a slight excess of anionic charge, leads to the conclusion that besides the charge ratio, other factors may play an important role in the mechanism of DNA release from lipid/DNA complexes. This conclusion is being further supported by Xu and Szoka's observation[28] that ionic water soluble molecules such as ATP, tRNA, DNA, poly(glutamic acid), spermidine and histone

do not displace DNA from the cationic lipid/DNA complex, even at a 100-fold charge excess (−/+). In their model for the post-endocytotic release of DNA from cationic carriers, they assume the formation of a charge neutral ion pair between cationic and anionic lipid, which ultimately results in the displacement of the DNA from the cationic lipid and the release of DNA into cytoplasm:

$$\text{liposome}^{(+)}/\text{DNA}^{(-)} + \text{liposome}^{(-)} \Leftrightarrow [\text{lipid}^{(+)}/\text{lipid}^{(-)}]\text{liposome} + \text{DNA}^{(-)}$$

According to this equation,[22] it seems obvious that an additional gain of free energy is obtained by hydrophobic interactions between anionic and cationic lipids, i.e. formation of charge neutral liposomes. Considering that there is no difference in the net charge between both sides of the equation, the mixed liposome formation should be the only driving force leading to DNA release from its lipidic carrier. Intriguingly, it was found earlier[13] that in physiological solutions, it is not possible to incorporate dequalinium into liposomes made of lecithin and lecithin/phosphatidylserine respectively. This indicates a very restricted ability of dequalinium to mix with phospholipids, which would cause the (assumed) equilibrium in the above equation to be on the left side. It was therefore concluded that the miscibility between the cationic lipid and the anionic agent used (by nature or by man) to displace the DNA is of significant importance.[22]

The general feasibility of the DQAsome-based strategy for transfecting mitochondria within living mammalian cells, involving pDNA-MLS peptide conjugates, has most recently been demonstrated utilizing confocal fluorescence microscopy.[30] It should be noted that the use of physico-chemical methods is, by far, still the only way to demonstrate the import of transgene DNA into the mitochondrial matrix in living mammalian cells. The complete lack of a mitochondria-specific reporter plasmid designed for mitochondrial expression, severely hampers all current efforts towards the development of effective mitochondrial expression vectors. While any new non-viral transfection system (i.e. cationic lipids, polymers and others) aimed at the nuclear-cytosolic expression of proteins can be systematically tested and subsequently improved by utilizing any of the many commercially available reporter gene systems, such a methodical approach to develop mitochondrial transfection systems is currently impossible. A series of papers by Charles Coutelle's laboratory describe the principal approach for the design of a mitochondria-specific reporter systems.[31–33] However, no such system has yet become commercially available. It should also be noted that the functional expression of Coutelle's mitochondria-specific expression systems inside the mitochondrial matrix has not been demonstrated yet. Thus, evaluating the effectiveness of mitochondria-specific systems in delivering DNA into mitochondria depends largely on the physical tracking of DNA.

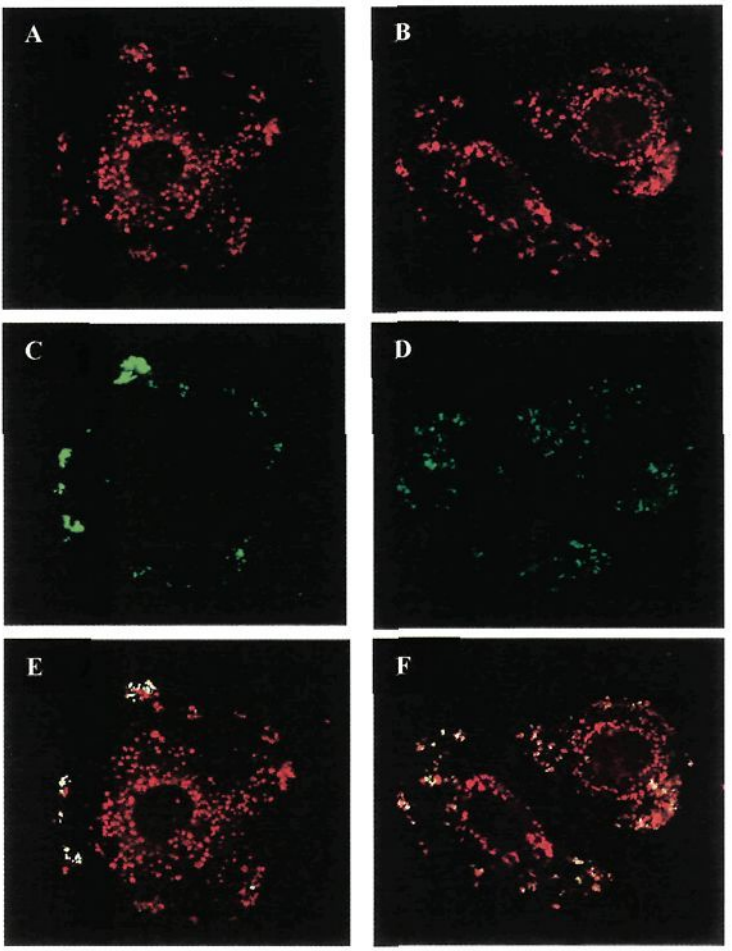


**Fig. 7.** Confocal fluorescence images of BT20 cells stained with mitotracker (red) after exposure for 10 hrs to DNA( green) complexed with C-DQAsomes. Left column: circular MLSpDNA conjugate, right column: linearized MLS-pDNA conjugate. Top row (A and B): red channel, middle row (C and D): green channel, bottom row (E and F): corresponding overlaid images.[30]

Figure 7 shows confocal fluorescence micrographs of cells incubated with MLS-pDNA conjugates, which were vectorized with vesicles made from the cyclohexyl derivative of dequalinium (C-DQAsomes). For the cell exposures imaged in the left column (panels A, C and E) the non-restricted, i.e. circular form of pDNA was used, while for the experiments pictured in the right column (panels B, D and F), the plasmid DNA was linearized before DQAplex formation. The characteristic red mitochondrial staining pattern (panels A and B) shows the functional viability of the imaged cells and the intracellular green fluorescence (panels C and D) demonstrates efficient cell internalization of the fluorescein labeled DNA. The green and

red fluorescence channels were then overlaid to produce the composite image seen in panels E and F, where the regions of true co-localization of red and green fluorescence were pseudo-colored in white for better visualization. Strikingly, in the overlaid images, there is hardly any green fluorescence detectable. Nearly all areas of green fluorescence in panels C and D appeared as white areas in panels E and F, strongly suggesting that almost the entire DNA has been delivered not only towards mitochondria, but also into the organelle. However, whether all or at least a portion of the pDNA has actually entered the mitochondrial matrix, i.e. has crossed both mitochondrial membranes, and therefore would potentially be accessible to the mitochondrial transcription machinery, remains yet to be determined.

## 4. DQAsomes as Carriers of Pro-apoptotic Drugs

Dysregulation of the apoptotic machinery is generally accepted as an almost universal component of the transformation process of normal cells into cancer cells and a large body of experimental data demonstrates that mitochondria play a key role in the complex apoptotic mechanism. Consequently, any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells is believed to have potential therapeutic effect.[34,35] Several clinically approved drugs such as VP-16 (etoposide), arsenite and vinorelbine, as well as an increasing number of experimental anticancer drugs (reviewed by Constantini *et al.*[36]), such as betulinic acid, lonidamine, ceramide and CD437 have been found to act directly on mitochondria, resulting in triggering apoptosis. In order to maximize the therapeutic potential of such anticancer drugs, which are known to act at or inside mitochondria, the use of DQAsomes as a mitochondria-specific drug delivery system has been proposed.[37]

Hypothetically, DQAsome-based anticancer chemotherapy entails features which would make it putatively superior to conventional chemotherapeutic approaches on the cellular, as well as the subcellular level:

Firstly, the delivery of drugs known to act directly on mitochondria may trigger apoptosis in circumstances in which conventional drugs fail to act, because endogenous, "upstream of mitochondria" apoptosis induction pathways are disrupted.[36]

Secondly, transporting the cytotoxic drug to its intracellular target could overcome multi-drug resistance by hiding the drug inside the delivery system until it becomes selectively released at the particular intracellular site of action, i.e. mitochondria.

Thirdly, many carcinoma cells, including human breast adenocarcinoma-derived cells, have an elevated plasma membrane potential relative to their normal parent cell lines in addition to the higher mitochondrial membrane potential.[29,39–43] They could provide the basis for a double-targeting effect of DQAsomes, i.e. on the cellular level (normal cells vs. carcinoma cells), and on the sub-cellular level (mitochondria versus nucleus).

First data involving the encapsulation of anticancer drugs into DQAsomes have been published most recently.[38] In this study, paclitaxel was chosen as a model compound. Paclitaxel is known as a potent antitubulin agent used in the treatment of malignancies.[44] Its therapeutic potential, however, is limited due to a very narrow span between the maximal tolerated dose and intolerable toxic levels. In addition, its poor aqueous solubility requires the formulation of emulsions containing Cremophor EL®, an oil of considerable toxicity by itself.[45] Recently, it has been demonstrated that clinically relevant concentrations of paclitaxel target mitochondria directly and trigger apoptosis by inducing cytochrome c release in a permeability transition pore (PTP)–dependent manner.[46] This mechanism of action is known from the other pro-apoptotic, directly on mitochondria acting agents.[47] A 24-hour delay between the treatment with paclitaxel[46] or with other PTP inducers,[47,48] and the release of cytochrome c in cell-free systems, compared with intact cells, has been explained by the existence of several drug targets inside the cell, making only a subset of the drug available for mitochondria.[46] Consequently, paclitaxel was considered a prime candidate to benefit from a mitochondria-specific drug delivery system such as DQAsomes. It was demonstrated that paclitaxel can be incorporated into DQAsomes at a stoichiometric molar ratio of 1 paclitaxel to 2 dequalinium. Considering the known spherical character of DQAsomes, the results of an electron microscopic (EM) analysis of dequasomal incorporated paclitaxel, however, seem rather surprising. The transmission EM image (Fig. 8, left panel) and the cryo-EM image (Fig. 9) of an identical sample show a remarkable conformity worm- or rod-like structures approximately 400 nm in length, the size of which could also be confirmed by the size distribution analysis shown in Fig. 8, right panel. The molecular structureof this worm-like complex remains to be determined; nevertheless,

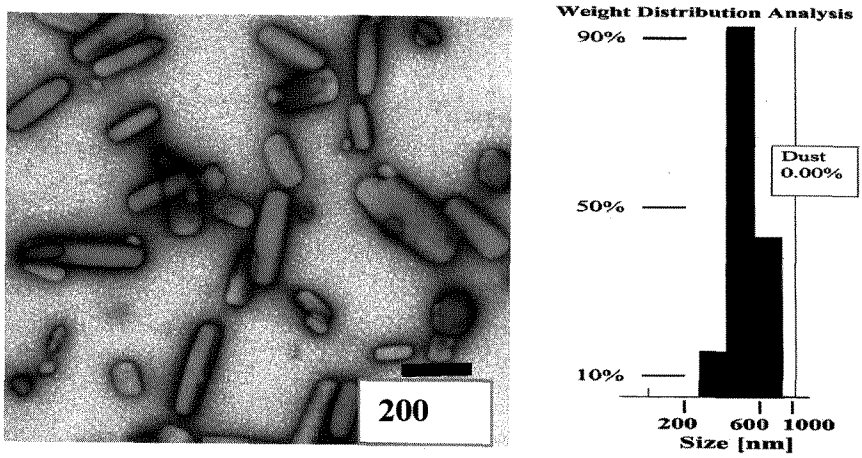


**Fig. 8.** Left panel: Transmission electron microscopic image (uranyl acetate staining) of DQAsomal incorporated paclitaxel (0.67 mol taxol/mol dequalinium); Right panel: Size distribution analysis of identical preparation shown in left panel.[38]

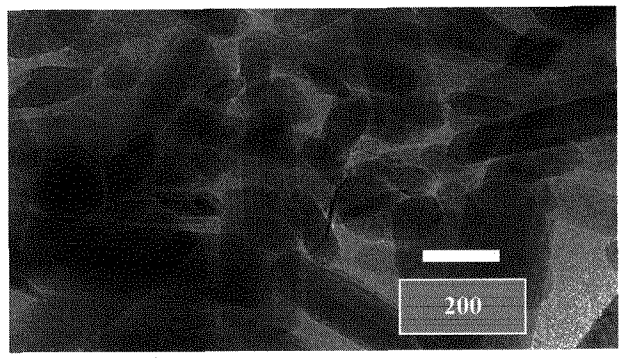


**Fig. 9.** Cryo-electron microscopic image of DQAsomal incorporated paclitaxel (0.67 mol dequalinium/mol paclitaxel).[38]

the formation of worm-like micelles as described for self-assembling amphiphilic block co-polymers[49] appears possible.

In a preliminary study, paclitaxel-loaded DQAsomes were tested for their ability to inhibit the growth of human colon cancer cells in nude mice.[38] For controls with free paclitaxel, the drug was suspended in 100% DMSO at 20 mM, stored at 4°C and immediately diluted in warm medium before use. In all controls, the respective dose of free paclitaxel and empty DQAsomes was adjusted according to the dose of paclitaxel and dequalinium given in the paclitaxel-loaded DQAsome sample. Due to the lack of any inhibitory effect on tumor growth, the dose was tripled after 1.5 weeks. Figure 10 shows that at concentrations where free paclitaxel and

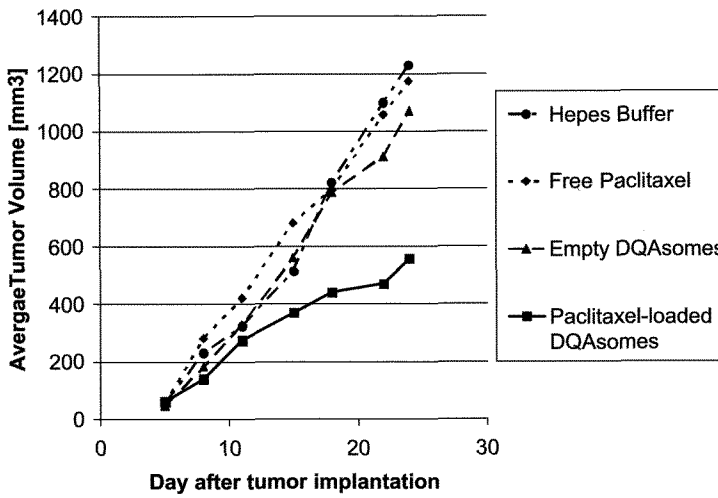


**Fig. 10.** Tumor growth inhibition study in nude mice implanted with human colon cancer cells. The mean tumor volume from each group was blotted against the number of days. Each group involved 8 animals. For clarity, error bars were omitted. Note that after 1.5 weeks the dose, normalized for paclitaxel, was tripled in all treatment groups.[38]

empty DQAsomes do not show any impact on tumor growth, paclitaxel-loaded DQAsomes (with paclitaxel and dequalinium concentrations identical to controls) seem to inhibit the tumor growth by about 50%. Correspondingly, the average tumor weight in the treatment group, after sacrificing the animals 26 days, later was approximately half of that in all controls.

Although this result seems to suggest that DQAsomes might indeed be able to increase the therapeutic potential of paclitaxel, the preliminary character of this first *in vivo* study has to be emphasized. Experiments to optimize the treatment protocol are ongoing in the author's laboratory.

## 5. Summary

Since their initial description in 1998, DQAsomes and DQAsome-like vesicles have been established as the first mitochondria-targeted colloidal delivery system, capable of transporting plasmid DNA as well as small drug molecules towards mitochondria within living mammalian cells. The further exploration of this unique mitochondriotropic delivery system will introduce new ways for the treatment of cancer and for the therapy of a multitude of mitochondrial diseases.

## Acknowledgments

I am grateful to Prof. V. P. Torchilin for many helpful discussions and for his strong and continuous support of my work. I also would like to sincerely thank my graduate students, Gerard D'Souza, Shing-Ming Cheng, Sarathi Boddapati and Eyad Katrangi, whose experimental work has made the writing of this chapter possible. I am obliged to the Muscular Dystrophy Association (Tucson, AZ), the United Mitochondrial Disease Foundation (Pittsburgh, PA), MitoVec, Inc. (Boston, MA) and Northeastern University (Boston, MA) for the financial support I received from these organizations during the last four years.

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# 20

# Liposomal Drug Carriers in Cancer Therapy

*Alberto A. Gabizon*

## 1. Introduction

In the last two decades, we have witnessed the development of implantable and injectable drug delivery systems for applications in the treatment of cancer and other diseases. These systems have arisen from various needs:

1. To provide depot forms of drug administration and more convenient dosing schedules. Examples are implantable biodegradable rods for slow release of peptides such as LH-RH partial agonists (e.g. goserelin depot), for blockade of gonadal production of androgens or estrogens.[1] This is a simple and pharmacologically effective approach developed for an implantable drug delivery system.
2. To provide for convenient vehicles of administration for poorly soluble drugs. These systems may or may not confer an advantage to the therapeutic index of the drug, but their basic "raison d'être" is to provide a vehicle of injection. An example is paclitaxel entrapped in polymerized albumin nanoparticles[2] for i.v. administration of paclitaxel in cremophor-free form. These should be distinguished from simple excipients used as solubilizers (e.g. cremophor in the case of paclitaxel), because, in the former, the drug and vehicle are physically in one single complex at least during the initial phase in circulation.
3. To improve the efficacy and reduce the side effects of new and old anticancer drugs. Examples include formulations of anthracyclines encapsulated in liposomes (e.g. Doxil, Myocet, Daunoxome) or conjugated to polymers.[3]

The objective here is to change the pharmacokinetics, biodistribution, and the bioavailability profile so as to achieve a positive impact on the drug pharmacodynamics. This is the most refined approach to drug delivery and includes intravenous administration of a drug stably associated to a carrier, with or without specificity to a target cancer cell molecule.

In this review chapter, we will focus on injectable particulate drug delivery systems of anticancer drugs (Fig. 1), particularly on liposomes, the most widely used drug nanocarrier in cancer. The physico-chemical properties of liposomes are discussed elsewhere in this book. Briefly, liposomes are vesicles with an aqueous interior surrounded by one or more concentric bilayers of phospholipids with a diameter ranging from a minimal diameter of ~30 nm to several microns. However, for injectable clinical applications, practically all liposome formulations are in the submicron ultrafilterable range (<200 nm size) and can be considered as nanosize particulate systems. Liposomes are formed spontaneously when amphiphilic lipids such as phospholipids are dispersed in water. The ensuing structures are physically stable supramolecular assemblies, and unlike polymerized particles,

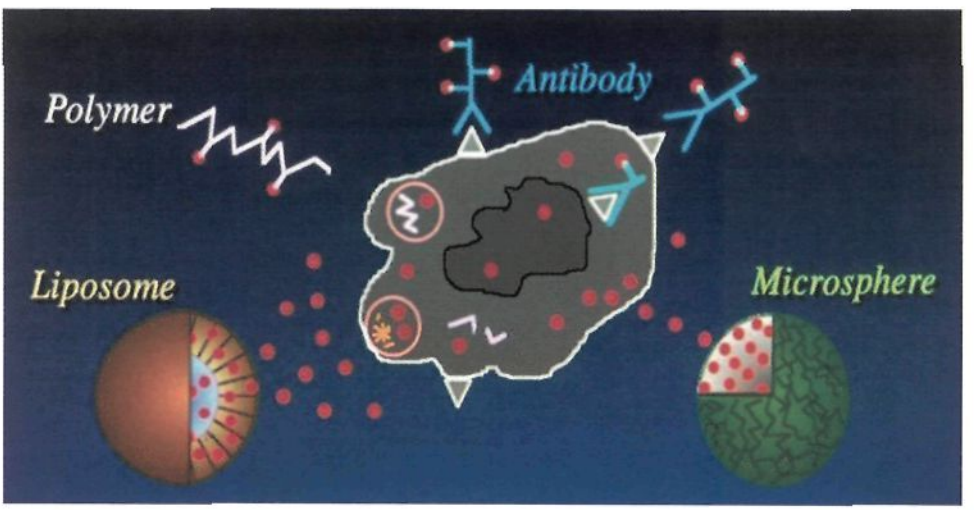


**Fig. 1.** Cancer therapy and drug delivery systems — Schematic drawing illustrating various approaches to smart cancer drug delivery. (1) Targeting of drugs conjugated to antibodies or ligands directed to tumor cell-specific surface receptors. (2) Controlled release of drugs entrapped in microspheres or nanospheres by diffusion and/or degradation of the particle matrix in extracellular fluid. (3) Release of drugs entrapped in phospholipids vesicles (liposomes) by leakage and/or endocytosis and liposome breakdown. (4) Delivery of drugs conjugated to polymers by endocytosis and intracellular drug release. These approaches may also be combined, for instance, in the case of liposomes targeted with ligands or antibodies to tumor cells. [*Note: relative scales are disproportional.*]

they are not covalently bound. Although liposome formation is actually a spontaneous process, the current trend is to classify them into a class of pharmaceutical devices in the nanoscale range engineered by physical and/or chemical means, and referred to as nanomedicines.[4] Nanomedicines are a direct result of the application of nanotechnology to medicine, and encompass in their wide context, molecular and supramolecular devices such as liposomes and other nanoparticulate carriers. In fact, liposomes are the first generation of nanosize drug delivery devices approved for the treatment of cancer (i.e. Doxil containing doxorubicin) and fungal infections (i.e. Ambisome containing amphotericin B). Current liposome formulations represent a basic form of nanomedicine involving a slow drug release system, and often a passive targeting process known as enhanced permeability and retention (EPR) that will be discussed later in this chapter. The field of nanomedicines is rapidly evolving and aims at increased sophistication of nanosize devices interacting with cellular targets at the nanoscale level.

## 2. The Challenge of Cancer Therapy

Our understanding of the molecular processes underlying the pathologic behavior of cancer cells has progressed enormously in the last decade.[5] Of particular relevance to cancer targeting is the fact that a number of receptors, mostly growth factor receptors, have been found to be overexpressed in tumor cells, and to play an important role as catalysts of growth. Receptor profiling of tumors may offer a potential Achilles heel for targeting specific ligands or antibodies, with or without delivery of a cytotoxic drug cargo.[6] In addition, the pathophysiology of tumor neovasculature and the interaction of tumor with stroma have been recognized as processes that play a major role in tumor development. Cancer is ultimately a disease caused by somatic gene mutations that result in the transformation of a normal cell into a malignant tumor cell. Eventually, the tumor cell phenotype progresses along three major steps[7]:

1. Increased proliferation rate and/or decreased apoptosis, causing an increase of tumor cell mass.
2. Invasion of surrounding tissues and switch on of angiogenesis. This is a critical step that differentiates *in situ*, non-invasive, tumors with no metastatic potential from invasive tumors with metastatic and life-threatening potential. Although there is considerable variability, tumors with angiogenic potential become vascularized when the cell load reaches an order of $10^7$ cells, equivalent to a nodule of ~2 mm diameter.
3. Metastases, i.e. abnormal migration of tumor cells from the primary tumor site via blood vessels or lymphatics to distant organs, with formation of secondary tumors. This is most commonly the process that causes death of the host due

to disruption of the function of vital organs or systems (i.e. brain, lung, liver, kidney, bone marrow, coagulation, intestinal passage, and others).

Despite formidable advances in clinical imaging, the diagnosis of a tumor mass[1] usually requires the presence of a nodule of ~10 mm diameter, representing a cluster of $10^9$ cells.[2] Since the lethal tumor burden is in the order of $10^{12}$ cells in most cancer patients, this implies that tumors have already gone through 75% of their doubling cell expansion process by the time of clinical diagnosis. As a result, significant heterogeneity and phenotypic diversity are already present in most diagnosed cancers, posing a major therapeutic challenge due to the development of metastatic ability and drug resistance.

Nowadays, drug-based therapy of cancer is applied in three possible settings:

- Primary treatment, which is also known as neo-adjuvant or pre-operative treatment. In this setting, anti tumor drugs are given prior to potentially curative local therapeutic modalities such as surgery or radiotherapy. These patients have a primary tumor, but no clinical evidence of distant metastases. Concomitant treatments of chemotherapy, or hormonal therapy, with radiotherapy can also be included in this category. The goal is to reduce tumor bulk, the risk of tumor seeding, and to facilitate surgery or radiotherapy of the primary tumor.

- *Adjuvant treatment.* The aim is to eradicate clinically undetectable residual tumor cells, presumably left over after surgical removal of the primary tumor. Adjuvant treatment is generally applied in patients with a high risk of micrometastases. To some extent, the adjuvant approach likens to a black box because all patients at high risk are treated without knowing for sure who are the patients harboring metastases and who are not. Also, we have no immediate way of knowing whether the treatment is effective or not. Only long follow-up periods will reveal if cancer will recur in a specific patient. Therefore, the proof of efficacy of adjuvant treatment is exclusively statistical. Despite these limitations, it has been demonstrated statistically that adjuvant treatment can cure subclinical, micrometastatic disease in a fraction of patients with breast cancer, colon cancer, and other tumors, who would not be amenable to cure if the disease is to become macroscopic and clinically detectable prior to treatment. The evaluation of adjuvant treatment effects is complicated by the poorly understood phenomenon of tumor dormancy in which tumor cells appear to remain as tiny, quiescent avascular clusters for

[1] This does not apply to superficial skin tumors which can be recognized sometimes when tumors contain cell clusters of $10^7$ reaching ~2–3 mm diameter.

[2] Occasionally modern imaging techniques (high resolution CT scan, MRI) can detect smaller (3–5 mm) findings with suspected cancer features in asymptomatic individuals. However, it is reasonable to assume that non-imaging techniques, for ex. proteomics based, will be needed to safely break through the $10^{8-9}$ cancer cell mass diagnostic threshold.

long periods of time. Small, microscopic, tumor cell clusters may get their nutrients by diffusion from pre-existing adjacent vessels of normal tissues. Therefore, adjuvant therapies specifically directed to tumor vasculature are unlikely to be effective against some micrometastases during the avascular phase.

- Treatment of metastatic disease or neoplastic conditions not amenable to surgical or radiotherapeutic eradication. In these cases, chemotherapy is potentially curative only in hematological and lymphoid neoplasms, and in a few cases of solid tumors such as testicular cancer and choriocarcinoma. In most instances, including the most common types of cancer namely breast, prostate, lung, and colon, chemotherapy is palliative, i.e. temporary tumor regression and prolongation of survival can be achieved, but cure is exceptional and most tumors ultimately recur and are lethal.

Let us now examine the currently available cancer drug armamentarium. Drug therapies of cancer can be divided into three major groups:

1. *Cytotoxic agents.* As the name implies, these agents are toxic to cells and lack tumor cell specificity. They can be divided into three major groups:

- Agents that damage the DNA template directly or indirectly.
- Agents that damage the microtubule-based spindle apparatus.
- Agents that inhibit DNA synthesis (antimetabolites).

Upon structural damage or arrest of the cell cycle, tumor cells undergo apoptosis which is the main form of cell death. Treatment with cytotoxic agents is usually referred to as cancer chemotherapy. The use of cytotoxic agents remains the mainstay of cancer therapy. It is this group of agents that urgently requires a delivery system to improve its tumor specificity, and/or reduce its damage to normal tissues. In addition to the lack of specificity of chemotherapeutic (cytotoxic) agents, a number of physiologic factors can seriously limit the efficiency of drug distribution from plasma to tumors and neutralize their effects. These include competition for drug uptake of well-perfused tissues such as liver and kidneys, rapid glomerular filtration and urinary excretion of low molecular weight drugs, protein binding with drug inactivation (e.g. cisplatin), and stability problems in biological fluids (e.g. hydrolysis of nitrosoureas, opening of lactone ring of camptothecin analogs).

2. *Hormonal agents.* They are used mainly against breast and prostate cancers. These tumors often require estrogen or androgen receptor activation for growth stimulation. The hormonal therapies currently in use are mostly based on synthetic compounds modeled to block the gonadal or peripheral production of estrogens and androgens (i.e. LH-RH partial agonists, aromatase inhibitors) or to compete for the tumor cell receptors of these hormones (i.e. anti-estrogens, anti-androgens).

Corticosteroids and somatostatin analogs can also be included in the category of hormonal agents.

3. *Non-cytotoxic agents modifying biological response.* These can be classified in at least three distinct groups:

- Antibodies blocking growth factors, growth factor receptors and other cell-membrane receptors of tumor cells or supporting stroma (e.g. bevacizumab, trastuzumab, cetuximab, rituximab).
- Agents blocking signal transduction kinases (e.g. gefitinib, imatinib).
- Cytokines with miscellaneous activities (e.g. interferon-$\alpha$, interleukin-2).

## 3. The Rationale for the Use of Liposomal Drug Carriers in Cancer

The rationale for the use of liposomes in cancer drug delivery is based on the following pharmacological principles,[8] which are also applicable to non-liposomal nanoparticulate drug carriers:

1. *Slow drug release.* Drug bioavailability depends on drug release from liposomes. Entrapment of drug in liposomes will slow down drug release and reduce renal clearance to a variable extent. Slow release may range from a mere blunting of the peak plasma levels of free drug, to a sustained release of drug mimicking continuous infusion. These pharmacokinetic changes may have important pharmacodynamic consequences with regard to toxicity and efficacy of the liposome delivered agents.

2. *Site avoidance of specific tissues.* The biodistribution pattern of liposomes may lead to a relative reduction of drug concentration in tissues specifically sensitive to the delivered drug. This may have implications with regard to the therapeutic window of various cytotoxic drugs, such as the cardiotoxic anthracyclines, provided that anti tumor efficacy is not negatively affected.

3. *Accumulation in tumors.* Prolongation of the circulation time of liposomes results in significant accumulation in tissues with increased vascular permeability. This is often the case of tumors,[9] especially in those areas with active neoangiogenesis. Tumor localization of long-circulating liposomes, such as pegylated liposomes, sometimes referred to as Stealth or sterically-stabilized,[10] is a passive targeting effect that enables substantial accumulation of liposome-encapsulated drug in the interstitial fluid at the tumor site,[11] a phenomenon sometimes referred to as enhanced permeability and retention (E.P.R.) effect (Fig. 2).

There are a number of differential effects of physiologic factors on clearance and biodistribution of low molecular weight drugs and nanoparticles (see also Table 1):

- *Protein binding.* Low molecular weight drugs may be inactivated and/or irreversibly bound by plasma proteins, thus reducing the bioavailability towards

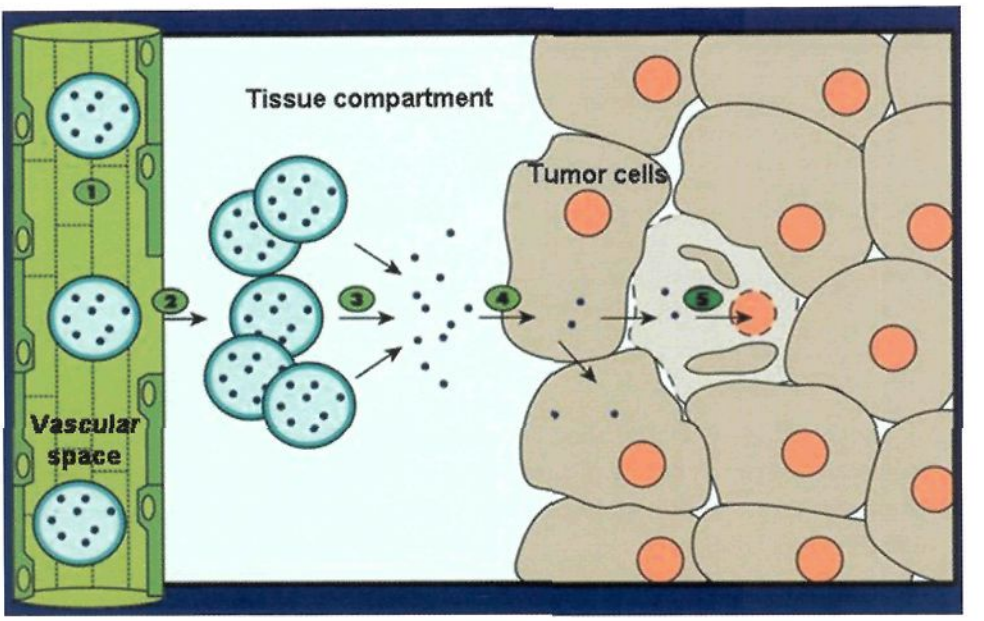


**Fig. 2.** Extravasation and release of liposomal drug cargo in tumor interstitial fluid compartment — Schematic drawing illustrating the concept of passive targeting of liposomes to tumors exploiting the EPR effect. The dots represent the drug molecules encapsulated in the liposome water phase. The various steps implied in the targeting process are numerically designated from 1 to 5. (1) Liposomes with long-circulating properties are required to increase the number of passages through the tumor microvasculature. (2) Increased vascular permeability in tumor tissue enables properly downsized liposomes to extravasate and reach the tumor interstitial fluid. (3) Because of their limited diffusion capacity, liposomes remain in close vicinity to blood vessels. (4) Drug is gradually released from liposomes accumulating in the interstitial fluid moving swiftly through the tumor cell layers and entering tumor cells. (5) The cytotoxic effect leading to tumor cell death is expected to follow the same mechanism known for free drug. [*Note: relative scales are disproportional.*]

cellular target molecules. Cisplatin, a widely used anticancer cytotoxic drug, is one such example. In the case of nanoparticles, plasma proteins can adsorb to their surface a process known as opsonization that results in tagging the particle for recognition and removing it by macrophages. In addition, protein binding to the liposome surface may de-stabilize the bilayer and accelerate the leakage of liposome contents. PEG coating (pegylation) of liposomes reduces opsonization and the effects associated with it.

- *Reticulo-endothelial system (RES) clearance.* It is unimportant for low molecular weight drugs, but plays a major role in the clearance of nanoparticles reducing the fraction available for distribution to tumor tissue. Kupffer cell macrophages lining the liver sinusoids remove opsonized liposomes and other nanoparticles from circulation, and represent a major factor in the clearance of particulate carriers.

**Table 1** Differential effects of physiologic factors on clearance and biodistribution of low molecular weight (MW) drugs and nanoparticles.

| Factor | Extravascular transport | Microvascular permeability (fenestrations) | Glomerular filtration | Protein binding | R.E.S. clearance |
|---|---|---|---|---|---|
| Low MW drugs | Diffusion | Not important | Filterable | Binding and inactivation | Unimportant |
| Nanoparticles | Convection | Critical for tissue targeting | Non-filterable | Opsonization and de-stabilization | Major clearance pathway |

- *Glomerular filtration.* Unless they become protein-bound, low molecular weight drugs can be filtered out by kidney glomeruli. In contrast, liposomes and other nanoparticles are non-filterable, because their diameter exceeds the glomerular filterable threshold size.

- *Microvascular permeability.* Enhanced microvascular permeability with fenestrations in capillaries and post-capillary venules is critical for the extravasation of nanoparticles from the blood stream to the interstitial fluid of the target tissues. The presence of fenestrations is irrelevant for tissue delivery of small molecules.

- *Extravascular transport.* Diffusion is the predominant mechanism of transport for small molecules. In contrast, convective transport plays a major role in the extravascular movement of nanoparticles, for which diffusion rates are very slow.[12] Large tumors tend to develop high interstitial pressure that reduces the rate of convective transport significantly. In fact, in an animal model, it has been shown that liposomes accumulate significantly less in larger tumors on a per gram tissue basis.[13] In agreement with this, large tumor size predicts poor response to liposome-delivered chemotherapy in ovarian cancer.[14] Table 2 lists a number of tumor and liposome factors that play important roles in the delivery of liposomal drugs. On the tumor side, a rich blood flow and a highly permeable microvascular

**Table 2** Parameters affecting delivery of liposomal drugs to tumors.

| Tumor factors | Liposome factors |
|---|---|
| • Blood flow | • Long circulation time |
| • Vascular permeability | • Stability (drug retention) |
| • Interstitial pressure | • Small vesicle size |
| • Phagocytic activity | • Saturation of the RES |

bed will increase the probability of liposome deposition, while a high interstitial fluid pressure is likely to reduce the movement of molecules and particles into the tumor compartment. On the liposome side, avoiding drug leakage and prolonging the circulation time will result in more liposomes reaching the tumor vascular bed with an intact drug payload, and a small vesicle size will facilitate extravasation through the endothelium gaps or fenestrations. There are also data indicating that saturation of the RES will prolong circulation time and indirectly enhance liposome deposition in tumors.[15]

## 4. Liposome Formulation and Pharmacokinetics — Stealth Liposomes

In 1971, Gregoriadis *et al.*[16] published the first research work in which liposomes were used as drug carriers for medical applications. This initial study led to growing interest in liposomes, and many laboratories began examining liposome pharmacokinetics and biodistribution in animals, as well as *in vitro* stability in serum. The early liposome work was mostly based on formulations composed of neutral egg lecithin (PC), often in combination with negatively or positively charged lipids. These liposomes were found to release rapidly a large fraction of their encapsulated contents in circulation. Furthermore, they were quickly removed from the circulation by macrophages of the RES. Reformulation with high phase-transition temperature (Tm) lipids (distearoyl-PC, dipalmitoyl-PC, sphingomyelin) and addition of cholesterol led to improved retention of liposome contents and prolongation of circulation time, especially when the vesicles were properly downsized to <100 nm diameter. However, these relatively improved liposome formulations would still accumulate largely in the RES and a greater improvement in circulation half-life appeared to be required for cancer targeting. Surface modifications of liposomes that could reduce the RES affinity were investigated based on the erythyrocyte paradigm, whereby a layer of carbohydrate groups prolongs circulation for nearly 3 months. A number of glycolipids such as monosialoganglioside (GM1), phosphatidyl-inositol, and cerebroside sulfate, were included in the formulations and extended liposome circulation time.[17,18,19] However, a major advance took place when the hydrophilic polymer polyethylene-glycol (PEG), which was known to reduce immunogenicity and prolong circulation time when attached to enzymes and growth factors, was introduced into liposomes in the early 90s. PEG, which is inexpensive due to easy synthesis and could be prepared in high purity and large quantities, had distinct advantages over the other glycolipid surface modifiers. Addition of a conjugate of PEG with a lipid anchor, distearoyl-phosphatidylethanolamine (PEG–DSPE) to the liposomal formulation was shown to prolong liposome circulation time significantly,[20,21,22] and formed

a pivotal element of the pharmaceutical development of the Doxil formulation described thereafter in this chapter. Due to their ability to avoid RES clearance mechanisms, PEG-coated liposomes have been coined "Stealth"[3] liposomes.[23] In parallel to the development of stable formulations with longer circulation half-lives, it was soon realized that a prolonged residence time in circulation was a critical pharmacokinetic factor for liposome deposition in tumors and that there was a strong correlation between liposome circulation time and tumor uptake.[18] A number of studies have addressed the mechanism of liposome accumulation in tumors. Microscopic observations with colloidal gold-labeled liposomes[24] and morphologic studies with fluorescent liposomes in the skin-fold chamber model[25] have demonstrated that liposomes extravasate into the tumor extracellular fluid through gaps in tumor microvessels and are found predominantly in the perivascular area with minimal uptake by tumor cells. Studies with ascitic tumors[26,27] demonstrate a steady extravasation process of long circulating liposomes into the ascitic fluid, with gradual release of drug followed by drug diffusion into the ascitic cellular compartment. The process underlying the preferential tumor accumulation of liposomes, as well as other macromolecular and particulate carriers, is known as EPR (enhanced permeability and retention) effect.[28] This is a passive and non-specific process resulting from increased microvascular permeability and defective lymphatic drainage in tumors creating an *in situ* depot of liposomes in the tumor interstitial fluid. Circulating liposomes cross the leaky tumor vasculature, moving from plasma into the interstitial fluid of tumor tissue, following convective transport and diffusion processes. Although convective transport of plasma fluid also occurs in normal tissues, the continuous, non-fenestrated endothelium and basement membrane prevents the extravasation of liposomes. EPR is a relatively slow process, in which long-circulating liposomes possess a distinct advantage because of the repeated passage through the tumor microvascular bed and their high concentration in plasma during an extended period of time.

For any intra-vascular drug carrier device to access the tumor cell compartment and interact with tumor cell receptors, it must first cross the vascular endothelium and diffuse into the interstitial fluid, since with few exceptions, tumor cells and their surface receptors are not directly exposed to the blood stream. Therefore, the EPR effect is not only important for the tumor accumulation of non-targeted liposomes, but it is also for that of ligand-targeted liposomes. This has led us to postulate that the extravasation process is the rate-limiting step of liposome accumulation in tumors.[29] Experimental data with targeted and non-targeted liposomes have so far lent consistent support to this hypothesis.[30]

[3]Stealth is a registered trademark of Alza Corp., Mountain View, CA.

In most instances, delivery of drug to tumor cells depends on the release of drug from liposomes in the interstitial fluid, since liposomes are seldom taken up by tumor cells, unless they are tagged with specific ligands. The factors controlling this process and its kinetics are not well understood and may vary among tissues, depending on the liposome formulation in question. In the case of remote-loaded formulations, e.g. anthracyclines, a gradual loss of the liposome gradient retaining the drug, in addition to the disruption of the integrity of the liposome bilayer by phospholipases, may be involved in the release process. Uptake by tumor-infiltrating macrophages could also contribute to liposomal drug release. In any case, once the drug is released from liposomes, it will diffuse freely through the interstitial tumor space and reach deep layers of tumor cells. This is an inherent advantage of this delivery system as opposed to covalently bound drug-carrier systems. It is also a critical factor for the success of the liposomal drug approach, since most of the liposomes appear to remain in interstitial spaces immediately surrounding the blood vessels,[25] and therefore would not be able to interact with more than one layer of tumor cells.

The EPR effect has been confirmed in a variety of implanted tumor models. Its validity regarding human tumors, and particularly, cancer metastases, is as yet unclear. One concern is that interstitial fluid pressure increases in most tumors once they grow beyond a certain size threshold,[31] thereby hindering extravascular transport and liposome delivery. Unfortunately, there is a paucity of imaging studies in cancer patients with radiolabeled liposomes. One of the few studies with radiolabeled pegylated liposomes demonstrated significant liposome accumulation based on tumor imaging findings in 15 out of 17 patients tested.[32] In another study, in which tumor metastases and normal muscle tissues of 2 breast cancer patients were examined for doxorubicin concentration after injection of pegylated liposomal doxorubicin (PLD), liposomal drug was found at 10-fold greater concentration in tumor, as compared with muscle.[33] Another important piece of work in this area is the study of Northfelt *et al.*,[34] that pointed to an enhanced deposition of drug in Kaposi's sarcoma skin lesions of patients receiving PLD, compared with the normal skin of the same patients and to doxorubicin concentration in Kaposi's sarcoma biopsies of patients receiving free doxorubicin. More imaging and drug-carrier biodistribution studies are needed to determine how important and frequent is the observation of human tumors with selectively enhanced uptake of liposomes. These studies would also enable to determine whether there is a correlation between liposome accumulation in tumors and anti tumor response, and a need to select patients for liposome-delivered drug therapy based on positive liposome tumor imaging.

## 5. Preclinical Observations with Liposomal Drug Carriers in Tumor Models

The drug most frequently tested in liposomal formulations is doxorubicin and related anthracyclines. The choice of doxorubicin by many of the early research groups examining the role of liposomes as drug carriers in cancer chemotherapy, stems from its broad spectrum of anti tumor activity on the one hand, and its disturbing cumulative dose-limiting cardiac toxicity on the other hand.

Anthracyclines such as doxorubicin and daunorubicin cause acute toxic side effects including bone marrow depression, alopecia, and stomatitis, and are dose limited by a serious and mostly irreversible characteristic cardiomyopathy.[35] The first study describing the encapsulation of anthracyclines into liposomes appeared in 1979.[36] Work from various research groups followed, supporting the general principle that liposomal formulations reduced the toxicity of anthracyclines in animal models.

Using the Stealth technology and an elegant loading mechanism based on an ammonium sulfate gradient, a formulation of pegylated liposomal doxorubicin (PLD) known as Doxil in the USA (Caelyx in Europe) has been developed. The loading mechanism, coined "remote (active) loading", leads to highly efficient accumulation of doxorubicin inside the aqueous phase (~15,000 doxorubicin molecules/vesicle), where the drug forms a crystalline-like precipitate, contributing to stable drug entrapment by remaining osmotically inert.[37,38,39] This loading technology provides substantial stability with negligible drug leakage in circulation, while still enabling satisfactory rates of drug release in tissues and malignant effusions.[40]

Studies in animal tumor models with doxorubicin encapsulated in pegylated and other long-circulating liposomes, established the following pharmacologic observations[41]:

- Increased anti tumor activity of liposomal drug, as compared with optimal doses of free drug in various rodent models of syngeneic and human tumors.
- Increased accumulation of liposomal drug in various transplantable mouse and human tumors, compared with free drug.
- Delayed peak tumor concentration and slow tissue clearance after injection of liposomal drug.

The most valuable pharmacokinetic advantage of the Stealth liposomal delivery system is the enhancement of tumor exposure to doxorubicin, as a result of the accumulation of Stealth liposomes in tumors, as demonstrated in animal models and in some forms of human cancer. When the tissue uptake of PLD was examined in a couple of syngeneic mouse tumor models, it was found that the tumor drug uptake correlated linearly with dose, while the liver drug uptake showed a

saturation profile. In the case of free doxorubicin, liver uptake increased linearly with dose, while tumor uptake increased marginally with dose. As a result, the delta of tumor drug concentration in favor of PLD was substantially greater at high doses.[15] These results suggest a passive process of liposomal uptake into tumor, with nonsaturable kinetics.

In preclinical therapeutic studies using a variety of rodent tumors and human xenografts in immunodeficient nude mice, PLD was more effective than free doxorubicin and other (non-pegylated) formulations of liposomal doxorubicin.[11] In a few instances, the activity of PLD preparations was matched but not surpassed by other non-pegylated, long-circulating preparations of liposomal doxorubicin.[42] In most of these studies, the improved efficacy of PLD was obtained at milligram-equivalent doses of the MTD of free doxorubicin, indicating that there was a net therapeutic gain per milligram drug, independent of toxicity buffering. An elegant study addressed this issue directly by examining the activity of escalating doses of PLD and doxorubicin against implants of the mouse 3LL tumor (Lewis lung carcinoma), and concluded that the activity of 1–2 mg/kg Doxil was approximately equivalent to 9 mg/kg doxorubicin, i.e. a 6-fold enhancement in efficacy.[43] Similar observations were made in the M109 model, pointing to a 4-fold advantage for PLD, compared with free doxorubicin, i.e. a dose of 2.5 mg/kg PLD was at least as effective as 10 mg/kg free doxorubicin.[15]

There is a large body of preclinical data on other liposome formulations of anticancer agents moving into clinical development, or already approved for clinical use. In many cases, it is likely that the added value of these formulations has not been or will not be sufficient to justify further development, despite positive preclinical data.

## 6. Liposomal Anthracyclines in the Clinic

The anthracycline antibiotic doxorubicin has a broad spectrum of antineoplastic action and a correspondingly widespread degree of clinical use. In addition to its role in the treatment of breast cancer, doxorubicin is indicated in the treatment of various cancers of the lymphatic and hematopoyetic systems, gastric carcinoma, small-cell cancer of the lung, soft tissue and bone sarcomas, as well as cancer of the uterus, ovary, bladder and thyroid. Unfortunately, toxicity often limits the therapeutic activity of doxorubicin and may preclude adequate dosing. Other common complications of conventional anthracycline therapy include alopecia and dose-limiting myelosuppression. Most importantly, cardiotoxicity limits the cumulative dose of conventional anthracycline that can be given safely.[44]

Encapsulation of anthracyclines within liposomes significantly alters their pharmacokinetic profiles and promotes selectively high drug concentrations in tumors.[45] In animal studies, these pharmacologic effects resulted in maintained

or enhanced anthracycline efficacy and safety in a variety of experimental tumor types.[46] Improved therapeutic index profiles in clinical trials of liposomal anthracycline therapy for Kaposi's sarcoma,[47] ovarian cancer,[48] breast cancer,[49] or multiple myeloma[50] have been reported. Liposomal anthracycline therapy should be preferred when conventional anthracycline therapy is likely to be effective, but the required course of treatment would lead to unacceptable risk of toxicity. The relative lack of cardiotoxicity with liposomal anthracycline therapy is an important asset of the liposomal approach.[51]

There are 3 commercial formulations of liposomal anthracyclines that have been approved for clinical use: Doxil, Myocet, and Daunoxome. Tables 3 and 4 present a comparative list of their tolerated doses and pharmacokinetic parameters[41,87–89] respectively. A summary of their main clinical highlights is presented below.

## 6.1. *Doxil*

As indicated before, Doxil® (known in Europe as Caelyx®) is a doxorubicin formulation in which the drug is encapsulated in PEGylated liposomes (Stealth

**Table 3** Comparative single and cumulative tolerated doses of free and liposomal anthracyclines based on their acute/subacute toxicity and cardiac toxicity respectively.[1]

| | Doxorubicin[2] | Doxil | Myocet | Daunorubicin | Daunoxome |
|---|---|---|---|---|---|
| Maximal Single Does | 60–75 mg/m$^2$ | 50–60 mg/m$^2$ | 75 mg/m$^2$ | 90 mg/m$^2$ | 100–120 mg/m$^2$ |
| Maximal Cumulative Dose[3] | ~450 mg/m$^2$ | Undetermined (>650 mg/m$^2$) | ~785 mg/m$^2$ | 900 mg/m$^2$ | Undetermined |
| Maximal Dose Intensity | 20–25 mg/m$^2$/week | 12.5 mg/m$^2$/week | 25 mg/m$^2$/week | 30 mg/m$^2$/week | 40 mg/m$^2$/week |
| Dose Limiting Toxicities | Neutropenia Stomatitis | Stomatitis, Skin toxicity | Neutropenia, Stomatitis | Neutropenia | Neutropenia, Mucositis |

[1]Other anthracyclines in clinical use: Epirubicin is an epimer of doxorubicin widely used in breast cancer with less cardiotoxicity but also less activity on a per mg basis, owing to faster glucuronidation and faster clearance. Its therapeutic index advantage over doxorubicin, if any, is marginal. Idarubicin, an analog of daunorubicin, is another clinically approved anthracycline but of less common use. Mitoxantrone, an antracenedione, is a drug related to anthracyclines with dose-limiting neutropenia and with cardiotoxic potential albeit after longer treatment periods than doxorubicin. It is also approved for clinical use but its added value is doubtful since it appears to be somewhat less active than doxorubicin in metastatic breast cancer.

[2]Doxorubicin cumulative dose may be substantially increased with co-administration of dexrazoxane, a cardioprotective agent.

[3]Dose associated with 5% risk of cardiotoxicity.

**Table 4** Comparative human pharmacokinetics parameters of free and liposomal anthracyclines.[1]

| | Doxorubicin | Doxil[2] | Myocet | Daunorubicin | Daunoxome |
|---|---|---|---|---|---|
| Distribution t½ (hr) | Rapid (min) | 72.9 | <1.0[3] | Rapid (min) | 5.6 |
| Terminal t½ (hr) | 42.9 | ND[4] | 16.4 | 20.6 | ND[4] |
| Clearance (mL/hr) | 46,100 | 49 | 5185 | 114,750 | 408 |
| Volume of Distribution (L) | 1447 | 4.3 | 58.3 | ~2000 | 3.2 |
| Dose ($mg/m^2$) | 60 | 60 | 60 | 75 | 100 |
| Reference | Swenson *et al.*[87] | Gabizon *et al.*[41] | Swenson *et al.*[87] | Riggs[88] | Bellott *et al.*[89] |

1. To normalize for body surface, values were corrected for an average body surface area of 1.7 $m^2$.
2. Median values of 4 studies are shown.
3. Not reported. Extrapolated approximation is shown.
4. Mono-exponential elimination of liposomal drug from plasma. Terminal clearance phase of released drug not detected.

liposomes), formulated with a hydrogenated (high phase transition temperature) PC and cholesterol. Doxil was granted market clearance in 1995 by the US Food and Drug Administration (FDA) for use in the treatment of AIDS-related Kaposis Sarcoma (KS), in patients with disease that has progressed on prior to combination chemotherapy and who are intolerant to such therapy. In 1996, it was granted market clearance by the European Union's commission for Proprietary Medicinal Products for the same indication. In 1999, Doxil was granted US market clearance for use in the treatment of recurrent carcinoma of the ovary in patients with disease that is refractory to paclitaxel-and platinum- based chemotherapy regimens. In January 2003, the European Commission of the European Union has granted centralized marketing authorization to Doxil, as monotherapy for metastatic breast cancer in patients who are at increased cardiac risk. In addition, phase II trials have been completed in the US and Europe, investigating the safety and efficacy of Doxil in multiple myeloma and in other solid tumors including sarcomas, carcinoma of head and neck, hepatocellular carcinoma, prostate cancer and the rest.

Doxil was already recognized 10 years ago as a liposomal doxorubicin formulation with unique pharmacokinetics and a dramatic change in the clinical toxicity profile. Clinical pharmacokinetic studies have indicated that Doxil prolongs the circulation time of doxorubicin dramatically, in agreement with preclinical studies. In 1994, we published the results of a pharmacokinetic study in which 15 patients

were given sequentially the same dose in drug-equivalents of free doxorubicin and Doxil.[52] A dramatic reduction in the drug clearance and volume of distribution, resulting in a 1000-fold increase in AUC with the liposomal formulation, was observed. It was also found that nearly all the drug circulating in plasma is in liposome-encapsulated form. Metabolites in plasma were undetectable or at very low levels. However, they were readily detected in urine 24 hrs or later after injection, indicating that the drug has become bioavailable. The following drug distribution picture has emerged from this initial study and from more recent ones[41]:

1. Drug circulates in plasma for prolonged periods of time (i.e. half-life in the range of ~50–80 hours) in liposome encapsulated form. Despite its prolonged presence in blood, the drug is not bioavailable, as long as it remains in the interior of a circulating liposome.
2. Most of the injected drug (>95%) is distributed to tissues in liposome-encapsulated form. Once in tissues, drug leakage and liposome breakdown with or without liposome internalization by cells gradually provides a pool of bioavailable drug. Metabolites are formed.
3. Rate of metabolite production is slower than the rate of renal clearance of metabolites. As a result, metabolites do not accumulate in plasma but can be detected in urine.
4. A small fraction of injected drug (<5%) leaks from circulating liposomes and is handled as a free drug with fast plasma clearance and rapid metabolism. This drug fraction is the source of small amounts of metabolites that can sometimes be detected in plasma.

In 1995, a phase I study of Doxil in patients with solid tumors[53] provided clear evidence of a major change in the toxicity profile, with muco-cutaneous toxicities as the major dose-limiting toxicities. In contrast, myelosuppression, and alopecia were minor and cardiotoxicity was conspicuously absent. The maximal tolerated dose was established as 60 mg/m$^2$, with mucositis being the dose-limiting toxicity. It was also found that the optimal dosing interval for retreatment was 4 weeks rather than the standard 3-week schedule of doxorubicin. The dose-schedule limiting toxicity was a form of skin toxicity known as hand-foot syndrome, also referred to as palmar-plantar erythrodysesthesia (PPE), which appears to be related to the long half-life of Doxil. Thus, it became well-established that the Doxil liposome formulation imparts a significant pharmacokinetic-pharmacodynamic change to the drug doxorubicin, unprecedented in magnitude for any intravenous drug delivery system. Later on, data gathered from phase II and III studies in metastatic breast cancer and recurrent ovarian cancer[48,49,54] brought down the recommended dose of Doxil to 40–50 mg/m$^2$ once in 4 weeks, i.e. 10–12.5 mg/m$^2$ per week. This dose

reduction was needed mainly to prevent skin toxicity resulting from successive courses of therapy at a dose intensity of 15 mg/m$^2$/week.

Kaposi's sarcoma (KS) is a multifocal tumor affecting the skin and sometimes the mucosas well known for its extremely high microvascular permeability. Profuse extravasation of colloidal gold-labeled Stealth liposomes in a transgenic mouse model of KS has been shown.[55] In AIDS patients, KS is frequent and has an aggressive course. Therefore, this condition was chosen for the initial clinical testing of Doxil in Phase II–III studies. Indeed, Doxil as a single agent therapy demonstrated a significantly greater efficacy and better safety than standard chemotherapy (i.e. combinations of bleomycin and vincristine, with or without doxorubicin) and was also effective as second line chemotherapy in pretreated patients. As a result of the extreme sensitivity of KS to chemotherapy, a low and relatively subtoxic dose of 20 mg/m$^2$ every 3 weeks is sufficient for effective treatment.[47]

Further to the successful application in KS treatment, there are three important benchmarks in the clinical research development of Doxil in solid tumors:

1. *Cardiac function in patients receiving Doxil.* Evidence of a major risk reduction of cardiotoxicity, compared with free doxorubicin historical data. A retrospective analysis of patients treated with large cumulative doses of Doxil did not reveal any significant cardiac toxicity, despite the fact that some of these patients were treated with 3 times as much as the maximal cumulative dose acceptable for free doxorubicin.[56] Two additional reports focusing on the cardiac biopsies of Doxil-treated patients at high cumulative doses confirmed the cardiac safety of Doxil.[57,58]

2. *Phase III study in recurrent ovarian cancer.* Significant increase in median survival with improved safety profile in the Doxil patient group versus the topotecan-treated patient group, where topotecan was the former standard therapy in this condition.[59] The use of Doxil was particularly beneficial in "platinum-sensitive" patients (i.e. patients in whom tumor recurrence occurred more than 6 months after the discontinuation of platinum-based front-line therapy). In this subgroup, the median survival of Doxil-treated patients was 107.9 weeks, compared with 70.1 weeks for topotecan-treated patients, a difference of ~9 months, equivalent to a 54% increase in survival. As a result, Doxil has become the standard therapy for recurrent ovarian cancer.

3. *Phase III study in metastatic breast cancer.* Equivalent anti tumor activity and reduced cardiotoxicity. In this study, it was found that treatment with Doxil 50 mg/m$^2$ every 4 weeks (dose intensity = 12.5 mg/m$^2$/week) had equivalent efficacy to free doxorubicin of 60 mg/m$^2$ every 3 weeks (dose intensity 20 mg/m$^2$/week), despite the lower dose intensity of the former.[60] In addition, cardiotoxicity, as well as alopecia, were dramatically reduced in Doxil-treated patients. Myelosuppression and nausea were also milder in Doxil-treated patients. However,

as seen in phase I–II studies, skin toxicity was prominent with Doxil and almost absent with doxorubicin treatment.

## 6.2. *Myocet*

Myocet™ (liposome encapsulated doxorubicin citrate complex) is a non-pegylated formulation of liposomal doxorubicin that has been approved for the treatment of metastatic breast cancer in Europe, but not in the United States. Myocet lipid composition consists of a fluid phase (low phase transition temperature) PC, as well as cholesterol with doxorubicin entrapped in the water phase. A unique feature of this formulation is that the drug is loaded into liposomes using a 3-vial kit, just prior to administration in the hospital pharmacy. The strategy employed is to decrease toxicity in a way that a net gain of therapeutic index is obtained. In the pivotal phase I trial of Myocet,[61] the maximum tolerated dose was between 75–90 mg/m$^2$, given on day 1 or split in 3 consecutive daily doses of each 3-week cycle. The major dose-limiting toxicity was neutropenia. G-CSF (granulocyte-colony stimulating factor) administration may increase the maximum tolerated dose, when higher doses of Myocet are desired by reducing the incidence of dose-limiting neutropenia.[62] Phase III studies in metastatic breast cancer, comparing Myocet to free doxorubicin, have shown similar anti tumor activity with significantly lesser cardiotoxicity for Myocet.[63,64] However, a study of high dose Myocet with G-CSF in patients with advanced breast cancer resulted in a disturbingly high incidence of cardiotoxicity (38%),[65] after a median cumulative dose of 405 mg/m$^2$ (range of 135–1065 mg/m$^2$), suggesting that the Myocet margin of cardiotoxicity gain over doxorubicin is limited.

As described above, the maximum tolerated doses of Doxil and Myocet differ as a result of their different formulations. Consequently, a more intense dose schedule is recommended for Myocet (75 mg/m$^2$ every 3 weeks) than for Doxil (50 mg/m$^2$ every 4 weeks). The pharmacokinetics of Myocet points to a small change in clearance and volume of distribution, when compared with free doxorubicin,[66] suggesting that, in contrast to Doxil, Myocet liposomes are cleared rapidly from circulation and their drug content also leaks substantially.

## 6.3. *Daunoxome*

Daunoxome® (daunorubicin citrate liposome injection), is approved for use in patients with advanced HIV-associated Kaposi's sarcoma at a recommended dose of 40 mg/m$^2$ every 2 weeks.[67] Daunoxome lipid composition consists of a solid phase (high phase transition temperature) PC, as well as cholesterol with daunorubicin encapsulated in the water phase. The maximum tolerated dose of Daunoxome was

evaluated in a phase I trial of 32 patients with solid tumors, using Daunoxome doses that were escalated in steps from 10 to 120 mg/m$^2$.[68] Dose-limiting neutropenia occurred in all patients who received 120 mg/m$^2$. The maximum tolerated dose of Daunoxome was established as 100–120 mg/m$^2$ in patients with solid tumors every three weeks. A more recent study with Daunoxome in breast cancer patients confirmed this dose level as MTD.[69] Two studies in patients with acute leukemia administered high dose Daunoxome in 3-day courses, up to a total dose of 450 mg/m$^2$.[70,71] Mucositis was the dose-limiting toxicity in these high dose Daunoxome studies. In one of the studies, 2 of 28 patients experienced fatal cardiotoxicity.[71] In a phase I study of 48 children with relapsed or resistant solid tumors,[72] the trial was prematuredly discontinued due to evidence of cumulative cardiotoxicity, including two deaths after 4 courses of Daunoxome treatment. In summary, neutropenia typically limits the maximum dose of Daunoxome in solid tumor patients, and mucositis limits the maximum dose that can be given in a single cycle to leukemia patients. Cardiotoxicity was reported in some patients, and appears especially problematic in children.

The pharmacokinetics of Daunoxome points to a major retardation of clearance and a small volume of distribution, when compared with free daunorubicin.[67] The distribution half-life of Daunoxome (7–8 h) is nearly 10 times shorter than that of Doxil, suggesting that the former is cleared faster by the RES, in agreement with the differences observed between pegylated and nonpegylated liposomes in preclinical studies. However, in contrast to Myocet, the Daunoxome pharmacokinetics data do not point at any significant drug leakage in circulation.

## 7. Clinical Development of Other Liposome-entrapped Cytotoxic Agents

Dose-finding safety studies have been performed with several other liposomal anthracyclines, including a cardiolipin-based liposome formulation of doxorubicin,[73] doxorubicin entrapped in negatively charged phosphatidylglycerol liposomes,[74] liposomal annamycin,[75] a pegylated form of liposomal doxorubicin manufactured only in Taiwan (PEG-distearoylPC-cholesterol),[76] and an immunoliposome-encapsulated form of doxorubicin (MCC-465), targeted with an antibody reacting with human gastric cancer.[77] Some of these formulations have not progressed any further to phase II–III studies. For those formulations still under investigation, further clinical trials are needed to establish their efficacy.

The most advanced compounds currently being developed in liposomes belong to two families of cytotoxic drugs: Camptothecin analogs with topoisomerase I inhibitory properties,[78,79] and Vinca alkaloids.[80] Both are cell cycle phase-specific cytotoxic drugs. Their anti-tumor activity tends to increase with liposome

encapsulation, as we extend time of exposure by exploiting the liposomal slow release features. An additional advantage for camptothecin analogs is the fact that their activity is better maintained in the lactone configuration which is stable in an acid environment, as is the case of liposomes with remote drug-loading techniques based on proton gradients. At this time, none of these compounds have yet been approved by a regulatory agency after going through phase III studies.

It should be noted that the development of liposomal formulations of cytotoxic agents has often failed for different reasons. The formulation of a liposomal anticancer agent is a complex process with at least three distinct variables that may affect the outcome and the risk of failure: the choice of the liposome carrier, the choice of the drug, and the method of drug encapsulation. One example is the formulation of cisplatin in pegylated liposomes known as SPI-77. These liposomes have long-circulating characteristics, retain the drug in plasma exceedingly well, reduce drug toxicity, and produce a high and long-lasting accumulation of drug in implanted tumors.[81] However, the anti-tumor activity is reduced by comparison to free drug in preclinical studies.[81] In clinical studies, SPI-77 was inactive and showed no dose-limiting toxicities, even at doses greater than 2-fold the MTD of free cisplatin.[82,83] It appeared that cisplatin release from these liposomes was minimal in *in vitro*[81] as well as *in vivo* systems, as indicated by the low occurrence of DNA adducts resulting in a major reduction of bioavailability.[84] As a result, the development of SPI-77 was discontinued at the Phase I clinical level.

## 8. The Future of Liposomal Nanocarriers

Although progress in the understanding of cancer biology at the molecular level will undoubtedly lead to more new drugs with exquisite selectivity, some of these agents may need an efficient system for *in vivo* delivery to yield optimal results. In addition, the use of broad-spectrum cytotoxic drugs will remain as a major tool in cancer therapy for decades to come, and, for cytotoxic drugs, various delivery systems may have a beneficial effect on the therapeutic index as already shown. Liposomes remain one of the most attractive platforms for systemic drug delivery, and an increased sophistication of these systems would be expected. The most immediate improvement is the coupling of a ligand to the surface of the liposome that will target the vesicles to a specific cell-surface receptor, followed in most cases by internalization and intracellular delivery of the liposome drug cargo. Examples in this direction are the targeting of Doxil to Her 2 expressing or folate-receptor, expressing cancer cells using a specific anti-Her 2 single chain Fv respectively or a folate conjugate anchored to the liposome surface.[30,85] Another example is the tumor vascular targeting by endothelium-specific peptides associated to liposomes.[86] A

major advantage of targeted liposomal nanocarriers over ligand-drug bioconjugates is the delivery-amplifying effect of the former, which may be able to provide to the target cell, a ratio of 1000 drug molecules per single ligand-receptor interaction.

Other future avenues that can be exploited using the liposome platform and have a definite preclinical basis include:

1. Association with a reporter, i.e. an imaging or tracing element, that will provide the possibility of tracking the fate of the liposome *in vivo* or even the occurrence of a pharmacological effect such as apoptosis.
2. Co-delivery of synergistic agents. The liposome platform offers the possibility of co-delivery in space and time of two drugs with different pharmacokinetics and biodistribution patterns, thus enabling the optimal exploitation of synergistic properties.
3. Association with a bio-responsive element, i.e. a temperature-sensitive or pH-sensitive component that could destabilize the liposome and drive a burst release of drug.

The fact that liposome technology has matured into an acceptable pharmaceutical technology, and the promising contributions of liposomes to sophisticated drug delivery methods, augur that liposome carriers are to remain in cancer therapy for the foreseeable future.

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# 21

# Nanoparticulate Drug Delivery to the Reticuloendothelial System and to Associated Disorders

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## 1. Introduction

Physicochemical methods for site-specific delivery of drugs in the form of polymeric and colloidal particulate carriers e.g. liposomes, micelles and nanoparticles, have been of great interest to researchers recently. Nanoparticles, particularly polymeric nanoparticles, have been investigated since the late 1970s as an alternative to liposomes which suffered from inherent problems such as low encapsulation, rapid leakage, poor stability and production difficulties. Nanoparticles are particles ranging from 10 nm to 1000 nm in diameter and are the collective names for nanospheres and nanocapsules. Nanospheres are solid particles which can be used as drug carriers, where the active principles can be dispersed in the polymeric matrix or adsorbed on their surfaces or both. Nanocapsules have a polymeric shell with an inner liquid core and the active substances can be incorporated within the core or loaded on the surface by physical adsorption or by chemical bonding. Nanoparticles can be administered by a variety of routes, depending upon the desired therapeutic outcome and can even be used for vaccine administration and diagnostic imaging.

Drug release from nanoparticles is a very important factor for developing successful formulations. For achieving this, various targeted and controlled release

drug delivery systems have been developed. Controlled release is one of the basic modes of drug delivery with the objective of releasing a drug into a patient's body at a predetermined rate, or at specific times, or with specific release profiles. Release profiles of the drugs from nanoparticles depend on the nature of the delivery systems. Such systems often use synthetic polymers as the carriers for the drug.

These particulate carrier systems can release the drug by (a) polymer degradation or chemical cleavage of the drug from the polymer, (b) swelling of the polymers and releasing the drug entrapped within them, (c) osmotic pressure effects creating pores and releasing the drugs, which can get dispersed within the polymeric network of the nanoparticles, and (d) simple diffusion methods. They represent an interesting carrier system for the specific enrichment in macrophage containing organs like the liver and spleen. Thus, injectable nanoparticle carriers have important potential application in site-specific drug delivery.

## 2. Reticuloendothelial System and Associated Disorders

The reticuloendothelial system (RES) represents a group of cells having the ability to take up and sequester inert particles and vital dyes. This includes macrophages and macrophage precursors, specialized endothelial cells lining the sinusoids of the liver, spleen and bone marrow, and the reticular cells of the lymphatic tissue (macrophages) and of the bone marrow (fibroblasts). Thus, the reticuloendothelial system or the mononuclear phagocyte system encompasses a range of cells capable of phagocytosis i.e. macrophages and monocytes. They are either freely circulating within the blood or fixed to various connective tissues. Examples of the sites of fixed cells include pulmonary alveoli, liver sinusoids, skin, spleen and joints. The RES primarily functions to (a) remove the senescent cells from circulation and (b) provide phagocytic cells for both inflammatory and immune responses.

There are several RES associated disorders involving both macrophages and monocytes. These diseases could be of two types — infectious and non-infectious. Among the infectious diseases, pulmonary tuberculosis, typhoid fever, leishmaniasis, trypanosomiasis and acquired immune deficiency syndrome (AIDS) are worth mentioning. Among the non-infectious type, ulcerative colitis, collagen disease, Hodgkin's disease and Gaucher's disease are equally common.

## 3. Uptake of Nanoparticles by the Reticuloendothelial System

### 3.1. *Sites of uptake*

It has been observed that nanoparticles, like other colloidal drug delivery systems e.g. liposomes, niosomes, microparticles etc., on intravenous injections, are rapidly

sequestered and retained by the organs comprising of the reticuloendothelial systems (RES), mainly the liver, spleen and the bone marrow. Thus, targeting of the nanoparticles to the RES is much simpler than to any other organ. In the liver, the particles are mainly retained by the scavenging periportal and midzonal Kupffer cells, while the hepatocytes and liver endothelial cells may play a secondary role under special pathophysiological conditions or for special physico-chemical characteristics of particles. In the spleen, the marginal zone and red pulp macrophages are the major scavengers, while peritoneal macrophages and dendritic cells have a minor contribution. In the case of bone marrow, the sequestering mechanism is rather complex and appears to be species specific. Briefly, it encompasses two routes, a transcellular route, mediated through the diaphragmed fenestrate of the endothelial walls, and an intercellular route, involving the formation of bristle-coated pits containing matter on the internal surface of the endothelium. The particles that reach the bone marrow parenchyma are engulfed by stromal or hematopoietic macrophages. In guineapig, the venous endothelium has similar phagocytic properties to the sinusoidal endothelium, while in some other species such as rabbit and marmoset, certain macrophage subpopulations named perisinal phagocytes, which penetrate the endothelium sending cytoplasmic processes into the lumen, are also involved.[1,2]

## 3.2. *Mechanism of uptake*

Two major mechanisms are involved in the sequestering of nanoparticles and other colloidal particles by macrophages. These are (a) opsonophagocytosis and (b) opsonic-independent recognition.

### *(a) Opsonophagocytosis*

Opsonophagocytosis and opsonorecognition of particles by macrophage receptors are greatly enhanced when the particle surface is coated with certain protein ligands. Such ligands are known as "opsonins" and the process of their adsorption on the particle surface is termed "opsonization". Opsonins act as a ligand on the particle surface and facilitate their recognition and initial attachment by the phagocyte receptors. Taking liposomes as a model system, the major classes of opsonins have been found to include various subclasses of immunoglobulins (e.g. IgM, IgG1 and IgG3 in humans), certain components of the complement system (e.g. C3b, iC3b, Clq), fibronectin, lipopolysaccharide binding protein and pentraxins (e.g. C-reactive proteins and serum amyloid P-components). More recently, it was shown that both thrombospondin and Von Willebrand factor can also act as opsonins and trigger phagocytosis of sulfatide-rich or coated particles. Their

modes of interaction have been reviewed in detail.[2] Some opsonic entities such as the tetrapeptide tuftsin, unlike other opsonins, bind to the phagocytic cell rather than to the particle and enhance the phagocytic ingestion two or three-fold.[3] Of these, IgG along with complement C3b and albumin are seen to play the most important roles. These opsonins, bound to the particle surface, form a bridge between the particles and the macrophages, facilitating phagocytosis of the particles. The Fc receptor plays an important role in the clearance of IgG-opsonized particles.

The complement system provides the first line of defense against foreign microbes and particles, ensuring their cytolytic and/or phagocytic clearance. As in the case of liposomes, activation of the complement pathway may occur if the opsonic components C3b and iC3b are deposited on nanoparticles. This may be antibody-mediated, or nanoparticles may also activate complement through non-antibody mediated mechanism via the classical and alterative pathways. However, it is known that non-covalent linkage of C3b and iC3b to a particle surface cannot promote phagocytosis by macrophages. Rather, the covalent bond between the reactive glutamyl residue of the C3 thiol ester and the constituents of the particles surface is the central mechanism of opsonization and the mediator of phagocytic recognition.[4] In addition, the covalently attached C3b or iC3b must be accessible to their corresponding receptors on phagocytic cell surface. Moreover, the complement receptors must be in a functional state. Kupffer cells in humans and rats have been found to contain receptors for C3b (CR1) and iC3b (CR3 and CR4), and may play an important role in the macrophage clearance of nanoparticles. In humans, CR1 receptors are also found in monocytes and erythrocytes, while it is found on platelets in rats. Hence, these cells also play an important role in the clearance of immune complexes via the C3b–CR1 interaction. In humans, the erythrocyte-bound immune complexes are transferred to macrophages during their passage through the liver and the spleen; due to the multitude of erythrocytes, this may be a major contributor to the complement-mediated clearance process.

In the case of liposomes, it has been demonstrated that the vesicle-blood protein interaction is largely determined by the fluidity and hydrophobicity of the vesicles, as well as by the vesicle size. Cholesterol free and cholesterol poor vesicles are rapidly captured by macrophages, mostly of the liver; the uptake is enhanced in the presence of serum. In contrast to hepatic phagocytes, bone marrow cells preferentially capture cholesterol-rich rather than cholesterol-poor vesicles primed with serum. On the other hand, serum stimulates the uptake of all cholesterol-containing vesicles by both splenic phagocytes and peritoneal macrophages, but its effect is significantly greater on the uptake of cholesterol-rich rather than cholesterol-poor vesicles. In addition, serum suppressed the uptake of vesicles prepared from

saturated phospholipids by liver phagocytes, but enhanced their uptake by spleen and bone-marrow macrophages. Thus, vesicle uptake by the liver, spleen, bone-marrow and peritoneal macrophages is determined by tissue-specific opsonins. Again, this is determined by the membrane fluidity and hydrophobicity which plays an important role in attracting the right opsonins which determine phagocytic activity. From the above observations, it has been suggested that a balance between the opsonins and dysopsonins (i.e. naturally occurring substances that inhibit phagocytic ingestion, usually by altering the surface properties of the phagocyte or particle or both, thereby interfering with opsonization or altering the metabolic activity of the phagocyte) may regulate the uptake of vesicles by phagocytes. Moreover, large vesicles (above 400 nm) are more readily cleared by liver macrophages probably by complement activation, while smaller ones (100–400 nm) localize in the spleen and bone marrow macrophages. Although, calcium at physiological levels is a prerequisite for the process of phagocytosis, it has been demonstrated that elevation of serum calcium levels above normal can inhibit, while a decrease below normal can facilitate the opsonophagocytosis of particles by Kupffer cells.[5,6]

### *(b) Opsonin-independent recognition*

Non-opsonic blood proteins could also play an important role in particle clearance. Non-opsonic proteins, after adsorption onto particle surfaces, could experience conformational changes. Such changes are likely to expose chemical groups that could either be recognized directly by certain receptors on the phagocytic cell surface, or could act as ligands for the subsequent recognition by the blood opsonins. The molecules involved include mannose-binding protein (MBP), a C-type lectin with specificity for mannose, and N-acetyl glucosamine, also known to activate complement through the activation of C1r2 C1s2 complex, and by opsonization through macrophage C1q receptor. Scavenger receptors (SR) on macrophage plasma membranes and endothelial cells can recognize modified lipoproteins, polyanionic macromolecules, bacterial polysaccharides, silica and possibly anionic liposomes. Fc$\gamma$RII-B2 and Fc$\gamma$RI are regarded as putative receptors for low-density lipoproteins functioning, independent of IgG. CD14 is a physiologically important receptor for lipopolysaccharide, which is also a ligand for SR-AI. It is strongly expressed on monocytes and granulocytes, but on Kupffer cells, it is only expressed in chronic and acute liver diseases. A putative stearylamine receptor on Kupffer cells may also play a minor role in the clearance of neutral and anionic vesicles. Co-operation between different macrophage receptors, (e.g. fibronectin or immunoglobulins with complement or $\alpha\gamma\beta3$ with CD36) may increase the efficiency of particle phagocytosis and the clearance from blood.

Alternatively, macrophages as well as hepatocytes and liver endothelial cells may phagocytose/endocytose liposomes via direct recognition of phospholipid headgroups. Phospholipid recognition may be mediated by a number of plasma membrane receptors such as lectin receptors, CD14, various classes of scavenger receptors (e.g. classes A, B and D), Fc$\gamma$RI and Fc$\gamma$RII-B2.[2] The recognition is specific for unsaturated phospholipids and fails for saturated phospholipids.[7]

## 3.3. *Factors influencing uptake*

Extrapolating the above discussion on liposomes to nanoparticles, it may be emphasized that some of the factors which affect their uptake by the cells of the RES include particle size, surface charge and surface hydrophobicity/hydrophilicity. Cholesterol-free nanoparticles of diameter above 200 nm, incorporated with unsaturated phospholipid headgroups, are expected to be preferentially sequestered by liver macrophages and endothelial cells, while priming nanoparticles that are smaller than 200 nm diameter, containing phospholipid and probably cholesterol, with serum, may enhance their uptake by the spleen and bone marrow macrophages. The hepatic phagocytosis may be facilitated by subnormal blood calcium concentration.

Although the above hypotheses have not yet been experimentally proven entirely, it has been demonstrated many times in our laboratory that poly-DL-lactide (PLA) and poly-DL-lactide co-glycolide (PLGA) nanoparticles of approximately 250 nm in diameter, containing a high percentage (58.8% w/w) of unsaturated phospholipids (phosphatidyl ethanolamine or phosphatidyl choline), are highly effective in reducing the spleen and liver parasite loads in the hamster models of experimental visceral leishmaniasis.[8–10] This lends credence to the above view.

In general, it appears that surface hydrophobic nanoparticles of size greater than 200 nm in diameter have a greater chance of being sequestered by macrophages of the liver and spleen. However, it has been reported that very small nanoparticles (< 70 nm diameter), consisting of poly-DL-lactide (PLA) — poly-ethylene-glycol (PEG) copolymeric micelles, can pass through the sinusoidal fenestrations in the liver and gain access to the liver parenchymal cells.[11] Moreover, the effect of surface charge on phagocytosis and the biodistribution of albumin nanoparticles have been reported by Roser *et al.*[12] It has been noticed that albumin nanoparticles, with a zeta potential close to zero, showed a reduced *in vitro* phagocytic uptake by primary mouse peritoneal macrophages and a human hematopoietic cell line U-937, in comparison to charged particles, especially particles with a positive zeta potential. However, this difference has not been reflected in their *in vivo* blood circulation times and organ distributions in rats. Moreover, the influence of surface characteristics

which include surface charge density and zeta potential, along with size and surface hydrophobicity, has already been noticed on plasma protein adsorption patterns on colloidal drug carriers after intravenous administration, thus influencing their *in vivo* organ distribution.[13]

## 3.4. *Role of surface modifications on uptake*

Various surface modifications of nanoparticles have been shown to facilitate their uptake by different components of the RES. Colloidal gold nanoparticles, after opsonization with autologous plasma, are found to accumulate in Kupffer cells, the predominant opsonizing factor being fibronectin.[14] Monocrystalline iron oxide nanoparticles (MION) were found to be readily captured by macrophages, and opsonization by the serum component C3, vitronectin and fibronectin resulted in a six-fold increase.[15] Poly-lactide nanoparticles are sequestered by monocytes by passive adsorption and energy-requiring receptor-mediated endocytosis and the uptake are enhanced by opsonization with lipoproteins.[16] Body distribution of fully biodegradable [$^{14}$C]–poly (lactic acid) nanoparticles coated with albumin, after parenteral administration to rats, was examined by Bazile *et al.*[17] As deduced from whole-body autoradiography and quantitative distribution experiments, the $^{14}$C-labelled polymer is rapidly captured by the liver, bone marrow, lymph nodes, spleen and peritoneal macrophages. Nanoparticle degradation was addressed following $^{14}$C excretion. The elimination of $^{14}$C was quick on the first day (i.e. 30% of administered dose), but slowed down subsequently.

The block co-polymers of poloxamine and poloxamer series play an important role in the surface modification of nanoparticles. Of these, poloxamine 908 is a poly-oxyethylene (POE)/polyoxypropylene (POP) block copolymer, which adsorbs on nanoparticles via the relatively hydrophobic POP segments, while the mobile POE chains extend outward, suppressing aggregation and providing stability. Poloxamer-407 is a block copolymer of POE/POP and a non-ionic surfactant. Polystyrene microspheres coated with the block copolymers of poloxamer and poloxamine series were observed to adsorb IgG, complement C3, transferrin and fibronectin in 50% serum, as well as fibrinogen in 50% plasma.[18] Poloxamine 908 activates the mononuclear phagocyte system so that the coated particles are sequestered by liver.[19] Poly (organophosphazene) nanoparticles coated with poloxamine 908 were mainly captured by the rat liver, while poly (organo phosphazene) nanoparticles coated with a novel poly (organophosphazene)-poly (ethyleneoxide) copolymer with a 5000 MW PEO chain were reported to be significantly targeted at the bone marrow.[20] It has been observed that the sequestration of surface-engineered polystyrene nanospheres by the liver and spleen could be greatly augmented by the modification of poloxamer 407 and poloxamine 908,

by introducing a terminal protonated amine group to each PEO chain.[21] Also, the phagocytic uptake of poloxamer and poloxamine coated polystyrene particles by mouse peritoneal macrophages was found to decrease with increasing adsorbed layer thickness, i.e. longer hydrophilic polymer chains of the coating agent, and consequently, a greater steric stabilization effect.[22]

Surface engineered sterically stabilized nanospheres were synthesized and found to have enhanced drainage into lymphatics, as well as enhanced uptake by macrophages of the regional lymph nodes.[23] Lymph node localization of biodegradable poly-lactide co-glycolide nanospheres could be enhanced by coating them with poloxamer and poloxamine.[24] Correlation was observed between the length of the stabilizing POE chains of the block co-polymers polyoxyethylene (POE)/ polyoxypropylene (POP) in the poloxamer/poloxamine and nanosphere drainage and the passage across tissue-lymph interface in dermal lymphatic capillaries in the rat-footpads. The longer the POE chains, the faster the particle drainage. In order for effective opsonization of the nanospheres to occur in the lymphatics, the POE chains of the block copolymers should be of 5–15 ethylene oxide units. If the dimensions of the stabilizing POE chains of the poloxamines and poloxamers exceed the range of the Van der Waals forces of attraction, opsonization fails to occur and the surface modified nanospheres escape sequestration by the macrophages of the regional lymph nodes, and are rapidly drained into the systemic circulation, where they persist for prolonged periods.[23] It has also been reported that polystyrene and poly-lactide-co-glycolide nanoparticles show enhanced localization in the lymph nodes when their surfaces are coated with polylactide (PLA) : poly-ethylene-glycol (PEG) or by producing co-precipitate nanospheres of PLGA and PLA : PEG, depending on surface characteristics.[25] PEG-coated magnetite nanospheres have also been utilized to target diagnostic agents to regional lymph nodes.[26]

It has been reported that small colloidal nanoparticles ($\leq$ 150 nm in diameter) can be targeted specifically to the sinusoidal endothelial cells of the rabbit bone marrow, following intravenous administration, by coating their surface with the block co-polymer poloxamer 407, a non-ionic surfactant.[27]

Influence of surfactant concentration on the body distribution of the nanoparticles was studied by Araujo *et al.*[28] They noticed that the rapid RES uptake of the nanoparticles after intravenous injection, especially by the liver, can be reduced and the body distribution can be altered by coating them with non-ionic surfactants, e.g. poloxamine 908 and polysorbate 80. Evaluation of the likely mechanisms that contribute to the prolonged circulation times of sterically protected nanoparticles has already been made.[29] Recent evidence showed that sterically stabilized particles are prone to opsonization, particularly by the opsonic components of the complement systems.

## 4. Active Targeting of Nanoparticles by Receptor Mediated Endocytosis

Active targeting of nanoparticles to the organs of reticuloendothelial system could be done by attaching appropriate ligands for the well identified receptors on the target cells belonging to this system. Taking advantage of the presence of mannosyl fucosyl receptors on the macrophage surface, mannose bearing polymeric delivery systems have been designed and used with appropriate antileishmanial drug for site-specific delivery in the hamster model of experimental leishmaniasis.[30] These modified polymeric vesicles have been developed by coupling the amino group of phosphatidyl ethanolamine (PE), an essential compound of polymeric vesicles (PLGA) with amino group of p-aminophenyl $\alpha$-D mannoside, in the presence of glutaraldehyde as a bridging agent (Fig. 1). The results demonstrate that because of receptor mediated endocytosis, nanoparticle entrapment of antileishmanial compound enhanced its effectiveness, an effect that seemed to be much greater when mannose bearing polymeric vesicles are used. Similarly, nanoparticles coated with the polymer mannan as ligand have been demonstrated to have a 50% enhanced uptake than uncoated nanoparticles by mannose-receptor positive mouse

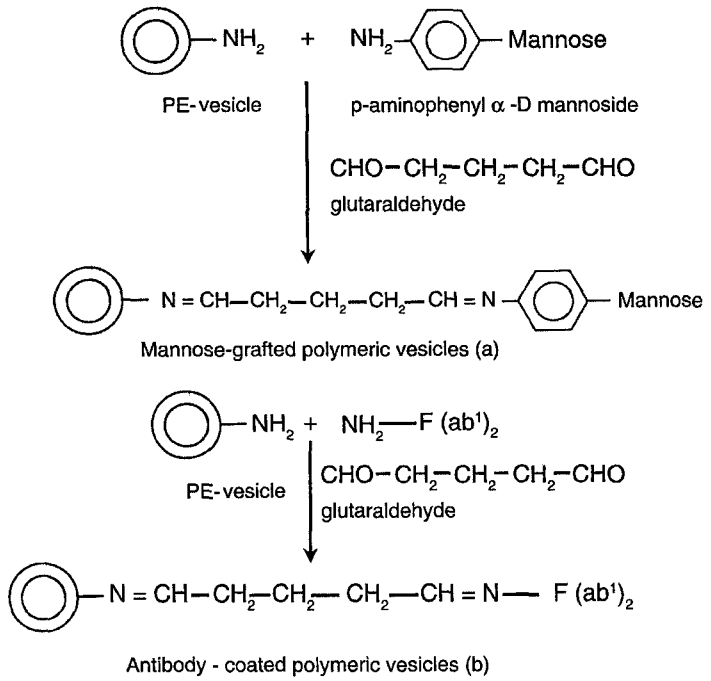


**Fig. 1.** Formation of mannose-grafted polymeric vesicles (a) and antibody-coated mannose-grafted polymeric vesicles (b).

macrophage cell line (J774E), by the process of receptor mediated endocytosis.[31] Alternatively, the possibility of grafting a monoclonal antibody raised against a parasite-specific antigen onto the polymeric vesicle surface cannot be ignored for active targeting of an antileishmanial compound. Besides the grafting of the synthetic mannoside or the coating with the polymer mannan, similar results could be obtained when indigenous glycosides, e.g. Bacopasaponin C and Arjunglucoside I, both isolated from the Indian medicinal plants, *Bacopa monniera* and *Terminalia bellerica* respectively, having glucose at the terminal end of glycosidic chain (Fig. 2), are incorporated in PLA nanoparticles and are subjected to test for antileishmanial property, using both free and nanoparticle-incorporated forms.[8,32] Much better therapeutic efficacy could be noticed with the polymeric vesicles incorporated with either of the two glycosides compared with the glycoside-unincorporated control vesicles. The unique presence of a glucose residue at the terminal end of the glycosidic chain, equipped the compounds to be self-targeting molecules that can be directed towards the glucose receptors present on the macrophage surface for facilitating a receptor mediated drug delivery to the target cells. Perhaps these are the

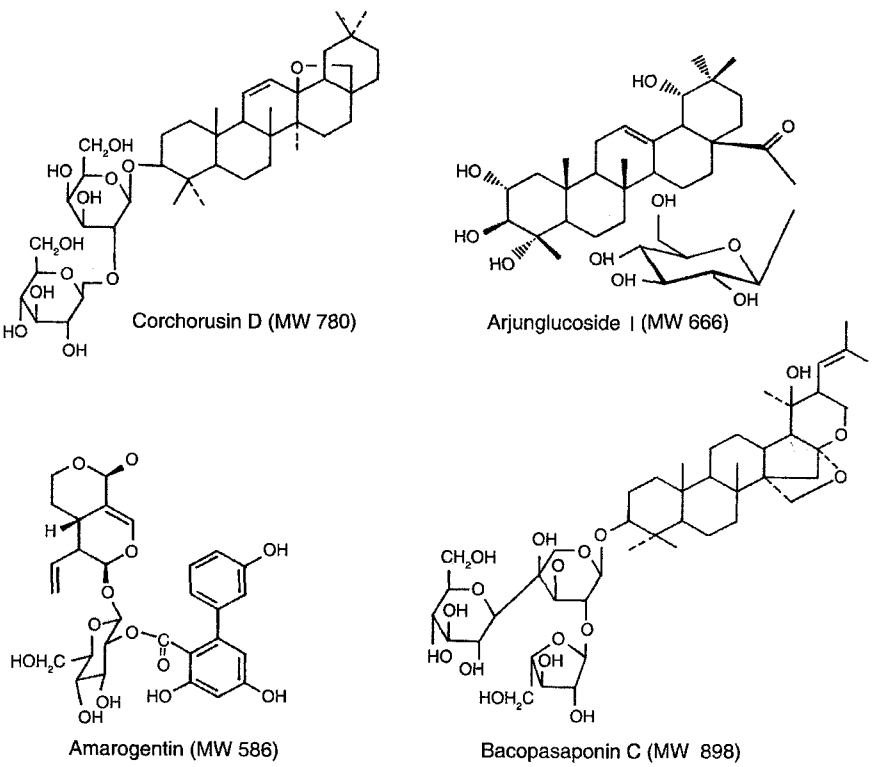


**Fig. 2.** Structures of some glycosides isolated from indigenous sources.

very first reports for the targeted delivery of antileishmanial compounds in experimental leishmaniasis, a RES-associated disorder, using polymeric vesicles as drug carriers.

## 5. Application in Chemotherapy

Among the major RES associated disorders, pulmonary tuberculosis is identified as a killer disease because its death toll every year is enormously high. With a view to develop appropriate delivery systems to test the efficacy of frontline antitubercular drugs *in vivo*, experimental tuberculosis was induced in murine models and nanoparticle-encapsulated antitubercular drugs were administered orally to them at every 10th day. When examined, no tubercle bacilli could be detected in the tissues after 5 such oral doses of treatment. Thus, nanoparticle encapsulated antitubercular drugs turned out to be a potential oral drug delivery system against murine tuberculosis.[33] Alternatively, subcutaneous nanoparticle based antitubercular chemotherapy was also tried. Injectable PLG nanoparticles were found to hold promise for increasing drug bioavailability and reducing dosing frequency for a better management of tuberculosis.[34] However, nebulization via the respiratory route of nanoparticle-based antitubercular drugs were reported to form a sound basis for improving drug bioavailability and reducing the dosing frequency for better chemotherapeutic control of pulmonary tuberculosis.[35]

The next major RES associated disorder is leishmaniasis, which causes substantial human morbidity and mortality in many parts of the world. In an attempt to probe the disease, several new drugs as well as new delivery systems were put forward with a view to increase the drug efficacy and to reduce the drug toxicity. Using nanoparticle-bound pentamidine in a *Leishmania major*/mouse model, ultra structural changes in parasites were noticed by Fusai *et al.*[36] In the parasites inside the Kupffer cells, transmission electron microscopy showed a swollen mitochondrion with a loss of cristae, destruction or fragmentation of the kinetoplast, loss of ribosomes and the destruction of parasite structures except for the subpellicular microtubules. The therapeutic efficacy of several indigenous antileishmanial agents e.g. Bacopasaponin C, isolated from the Indian medicinal plant *Bacopa monniera*, Quercetin, isolated from *Fagopyrum esculentum* and Harmine, isolated from *Peganum harmala*, were not only studied but compared after incorporating them in different vesicular delivery systems against experimental leishmaniasis in hamster models.[8–10] At equivalent quercetin[9] concentration, the nanocapsulated quercetin was found to be the most potent in reducing the parasite burden in the spleen as well as in reducing hepatotoxicity and renal toxicity, compared with free drug or drug in other vesicular forms. Similarly, Bacopasaponin C[8], at an equivalent dose of 1.75 mg/kg body weight and Harmine,[10] at an equivalent dose of 1.5 mg/kg body

weight, were found to be very active in all the vesicular forms, but the best efficacy in the lowering of spleen parasite load was found with the nanocapsulated form. Thus, in each case, the nanoparticle-loaded antileishmanial agent was found to be most efficient in the lowering of spleen parasite load and the efficacy was found to vary in the following order:

Nanoparticles > niosomes > liposomes > microspheres > free drug

and the hepatotoxicity, as well as the renal toxicity was found to follow in the reverse order as shown above. *In vitro* antileishmanial activity of amphotericin B loaded in poly (epsilon-caprolactone) nanospheres was also noted, but the nanospheres did not show any improvement of amphotericin B activity against the resistant strain.[37] Attempt was made to deliver piperine to treat experimental visceral leishmaniasis in mice model using oil in water emulsions known as lipid nanospheres (LN) or fat emulsions. A single dose of 5 mg/kg of lipid nanospheres of piperine was found to significantly reduce the liver and splenic parasite burden.[38] Therapeutic evaluation of free and nanocapsule encapsulated atovaquone was made in the treatment of murine visceral leishmaniasis by Cauchetier *et al.*[39] The liver parasite burdens, evaluated by using the Stauber method, indicated that the atovaquone-loaded nanocapsules were significantly more effective than the free drug.

Trypanosomiasis, another deadly disease caused by the parasite *Trypanosoma cruzi* was also challenged by using nanoparticles of polyalkylcyanoacrylate as a targeted delivery system for nifurtimox. The drug-loaded nanoparticles significantly increased trypanocidal activity compared with the empty one.[40] The use of poly (lactic-coglycolic acid) nanoparticles for targeted oral drug delivery to the inflamed gut tissue in the inflammatory bowel disease was examined.[41] Such a strategy of local drug delivery was considered to be a distinct improvement, compared with existing colon delivery devices for this disease. Efficacy of nanoparticles as carrier systems for antiviral agents in human immunodeficiency virus-infected human monocytes/macrophages was evaluated *in vitro* by Bender *et al.*[42] In the same year, macrophage targeting of antivirals, e.g. azidothymidine, was evaluated *in vivo* as a promising strategy for AIDS therapy.[43] The authors, after analyzing the results, concluded that nanoparticles could be considered as a promising drug targeting system for azidothymidine to the RES organs. They also hypothesized that an increase in drug availability at the sites containing abundant macrophages might allow a reduction in dosage in order to avoid systemic toxicity.

For targeted gene delivery, calcium phosphate nanoparticles were found to be a unique class of non-viral vectors, which can serve as efficient and alternative DNA carriers. Moreover, the surface of these nanoparticles was suitably modified by absorbing a highly adhesive polymer e.g. polyacrylic acid and these surface

modified calcium phosphate nanoparticles were used *in vivo* to target genes specifically to the liver.[44] Chitosan-DNA nanoparticles were designed as gene carriers using a complex coacervation process. The transfection efficiency was found to be cell-type dependent.[45] Conjugation of PEG on the nanoparticles allowed lyophilization without aggregation and without the loss of bioactivity. The clearance in mice following intravenous administration was found to be slower than unmodified nanoparticles, with a higher deposition in kidney and liver. Use of sodium chloride modified silica nanoparticles (SNAP) as a novel non-viral vector with a high efficiency of DNA transfer into cells has already been reported.[29] Previous gene transfer methods using non-viral vectors, such as liposomes or nanoparticles, resulted in relatively low levels (35 to 50% approx.) of gene expression. SNAP showed a better efficiency (about 70%) of DNA transfection into cells, as well as a better protection of DNA against degradation. Intravenous and/or intra-abdominal administration of the SNAP to mice revealed the accumulation of SNAP in the cells of the brain, liver, spleen, lung, kidney, prostate and testis, without any pathological cell changes or mortality, suggesting that they passed through the blood-brain, blood prostate and blood-testis barriers.

Sponge-like alginate nanoparticles were found to be a new potential system for the delivery of antisense oligonucleotides. The aim of this study was to design a new antisense oligonucleotide (ON) carrier system based on alginate nanoparticles, and to investigate its ability to protect ON from degradation in the presence of serum. From the results, such nanosponges were found to be promising carriers for specific delivery of ON to the lung, liver and spleen.[46]

## 6. Summary

During the last few decades, numerous approaches have been explored to modify the biodistribution and bio-availability of the drugs by using carriers systems of colloidal dimensions, e.g. liposomes, micelles and nanoparticles. From the various studies reported in the literature, it can be concluded that the factors responsible for particle uptake are the particle size, their surface charge, surface hydrophobicity and the presence and/or absence of surface ligands. Keeping these key factors in mind, the designing and production of polymeric nanoparticles has been investigated since the late 1970s. The major challenge in the development of particulate carriers for targeting at specific body sites is the preparation of the particles of optimum size with hydrophilic surfaces so as to have long circulation time in the blood and escape from RES scavenging. The body's RES, mainly the Kupffer cells in the liver usually take up polymeric nanoparticles with hydrophobic surface. Therefore, the residence time of these nanoparticles in the blood is considerably small. However, as it has been observed that nanoparticles such as other colloidal drug

delivery systems, on intravenous injection, are rapidly sequestered and retained by the organs comprising the reticuloendothelial system (RES), so that the targeting of nanoparticles to RES is a much simpler process than the targeting to any other organ.

The major defense system of the body, i.e. the reticuloendothelial system or more correctly, the mononuclear phagocyte system can recognize any foreign elements (here the injected nanoparticles) through the opsonization process. The Kupffer cells (macrophages) of the liver and of course to a lesser extent, the macrophages of the spleen and the circulating macrophages play an important role in removing the opsonized particles. Particle size and surface properties of the particles can modulate the process of particle capture. Particles that have large hydrophobic surface are efficiently coated with plasma components and are rapidly removed from circulation. Thus, injected nanoparticles are covered by plasma proteins immediately. The larger particles are trapped in the liver but the smaller ones can reach the general circulation and the modified surfaces can be directed to the inflammation sites, endothelial cells or spleen. Targeting, usually achieved by injecting nanoparticles *in vivo*, is mainly passive, although active targeting is being done very recently. An excellent example of passive targeting is the uptake of nanoparticles by the Kupffer cells of liver. In many cases, this targeting can be exploited to help treatment in disease conditions e.g. leishmaniasis and candiasis, where macrophages are directly involved in the disease processes. For greater specificity, the active targeting of the nanoparticulate delivery systems can be achieved by attaching the targeting ligand, appropriate to the receptors on the target cells, to the surface of the particle conjugate. Monoclonal antibodies and sugar residues are the possible ligands. The hepatocytes in the liver is an important target site for some diseases such as hepatitis, as well as in gene therapy. In gene therapy, the liver can be used as "bioreactors" where the administered gene can be used to express the missing factors such as growth hormones and blood factors. In the liver, the endothelial lining of the blood vessels (sinusoids) have gaps or fenestrations, through which nanoparticle can pass and there is no intact basement membranes below these fenestrations. Thus, the nanoparticles can have a close interaction with the liver hepotocytes. The size of the gap was estimated to be between 100 nm and 150 nm. Hence, recent work in the field has suggested that the size of the nanoparticles should be less than 50 nm in diameter for better interaction with the hepatocytes. The polymeric nanoparticles, besides being biocompatible and biodegradable and having longer circulation time in blood, remain unaffected by circulating lipases that protect the drug from the bioenvironment. In an attempt to acquaint the readers with the sequence of events that are associated with nanoparticulate drug delivery to the RES-associated disorders, this chapter first identifies the reticuloendothelial systems (RES), discusses about the possible mechanisms of the uptake of

nanoparticles by them, and finally, updates the application of drug-loaded nanoparticles in the chemotherapy of diseases associated with RES. Moreover, this chapter contributes to the furtherance of our present knowledge in the area of targeting by suggesting that the composition, surface characteristics and the size of the delivery vesicles are the three important parameters that must be considered when drawing a strategy for efficient delivery.

## Acknowledgment

The authors gratefully acknowledge the financial support provided to M.K.Basu by the Council of Scientific and Industrial Research (CSIR), Government of India, in the form of the Emeritus Scientist scheme.

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# 22

# Delivery of Nanoparticles to the Cardiovascular System

*Ban-An Khaw*

## 1. Introduction

Nanoparticles have become one of the highly desirable drug delivery vehicles in recent years,[1] not only due to the capacity but also due to their longevity. Most nanoparticles in use today are solid nanoparticles. Their applications in biological systems have both advantages as well as adverse effects.[2] However, biocompatible nanoparticles such as liposomes or micells have circumvented some of these adverse consequences.[1] Hood *et al.* reported the use of lipid based nanoparticles (40–50 nm), targeted with organic $\alpha v\beta 3$ ligands, to target the endothelium of tumor vasculature to induce anti-angiogenesis, following the delivery of mutant *Raf* gene.[3] In 2003, Kralj and Pavelic wrote, "the main interest currently lie in improving diagnostic methods and in developing better drug delivery systems to improve disease therapy" relative to the application of nanotechnology.[4] The current chapter will be restricted to review of the application of nanoparticles, primarily nano-lipid vesicles subsequently referred to by the original name, liposomes, to the cardiovascular system, from diagnostic to therapeutic applications including novel cell membrane lesion sealing to gene delivery.

## 2. Targeting the Myocardium with Immunoliposomes

The interest in the use of nanoparticles, such as liposomes, for targeting the cardiovascular system has increased dramatically in recent years. The first application

of non-target specific liposomes for localization in experimental myocardial infarction was reported by Caride *et al.*[5] They showed that plain cationic liposomes localized in the infarct better than neutral or anionic liposomes. However, the first targeted delivery of liposomes in cardiovascular application was reported by us in 1979.[6] Although the exact size of the immunoliposomes used in that study was not determined, both multilamellar and unilamellar immunoliposomes were generated. These liposomes were target-specific and were demonstrated to be able to target cardiac myosin, exposed to the extracellular milieu following experimental acute myocardial infarction. Using the canine model, In-111 labeled antimyosin immunoliposomes were demonstrated to localize in the infarct by gamma scintigraphic imaging, after catheter infusion of the immunoliposomes into the infarcted region. This study demonstrated the first potential application of liposomes as targeted nano-lipid vesicles for the delivery of various pharmaceuticals.

Despite this potential for *in vivo* targeted drug delivery, it was observed that these immunoliposomes also had high non-target organ activities *in vivo*. Organs such as the liver and bone with high reticuloendothelial distribution were prime non-target organs for non-specific immunoliposomes sequestration. Therefore, we reasoned that if the antimyosin immunoliposomes were made to mimic normal cells such as lymphocytes, then these modified immunoliposomes might circumvent the affinity for the reticuloendothelial system. To mimic normal cells, sialoglycoprotein, fetuin, was attached to liposomes by either glutaraldehyde cross-linkage or cholate dialysis method in the presence or absence of immunoglobulins.[7] Although the initial studies were tantalizing, unequivocal demonstration of this phenomenon was not achieved. The only clear cut advantage of sialoglycoprotein modified liposomes over plain liposome in mice was the increase retention of the liposome in the blood of mice at 15 min post intravenous administration ($54.7 \pm 11.0$ vs $41.8 \pm 5.2\%$ injected dose per gram, respectively).

Subsequently, Klibanov and co-workers[8] developed a method to prolong *in vivo* circulation time of liposomes, by coating liposomes with polyethyleneglycol. Torchilin *et al.* applied this method of polyethyleneglycol protection from sequestration by the reticuloendothelial system on antimyosin immunoliposomes, and demonstrated that PEG-antimyosin-immunoliposome with 10% mole PEG had slower blood clearance than PEG-antimyosin immunoliposomes with 4% mol PEG or just immunoliposomes.[9] The half life ($T_{1/2}$) of immunoliposomes in rabbits with experimental acute myocardial infarction was 40 min, whereas the $T_{1/2}$ of PEG-coated immunoliposomes at 10% mole PEG was about 1000 min (16 hrs 40 min) and 4% mol PEG was 200 min. This increase in circulation time enabled enhanced targeting of radiolabeled PEG-immunoliposomes in acute myocardial infarcts. The maximum ratio of infarct to normal tissue for plain liposomes was about 4:1, whereas that of 4% mol PEG-immunoliposomes was 20:1 and that of

10% mole PEG-immunoliposomes was 12:1 at 6 hrs post intravenous liposome or immunoliposomes delivery. The reduction in the uptake ratio of 10% mole-PEG-immunoliposomes is consistent with higher blood activity at the time of sacrifice than with 4% mole PEG-immunoliposomes. If the experiments were carried on longer, absolute uptake in the infarct, as well as the ratios of infarcted tissue to normal with 10% mole PEG-immunoliposomes should become greater than the values for the 4% mole PEG-immunoliposomes.

Torchilin *et al.* later reported that size also affected the targeting potential of PEG-modified immunoliposomes in rabbits with experimental myocardial infarction.[10] It appeared that small PEG-modified-antimyosin immunoliposomes of about 135 (120–150)nm diameter size had the highest accumulation of the intravenously administered immunoliposomes in the target ($0.25 \pm 0.14\%$ injected dose per gram of tissue $\pm$ SD). Non-specific uptake of the same PEG-antimyosin immunoliposomes in normal myocardium was only $0.02 \pm 0.1\%$ ID/g. Unmodified plain liposomes and PEG-modified plain-liposomes had $0.02 \pm 0.01$ and $0.13 \pm 0.10\%$ ID/g respectively in the infarcted myocardium. Normal myocardial activity was respectively $0.004 \pm 0.001$ and $0.017 \pm 0.006$. Antimyosin-liposome injection resulted in $0.14 \pm 0.05$ and $0.007 \pm 0.002\%$ ID/g localization in the infarct and normal myocardium respectively. This resulted in the target to normal myocardial activity ratios of $5.17 \pm 2.35$ for plain liposomes, $8.05 \pm 5.03$ for PEG-plain liposomes, $22.70 \pm 2.38$ for antimyosin liposomes and $14.10 \pm 7.15$ for PEG-antimyosin immunoliposomes. The lower target to non-target ratio of PEG-antimyosin immunoliposomes, relative to anti-myosin-immunoliposomes in the infarct, is due to the higher blood activity of the former at 5 hrs post intravenous administration of liposome preparations ($0.35 \pm 0.11$ vs 0.060.01% ID/g respectively).

When larger liposome preparations (350–400 nm diameter) were used, it was observed that plain liposomes had the same infarct localization activity ($0.02 \pm 0.01$) as the small plain liposomes. However, modification with antimyosin, or with both antimyosin and PEG, resulted in lower target activity (0.09 + 0.04, 0.0.15 + 0.02 respectively) but similar background activity (0.003 + 0.001 and 0.02 + 0.004 respectively). It was reasoned that the lower targeting potential with the larger immunoliposomes was due to the limitation of these larger nanoparticles to extravasate into the extracellular interstitial matrices, even though the blood activities at 5 hrs were similar (larger PEG-immunoliposomes = 0.41 + 0.08 and small PEG immunoliposomes = $0.35 \pm 0.11$). The larger PEG-modified plain liposomes appeared to have similar non-target organ activity as the smaller PEG-liposomes. The mechanism of non-specific accumulation with PEG-modified plain-liposomes may be related to blood activity that allowed longer contact with non-target tissues when PEG modified large and small liposomes were used (0.38 + 0.02 and

0.50 + 0.11 respectively). Both large and small liposomes had $T_{1/2}$s of 10 to 15 min. When they were modified with antimyosin, the $T_{1/2}$s were also similar between 15–20 min. Small PEG-modified liposomes had a $T_{1/2}$ of >1000 min, whereas larger PEG-modified liposomes or small and large PEG-immunoliposomes had $T_{1/2}$s $\geq$ 600 min. It appears that this increase in blood circulation activity raised the background activity, as well as the absolute target activity, when PEG-modified antimyosin immunoliposomes were used. It was concluded that for diagnostic applications where high target to non-target activity is desirable, immunoliposomes would be the best candidate for use. However, in therapeutic applications where absolute concentration of the targeting agent determines the efficacy of the intervention, small PEG-immunoliposomes would be preferable. However, large PEG-immunoliposomes may also be useful due to the larger pay-load capacity of the larger liposomes, despite their lower target activity.

## 3. Other Nanoparticle-Targeting of the Cardiovascular System

Although nanoparticles have been used as targeting agents for tumors, blood and lymphatic vessels, the ultimate utility of such agents in the cardiovascular system is just beginning, even though the first *in vivo* demonstration of the feasibility of immunoliposome-application in the cardiovascular system was reported in 1979.[6] Recently, Lanza and colleagues[11] reported targeting of antiproliferative drugs, such as doxorubicin and paclitaxel, to the vascular smooth muscle cells in cell cultures with a magnetic resonance imaging nanoparticle contrast agent. In this study, the investigators prepared perfluorcarbon nanoparticles containing gadolinium-DTPA-bis-oleate in 2% surfactant comixture of lecithin and cholesterol. The resultant nanoparticles had a mean diameter of 250 nm. These nanoparticles were targeted using a three step procedure.[12] Initial targeting was achieved with biotinylated monoclonal antibody to tissue factor (TF), followed by administration of avidin that bound to biotin. The third step consisted of administration of biotinylated non-gaseous, lipid-encapsulated, perflurocarbon emulsion nanoparticles loaded with doxorubicin or paclitaxel. The study showed that TF-targeted doxorubicin or paclitaxel loaded nanoparticles were more efficient antiproliferative agents than control targeted or non-targeted nanoparticles without drug loading. The same group also showed that *in vivo* targeting with antifibrin antibody enabled visualization of the fibrin clots in canine femoral arteries by intravascular ultrasound imaging.[12]

Another application of targeted nanoparticles in the cardiovascular system was reported by Spragg *et al.*[13] Their study showed that immunoliposomes sporting monoclonal antibody specific for an extracellular domain of E-selectin targeted

human umbilical vein endothelial cells (HUVEC) only after activation of these cells with recombinant human interleukin 1$\beta$. Localization of the targeted immunoliposomes was 13 to 275 fold higher in IL-1$\beta$ activated HUVEC than in unactivated ones. Other investigators have also shown that targeting of other adhesion molecules, such as ICAM-1, with antibodies to ICAM-1 was feasible *in vitro*.[14] Echogenic immunoliposomes targeted with antibodies to ICAM-1, VCAM-1, fibrin and tissue factors have recently been reported for imaging of atheroma in Yucatan miniswine model of endothelial denudation by intravascular ultrasound imaging.[15] Expression of ICAM-1, VCAM-1 and tissue factor, as well as fibrin deposition, were visualized within 5 min of antibody-targeted echogenic immunoliposomes administration.

## 4. Novel Application of Nano-Immunoliposomes

Although most of the applications of nanoparticle size immunoliposomes in the cardiovascular system have been in imaging or targeted drug delivery, in 1995, we reported a novel application of antimyosin immunoliposomes for cell membrane lesion sealing of hypoxic cardiocytes.[16] In this application, we reasoned that cell membrane lesions that develop in myocardial injury and ischemia *in vivo* or hypoxia *in vitro* result in irreversible myocyte death. However, if these cell membrane lesions were sealed prior to extensive loss of intracellular contents, and hypoxia or ischemia is removed, then the injured cells, with the lesions now sealed with a membrane sealing agent, should be able to remain viable and undergo membrane repair. This hypothesis is demonstrated in Fig. 1. The agent of cell membrane lesion sealing was proposed to be antimyosin immunoliposomes.[16] The concept of cell membrane lesion sealing as a repair mechanism is not exclusive to our hypothesis. Many

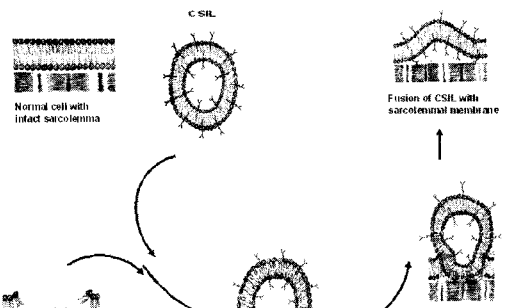


**Fig. 1.** Diagrammatic representation of the process of cell membrane lesion sealing with antimyosin immunoliposomes (CSIL).

cells, including mammalian cells, undergo rapid self-sealing of the ruptured cell membrane.[17–21] This is an innate property of many cells that responds to exposure to higher physiological concentration of Ca++ in the extracytoplasmic environment when lesions develop in the cell membrane, utilizing intracellular membrane vesicles such as lysosomes and endosomes to seal the lesions. This innate mechanism, although highly useful, is not sufficient when development of cell membrane lesions is more extensive.

In our initial report, embryonic cardiocytes in tissue culture were used to demonstrate the role of antimyosin-immunoliposomes as **C**ytoskeletal-antigen **S**pecific **I**mmuno**L**iposomes (CSILs) for sealing of cell membrane lesions induced by vigorous process of induction of hypoxia.[16] Cells ($2 \times 10^6$) in sterile 25 ml culture flasks with or without CSILs were flushed with $N_2$ gas for 4 min vigorously into the media dislodging the cells. The caps were closed tight and the flasks were incubated in a 37°C 5% $CO_2$ incubator for 24 hrs. The viability of the cells were either assessed by trypan blue exclusion method or by [$^3$H]thymidine uptake, after an additional 24 hrs of normoxic culture of the experimental cultures.[16] Figure 2 (left and right) shows that the viability of hypoxic cells treated with immunoliposomes (CSILs) ($96.17 \pm 1.24\%$ by trypan blue exclusion or $3.26 + 0.483 \times 10^6$ cpm by [$^3$H]thymidine uptake) was not significantly different from the viability of normoxic cultured controls ($98.3 \pm 0.58\%$ or $3.68 \pm 0.328 \times 10^6$ cpm respectively), whether viability was assessed by the dye exclusion or [$^3$H]thymidine uptake method. Viability of the CSIL treated cells was significantly greater than the viability of hypoxic embryonic cardiocytes treated with plain liposomes (PL-Hypoxia, $42.3 \pm 3.11\%$ or $1.14 \pm 0.577 \times 10^6$ cpm), IgG-liposomes (IgGL-Hypoxia, $42.85 \pm 6.24\%$), or hypoxia alone ($13.97 \pm 1.77\%$ or $0.115 \pm 0.155 \times 10^6$) (Fig. 2 left panel). Viability of controls

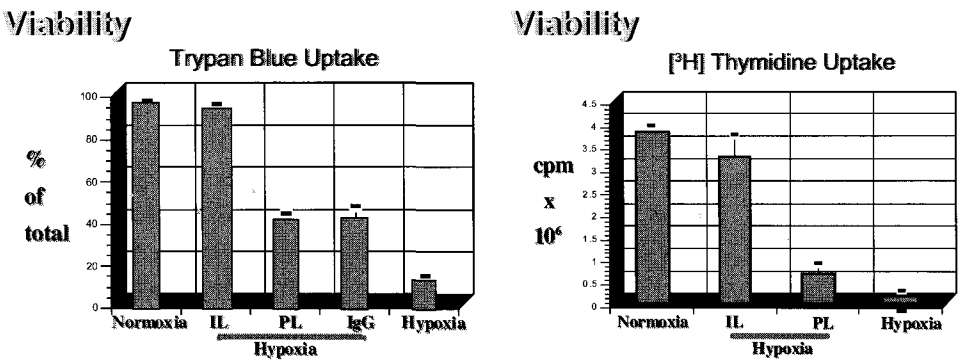


**Fig. 2.** Viability of hypoxic cardiocytes treated with Immunoliposomes (IL), plain liposomes (PL), IgG-liposomes (IgG-L) and normoxic and hypoxic conditions determined by trypan blue dye exclusion (left panel) or with Tritiated thymidine uptake criteria (right panel).

by the dye exclusion method was higher than by [$^3$H]thymidine uptake assessment (Fig. 2 right panel). Although the pattern is similar, the absolute difference may be due to the less stringent approach for the assessment viability by the trypan blue method.

Inclusion of rhodamine labeled lipids into the formulation of the immunoliposomes, enabled visualization of the attachment of liposomes on the surface of hypoxic cardiocytes in culture by epifluorescence (Fig. 3) or by confocal microscopy (Fig. 4). Only hypoxic cells treated with rhodamine liposomes showed epifluorescence (Fig. 3, left), whereas PL treated cells showed no epifluorescence (Fig. 3, right). Similarly, confocal micrographs showed that there were discrete regions of epifluorescence, the diameter of which corresponded to those of the liposomes ($\simeq$ 200–280 nm).[22] Assessment of the number of intact immunoliposomes

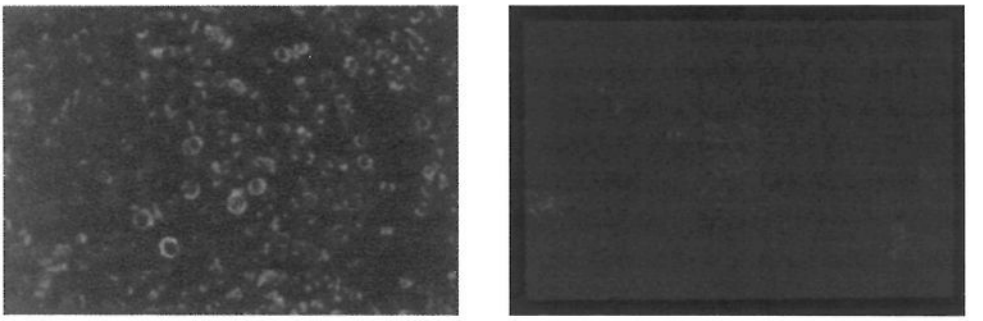

**Fig. 3.** Epifluorescent micrographs of hypoxic H9C2 cardiocytes treated with rhodamine-antimyosin immunoliposomes (left) and rhodamine-plain liposomes (right).

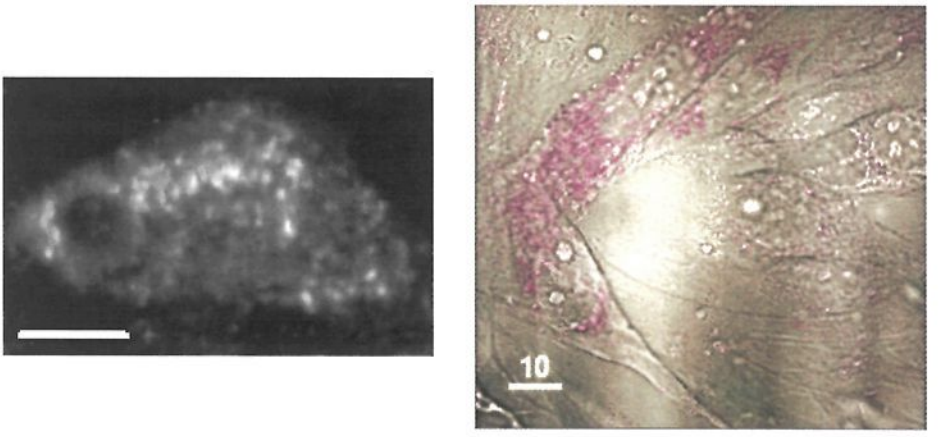


**Fig. 4.** Black and white confocal micrograph showing localization of intact liposomes (left). Pseudocolor of another confocal micrograph showing a pink hue underlying structures which appear to be individual liposomes. The bars represent 10 $\mu$m.

on hypoxic cardiocytes with normal cell morphology resulted in $80 \pm 20$ liposomes per cardiocytes (Fig. 4, left). However, there also appears to be diffused fluorescence in the cell membrane, indicative of the incorporation of the fluorescent lipids from the liposomes into the cell membrane (Fig. 4, right), probably resulting from the fusion of the immunoliposome membrane with that of the cell membrane. The incorporation of the fluorescent lipid from the immunoliposomes to the cardiocytes is not due to the action of lipid transferases, since there are no transferases in the culture medium. However, in *in vivo* situations, such transfer of liposomal lipids to normal cell membrane lipid bilayer could occur.

Preservation of myocardial viability by cell membrane lesion sealing with CSILs was also feasible in adult intact hearts.[23] In this study, immunoliposomes and control liposomes had an average diameter of $200 \pm 35$ nm. Isolated adult rat (CD-1) hearts were perfused with oxygenated Krebs-Henseleit bicarbonate buffer at 37°C, after instrumentation on a Langendorff perfusion apparatus.[23] Hearts were perfused under constant pressure of 80 mm Hg. Each heart was immersed in 0.9% NaCl solution maintained at 37°C and was paced at 300 beats/min (5 Hz). The left ventricular end-diastolic pressure was set at 10 mm Hg, utilizing a water-filled balloon-tipped catheter attached to a pressure transducer. The baseline hemodynamic parameters were recorded using a strip-chart recorder after 10 min of stabilization period. Global ischemia was induced by decreasing the perfusion pressure to zero within 60 seconds. Then, a 2 ml aliquot of freshly prepared 1 mg NGPE modified antimyosin immunoliposomes (CSILs), 1 mg NGPE modified non-specific IgG-liposomes (IgG-L) or placebo (PBS) was infused at various times of global ischemia. Various preparations of liposomes or placebo were administered into the aorta via a three-way stopcock placed 8 cm above the aorta, enabling administration of various agents without turning on the perfusion pump. This process enabled maintenance of global ischemia for the duration of the ischemic period. In all studies, a total global ischemia was maintained for 25 min followed by reperfusion for an additional 30 min. During the reperfusion period, the end systolic and end-diastolic pressures were determined and the difference represented as left ventricular developed pressure (LVDP). LVDP of each heart was then compared with the baseline LVDP and % LVDP of the baseline was determined. When globally ischemic hearts were treated with CSILs at 1 min of ischemia, the recovery of function (mean LVDP $= 98 \pm 14\%$) during reperfusion was near normal LVDP of sham operated hearts ($p =$ NS) (Fig. 5, left), and was highly and significantly greater than the LVDP of hearts treated with placebo ($12 \pm 7\%, p = 0.01$). The total time function curve of the LVDP of hearts treated with CSIL at 1 min of global ischemia was $87 \pm 6\%$ ($p =$ ns versus sham LVDP), but was greater than that of placebo treated hearts ($12 \pm 2\%, p = 0.01$). Injury to hearts after 25 min of global ischemia that were treated with CSIL or placebo, compared with sham operated heart by histochemical

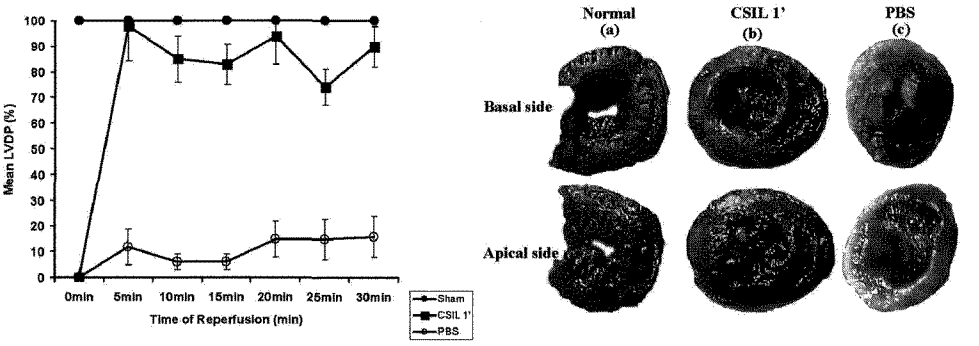


**Fig. 5.** LVDP of globally ischemic or normal hearts treated with CSIL (■), sham operation (•) and placebo (○) during reperfusion for 30 min (left panel), and the corresponding nitroblue tetrazolium chloride stained heart slices showing normal myocardium (stained brown) and infarcted myocardium (no staining, light color).

staining with nitroblue tetrazolium, is shown in Fig. 5, right. Nitroblue tetrazolium stains for dehydrogenase enzyme activity and is seen as brown to purplish brown stained tissues. These enzymes are lost following myocardial or cellular necrosis, resulting in no staining of the infarcted tissues seen in Fig. 5 (right), as light colored regions in the placebo treated hearts. Quantitative assessment also demonstrated that the size of the injury of CSIL treated hearts ($4 \pm 1\%$ of total ventricles) was the same as that of the sham operated hearts ($3 \pm 2\%, p = \text{ns}$).

If interventions were instituted almost immediately after the onset of global ischemia, then preservation of structure and function of the myocardium would be 100%. However, in real-life situations, time of initiation of injury to intervention cannot possibly be that short in most circumstances. Therefore, studies were also undertaken to determine whether there is a time dependency on myocardial function and structural preservation relative to CSIL administration. Thus, Langendorff instrumented hearts were subjected to global ischemia as before, however, administration of CSIL or control non-specific IgG-L was instituted at 5, 10 and 20 min of global ischemia. Reperfusion was instituted at 25 min and reperfusion sustained for 30 min. In globally ischemic hearts with CSIL administration at 5 min of ischemia, return to near normal LVDP was achieved at 10 min of reperfusion (Fig. 6, top left panel); when CSIL was administered at 10 min of global ischemia, return to near normal function was at 15 min of reperfusion (Fig. 6, top right panel). However, when CSIL was administered at 20 min of global ischemia, recovery of function was only $50 \pm 7\%$ of baseline LVDP (Fig. 6, bottom left panel), which was still greater than the LVDP of hearts injected with IgG-L ($29 \pm 5\%, p = 0.01$). Yet, the mean LVDP of all hearts treated with CSIL was statistically greater than the LVDP of hearts treated with non-specific IgG-L at corresponding times [Fig. 6 (bottom right panel)]. Infarct sizes determined by computer planimetry of the nitroblue

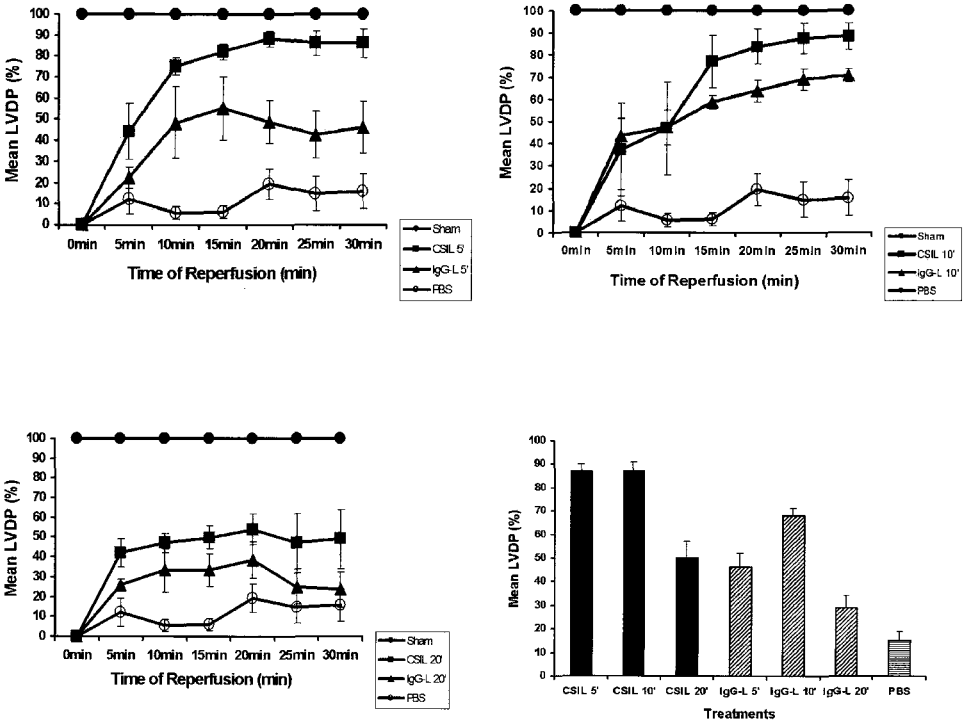


**Fig. 6.** LVDP of globally ischemic adult rat hearts treated with CSIL (■), IgG-L (▲) or placebo (○) relative to sham instrumented control hearts (●) at 5 (top left panel), 10 (top right) and 20 min (bottom left panel). Mean LVDP of hearts from 20 to 30 min of reperfusion are shown in the bottom right panel.

tetrazolium chloride stained heart slices showed that hearts treated with CSIL at 1, 5 and 10 min had similar injury as that of the sham operated hearts ($4 \pm 1\%$, $8 \pm 3\%$ and $6 \pm 2\%$, and $3 \pm 2\%$ respectively. $P$ = NS). The infarct size of hearts treated with CSIL even at 20 min of global ischemia was $19 \pm 3\%$ of the ventricles. This was significantly smaller than its corresponding control ($p \leq 0.05$), whereas hearts treated with control IgG-L at 5, 10 and 20 min of global ischemia were $39 \pm 4\%$, $35 \pm 7\%$ and $45 \pm 6\%$ respectively (Fig. 7, left panel). The corresponding nitroblue tetrazolium chloride stained heart slices are shown in Fig. 7, right panel.

Another parameter of myocardial injury that was determined was mitochondrial size. Although mitochondrial swelling is a hallmark of ischemic injury, irreversible injury cannot be directly extrapolated from just observation of mitochondrial size. Nevertheless, in view of the myocardial functional and histochemical evidences, mitochondrial size assessment from transmission electron micrographs add additional support for myocardial preservation in CSIL treated hearts, relative to IgG-L or placebo treated hearts. Figure 8 shows the comparison of

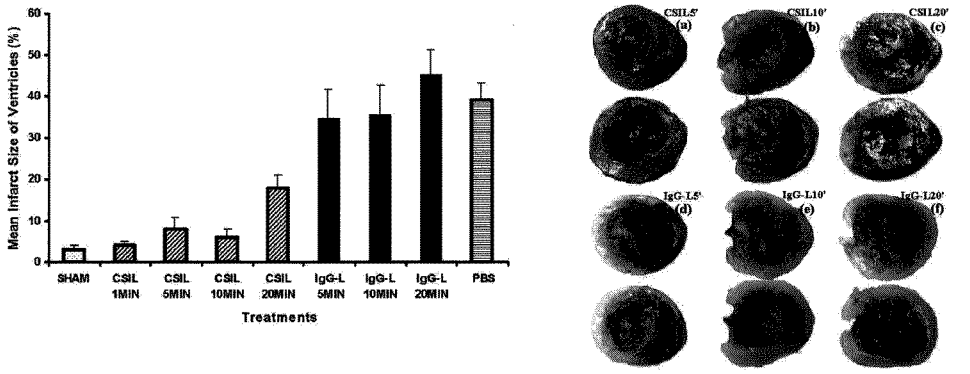


**Fig. 7.** Mean infarct sizes of rat hearts treated with CSIL or IgG-L or placebo at 1, 5, 10 and 20 min of global ischemia (left panel). The corresponding nitroblue tetrazolium chloride stained mid slices of rat hearts treated with CSIL or IgG-L at 5, 10 and 20 min of global ischemia. Minimal injury was seen in 5 and 10 min CSIL treated hearts, but injury was evident in the 20 min CSIL treated heart slice. Injury is evident in all heart slices treated with IgG-L (right panel, bottom two rows).

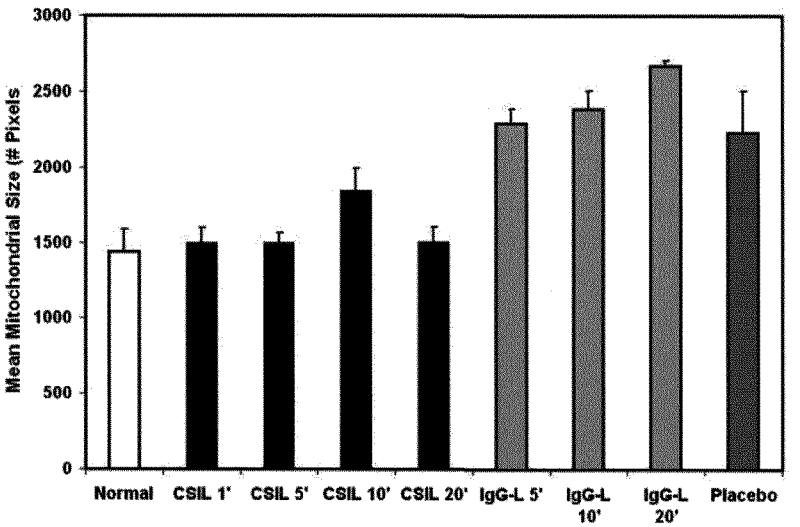


**Fig. 8.** Mean mitochondrial size of normal, CSIL, IgG-L or placebo treated hearts. Treatment was as indicated in the text.

mitochondrial size of normal hearts, CSIL treatment at 1, 5, 10 and 20 min of global ischemia, as well as IgG-L treated hearts at 5, 10 and 20 min of global ischemia and with placebo. No difference in mitochondrial size was observed between normal myocardium (1441 $\pm$ 146 mean number of pixels $\pm$ SEM) and myocardium treated at 1, 5, 10 and 20 min of global ischemia (1496 $\pm$ 103, 1496 $\pm$ 66, 1845 $\pm$ 147 and 1504 $\pm$ 101 respectively) ($p$ = NS). However, mitochondria of hearts treated with

IgG-L at 5, 10 and 20 min of global ischemia or placebo ($2294 \pm 95$, $2387 \pm 119$, $2667 \pm 37$ and $2234 \pm 270$ respectively) were larger than mitochondria of CSIL treated hearts ($p < 0.05$). These studies showed that myocardial viability preservation is not restricted to embryonic cardiocytes in cultures. Adult hearts are also amenable to structural and functional preservation, following cell membrane lesion sealing in a time-dependent manner during ischemia. This method of cell membrane lesion sealing has also been reported to preserve the integrity of vascular endothelium with antiactin-immunoliposomes.[24]

A question that remains concerning the utility of CSIL is whether immunoliposomes can retain the protective functions in the presence of plasma proteins *in vivo*, since experiments have demonstrated that cells in culture and adult hearts perfused with non-protein oxygenated buffer were prevented from undergoing myocardial necrosis, following cell membrane lesion sealing intervention with cytoskeletal-antigen specific immunoliposomes. To demonstrate that cell membrane lesion sealing also occur *in vivo*, rabbits with experimental myocardial infarction were used. In this study, rabbits were injected with anti-myosin CSIL, plain liposomes or saline at the time of left circumflex coronary artery occlusion by intracoronary infusion.[25] The occlusion was kept for 45 min followed by 6 hrs of reperfusion. The hearts were excised, sliced into $\simeq 5$ slices parallel to the short axis and stained with nitroblue tetrazolium chloride. The infarct was approximately 5 to 10% of the infarcts of the control plain liposome or saline treated rabbit hearts.[25] Subsequently, comparison to IgG-liposome treated hearts with acute myocardial infarction demonstrated that the CSIL treatment resulted in significantly smaller infarct size, as was observed in comparison to plain liposome or saline treated hearts.

Thus, cytoskeletal-antigen specific immunoliposomes, consisting of antimyosin or antiactin-immunoliposomes, were demonstrated to be able to preserve cell viability and integrity. Its potential utility in the cardiovascular system would be enhanced once its efficacy following intravenous delivery has been demonstrated. However, the study of Asahi *et al.* showed that intravenous delivery of the antiactin-immunoliposomes enabled preservation of the integrity of the endothelial cells of the cerebral vessels.[24]

## 5. CSIL as Targeted Gene or Drug Delivery

Due to the proposed mechanism of cell membrane lesion sealing, we also proposed that if drugs or gene constructs were to be included in the immunoliposomes such as CSILs, then these drugs or gene constructs should be delivered directly into the cytoplasm (Fig. 9). This route should bypass the endocytic route of drugs or gene construct delivery, thereby reducing destruction of the delivered cargo by the lysosomal enzymes, after formation of endolysosomes. Using silver grains as model

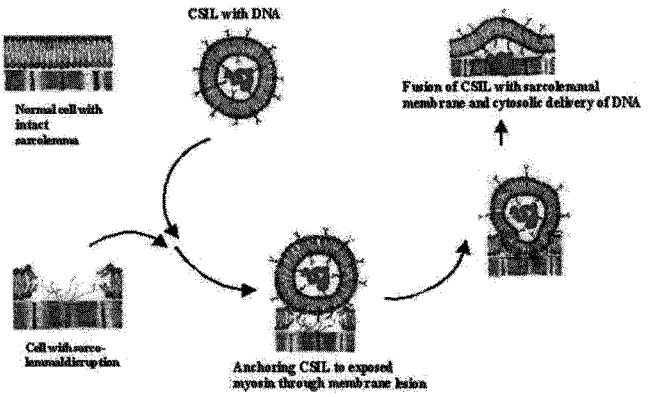


**Fig. 9.** Diagrammatic representation of delivery of intraliposomally entrapped genetic construct or drugs directly into the cytoplasm of target cell.

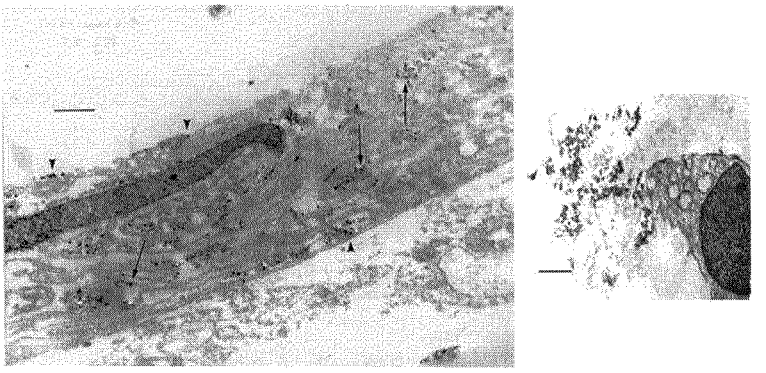

**Fig. 10.** Transmission electron micrographs of embryonic cardiocyte treated with silver grains impregnated CSIL (left) and plain liposome impregnated with silver grains (right). — = 1 $\mu$m.

drugs, we demonstrated that these drugs can be delivered directly into the cytoplasm of hypoxic cardiocytes treated with silver grains loaded CSILs.[16,25] Figure 10 (left) shows a transmission electron micrograph of a cardiocyte treated with silver grains impregnated CSILs. Silver grains in groups of concentration at about 200 nm were observed. However, in cells treated with silver grains impregnated plain liposomes, very few cells were viable. Of one such cell detected by transmission electron microscopy, the silver grains were observed in the extracellular space [Fig. 10 (right)].[25]

When the silver grains were replaced with genetic constructs, pGL2 and pSV-$\beta$-gal vectors, hypoxic cardiocytes treated with CSIL impregnated with either vectors showed luciferase activity or bacterial $\beta$-galactosidase activity. The successful transfection of the hypoxic cardiocytes with pGL2, a vector for fire-fly luciferase enzyme,

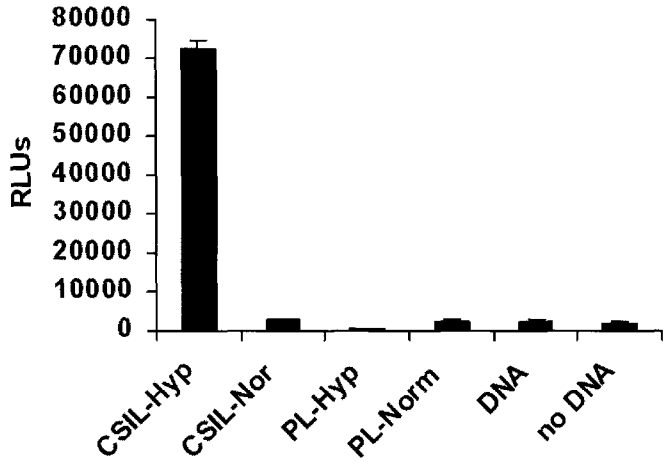


**Fig. 11.** Relative light units of luciferase activity of cardiocytes treated with various preparations and controls.

in CSILs is shown in Fig. 11 as relative light units (RLUs). RLUs were determined by the use of a luminometer.[25] As can be seen, only hypoxic cardiocytes treated with pGL2-CSILs showed increased RLUs significantly above normal cells with treatment with no vectors. Similarly, normoxic cardiocytes treated with pGL2-CSIL, hypoxic cardiocytes and normoxic cardiocytes treated with plain liposomes, or with only vectors, showed no significant gene transcription and expression. When hypoxic cardiocytes were treated with CSIL with entrapped pSV-$\beta$-gal vectors, almost all cells in the field of view under light microscopy exhibited bacterial-$\beta$-galactosidase enzyme activity, following reaction with X-Gal (0.2% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 2 nM $MgCl_2$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$ in phosphate buffered saline pH 7.4) [Fig. 12(A)]. When this mode of gene expression was compared with transfection of pSV-$\beta$-gal vector with cationic liposomes, cationic liposome transfection according to the manufacturer's protocol resulted in transfection of only a few cells per field of view [Fig. 12(B)]. In this micrograph, two cells with intense $\beta$-galactosidase activity were observed. Quantitation of the number of cells in the field of view that was successfully transfected with pSV-$\beta$-gal vector in CSIL, cationic liposome, IgG-liposomes, plain liposome and vector alone are shown in Fig. 12(C). Only CSIL and cationic transfection showed gene expression. CSIL-transfection or Csilfection was more than 40 times more efficient in transfecting cells than cationic liposomes. Although the intensity of gene expression was low with Csilfection, using the initial vector concentration of 75 $\mu$g of vectors in 13.5 mg lipids in 3 ml, when the vector concentration was increased to $\simeq 200\ \mu$g, also in 13.5 mg lipids in 3 ml, the intensity of gene transfection was increased [Fig. 12(D)]. This study showed that approximately $3 \times 10^{-12}\ \mu$g

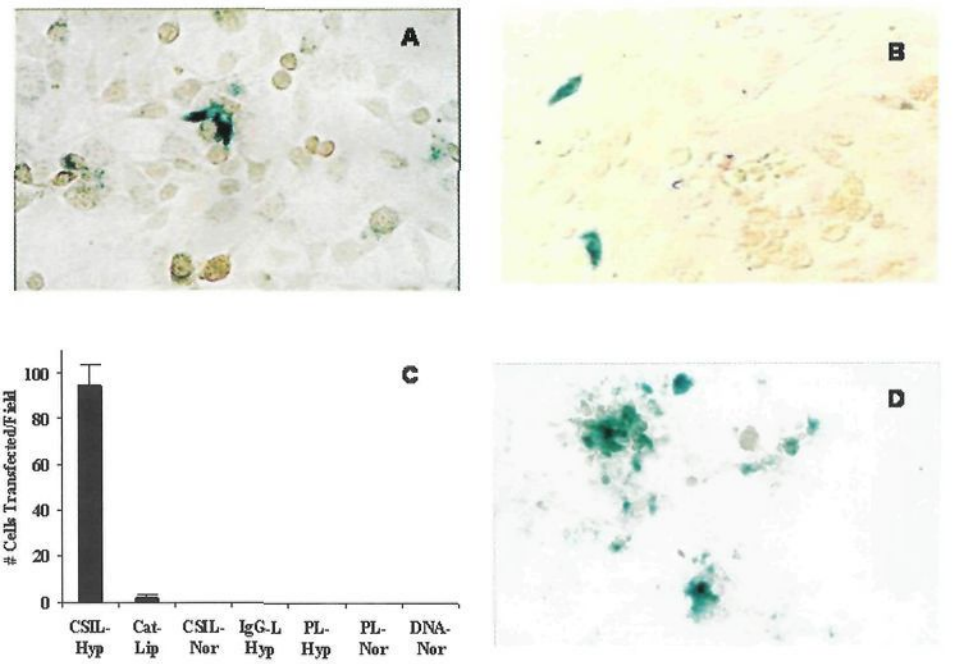


**Fig. 12.** **A**. Cardiocyte transfection with psV-$\beta$-galactosidase-CSILs at 50 $\mu$g/ml vector concentration. Magnification $\times$ 400. **B**. Cationic liposome transfection with psV-$\beta$-galactosidase vectors as per package insert. Magnification $\times$ 400. **C**. Number of cells transfected with psV-$\beta$-gal vectors by CSIL, cationic liposomes or plain liposomes. **D**. Csilfection with 140 $\mu$g/ml psV-$\beta$-galactosidase vectors. Magnification $\times$ 100.

of DNA were delivered into the cytoplasm of each cardiocytes, whereas cationic liposomes delivered approximately $9.5 \times 10^{-6}$ $\mu$g DNA per cell.[25] Yet, by increasing the DNA content by 3 times in the CSILs, the intensity of $\beta$-galactosidase expression was increased to the level of cationic liposome transfection, with at least 40 times more cells transfected.

## 6. Conclusion

The application of nanoparticles in the cardiovascular system is finally becoming desirable. As this chapter has shown, the initial foray into this field occurred in 1979, even though the term nanotechnology was not coined for at least a decade. Nevertheless, its potential as drug delivery and targeting for therapy and diagnosis were recognized earlier on. To date, the application of targeted nanoparticles in the cardiovascular system has included targeting the endothelium of atherosclerotic lesions and other inflammatory processes, gene delivery to ischemic cardiocytes and cell membrane lesion sealing with cytoskeletal-antigen specific immunoliposomes. Other applications such as targeted drug release from nanoparticles, after

targeted drug localization in the cardiovascular system, is envisioned for future therapy.

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# 23

# Nanocarriers for the Vascular Delivery of Drugs to the Lungs

*Thomas Dziubla and Vladimir Muzykantov*

The lungs perform a vital multifunctional physiological role. Yet, the pulmonary vasculature is susceptible to a host of pathologies, which contribute to morbidity and mortality. Many medical interventions can improve the course and outcome of these disease conditions, provided they can be delivered in an effective, localized and safe manner. Venous administration is a suitable route for drug delivery to the pulmonary vasculature, but most drugs do not have the pharmacokinetic features required for optimal pulmonary delivery. In theory, this problem may be overcome through the use of nanocarriers, which can act to improve the localization of drugs in the pulmonary vasculature and allow for a more controled release/activity profile for drugs that are otherwise cleared or inactivated rapidly. Several types of nanocarriers are potentially useful for this purpose including protein conjugates, liposomes and polymer nanocarriers. Stealth coats improve carrier circulation, while affinity ligands provide targeting. Yet, despite these promises and many experimental advances, significant obstacles must be overcome to permit clinical utility. This chapter gives a background of the biomedical aspects of lung targeting, introduces basic elements of current design of systems for vascular drug delivery to the lungs, and discusses specific applications where nanocarriers can improve current therapies, as well as the limitations of existing nanocarrier technologies in this context.

## 1. Introduction

Due to its critical, diverse physiological roles and high vulnerability to pathological processes, the pulmonary vasculature represents an important pharmacological target. In order to manage lung pathologies, a plethora of diagnostic and therapeutic treatments including contrast agents, isotopes, anti-inflammatory, anti-thrombotic and antioxidant agents, anticancer and anti-proliferative agents, enzyme replacement therapies (ERT), has been proposed. Yet, due to unfavorable natural pharmacokinetic properties, many of these strategies are currently not in use. For instance, despite the diversity of the chemical classes of these therapeutic agents, many of which are bio-therapeutics, e.g. proteins, most of them do not naturally accumulate in the lungs after intravascular injection, thereby greatly limiting their effectiveness and specificity.[1]

Many of these limitations may be overcome by the use of nanocarriers, which can improve drug delivery to the therapeutic site by passive and active targeting. Furthermore, nanocarriers can optimize the pharmacokinetic properties of drugs by: (1) increasing the delivery potential of poorly water-soluble drugs (2) providing extended release of drug in localized areas (3) enhancing the circulation life-time and (4) isolating sensitive/bioactive drugs from the blood, protecting from premature inactivation and systemic adverse effects. This chapter focuses on nanocarriers designed for drug delivery to the pulmonary vasculature. It begins with a brief background of the lungs as a therapeutic target and describes nanocarriers design, potential applications, current limitations and avenues for optimization and translation into the clinical domain.

## 2. Biomedical Aspects of Drug Delivery to Pulmonary Vasculature

While gas exchange, providing blood oxygenation in the vascular system, is the most important pulmonary function, the lungs serve a variety of other vital functions.[2] For instance, the pulmonary vasculature, a unique anatomical and functional compartment itself, acts as an anatomical filter for thrombi, aggregates activated or damaged blood cells and other types of emboli (e.g. lipid, gas) in the venous blood, which would otherwise embolize cerebral vasculature, resulting in stroke. In addition, with enzymes exposed on the luminal side of the vascular walls, it functions as a reactor bed for the blood, converting circulating agents (e.g. peptides, mediators and hormones), and thereby affecting systemic signaling and physiology.

The pulmonary vasculature is the primary interface between the systemic circulation and the exterior environment. Hence, it is vulnerable to the damaging effects of extraneous (e.g. inhaled pollutants, particulates, and pathogens) and endogenous

pathological factors (e.g. circulating thrombi, pathogens, tumor metastases). In particular, the pulmonary vascular endothelium, a cellular monolayer lining the luminal surface of blood vessels, is involved in many pathological conditions, local and systemic, and represents an important target for diverse diagnostic, prophylactic and therapeutic interventions. This chapter will focus on advanced drug delivery systems designed to achieve this specific goal.

## 2.1. *Routes for pulmonary drug delivery: Intratracheal vs vascular*

Drugs can easily reach lung tissue through either intratracheal (IT) or intravenous (IV) administration. Upon considering pulmonary drug delivery, IT administration (e.g. aerosols, inhalants) is the first to come to mind. It provides a route for non-invasive means of drug delivery to airway compartments (e.g. bronchial epithelium and interstitium)[3] and beyond, into the systemic circulation. As such, this is an ideal situation for drugs such as asthma medications, where bronchiolar delivery is required, or for systemic delivery of drugs (e.g. hormones), which can pass via the epithelial cells and other components of gas-blood barrier.[4–6]

However, for diseases where delivery to pulmonary endothelial cells is needed, IT administration effectiveness is limited. This route provides patchy delivery with inconsistent alveolar reach.[7] Since the alveoli are the area of greatest vasculature density with slowest perfusion, it is the key, yet relatively difficult to reach, site for the transport of drugs from the airways to circulation. Furthermore, once transport to the vascular space does occur, nothing keeps drugs from fleeing into the systemic circulation, thereby resulting in insufficient local residence time and concentration, thus limiting therapeutic effects in the pulmonary endothelium.

In contrast to the intratracheal route, IV is naturally designed to aid the delivery of circulating compounds to the pulmonary endothelium. The pulmonary vasculature is the first major microvascular network, which represents one third of the entire vascular surface area, encountered by IV injected drugs. In addition, the lungs receive half of the cardiac output at each systole (i.e. entire venous blood), whereas all the other organs share the other half (i.e. arterial blood). Also, the rate of blood perfusion through the high-capacity, low pressure vascular system in the lungs is relatively slow (see below), favoring interactions of circulating ligands with pulmonary endothelium. For these reasons, this review will focus on vascular targeting of the pulmonary endothelium by IV injection.

## 2.2. *Pulmonary vasculature as a target for drug delivery*

To design systems for pulmonary vascular drug delivery, one has to know pertinent features of lung vasculature physiology. For example, in order to cope

with bouts of high cardiac blood output and to satisfy oxygen demands, the lungs possess a high transient perfusion capacity. Hence, blood pressure and the rate of perfusion in the lungs are significantly lower, compared with systemic vasculature.

Several mechanisms regulate perfusion via pulmonary vasculature to adjust to changing cardiac blood output and ventilation rate. The lower lobes of both left and right lungs are perfused more effectively than the apical lobes. This inequity is matched by a similar ventilation pattern that exists between basal and apical areas of the lungs. Hence, lower lobes will receive more injected or inhaled drugs (Fig. 1).

A substantial fraction of pulmonary capillaries are only transiently perfused and they get recruited in physical stress for a greater blood volume exchange, optimizing the rate of gas exchange. These transiently perfused vessels, forming a reserve perfusion capacity to suit physical stress, can also be recruited to cope with the redistribution of blood flow in cases of localized and systemic pathologies (e.g. heart diseases). For instance, when vessels are partially or fully occluded (e.g. by fibrin thrombi or activated white blood cells), the adjacent vasculature is

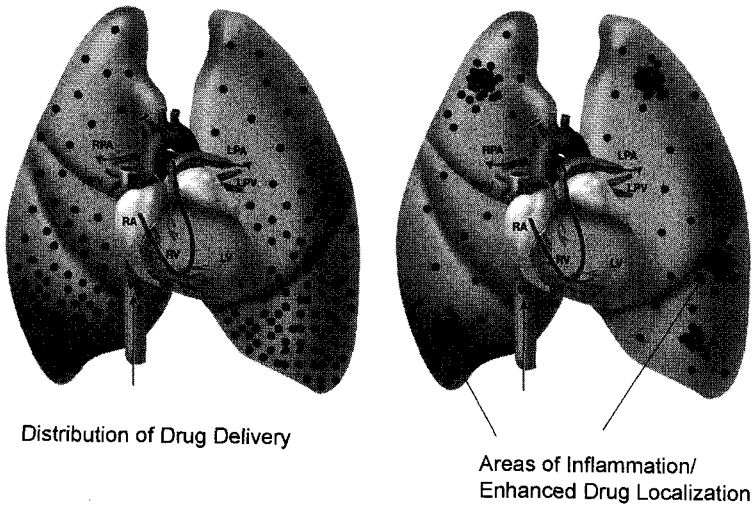


**Fig. 1.** Normal and pathological pulmonary blood perfusion patterns affect distribution of delivered nanoparticles. Under normal conditions, due to preferential perfusion and ventilation of lower lobes, this area of lungs will accumulate higher loads of nanocarriers (left). Enhanced permeability of pulmonary vasculature will drive preferential delivery of nanoparticles to sites of acute inflammation, hence passive targeting (right). Areas of inflammation may be reached by EPR effects or by active targeting of nanocarriers with coated with antibodies to cell adhesion molecules expressed preferentially in areas of inflammation. This allows for both the treatment of lung inflammation and non-invasive visualization areas of lung pathology.

recruited to meet perfusion demand and compensate for the pathological deficit. This ability to rapidly respond and alter flow patterns, allows the lungs to function as a filter for debris that would otherwise embolize the brain and the other organs.

Pulmonary perfusion changes under pathological conditions, thus affecting drug delivery. In addition to focal perfusion changes caused by thrombosis or inflammation (Fig. 1), generalized pulmonary vascular pathologies markedly alter hemodynamics in this organ. For example, primary pulmonary hypertension, depending on the phase of the disease, might lead to either acceleration or deceleration of pulmonary perfusion. Congestive heart disease, defects of the right heart valves and the insufficiency of pumping function of the right ventricle, may all result in blood pooling and stagnation in the pulmonary vasculature. All these factors may affect pulmonary delivery of injected drugs.

## 3. Pulmonary Targeting of Nanocarriers

Selective localization of drugs in the site of interest can be achieved by passive and/or active targeting. Passive targeting refers to the accumulation of carriers not involving specific recognition of the target compartment in the body, and includes mechanical and charge retention, and the enhanced permeation and retention (EPR) effect. In most cases, active targeting that employs recognition moieties possessing specific affinity to target determinants (e.g. antigen-antibody[8–10] or receptor-ligand,[11–14]) affords greater specificity of drug delivery. This section reviews these strategies for delivery nanocarriers to the pulmonary vasculature.

### 3.1. *Effects of carrier size on circulation and tissue distribution*

Whether passive or active targeting is used, nanocarrier size can affect its distribution, circulation and subcellular localization (Fig. 2). When carrier size is $< 100$ nm, permeation across endothelial and epithelial barriers is possible via transcellular and peri-cellular pathways.[15] Sub-micron carriers are less likely to pass through intercellular junctions in endothelial and epithelial cells, with the exception of organs with fenestrated endothelium having large, few micron openings, such as in the liver and the spleen. However, even relatively large carriers of $\sim 500$ nm in diameter, are still capable of being internalized either via receptor-mediated (e.g. endocytosis) or constitutive (e.g. macropinocytosis) pathways. Cellular internalization allows for a more precise level of control of subcellular destinations including lysosomes, other intracellular compartments and cytosol, or even beyond the endothelial cells.[15]

Micron carriers still allow circulation without embolism, although the likelihood of either barrier penetration or cellular internalization is greatly limited.

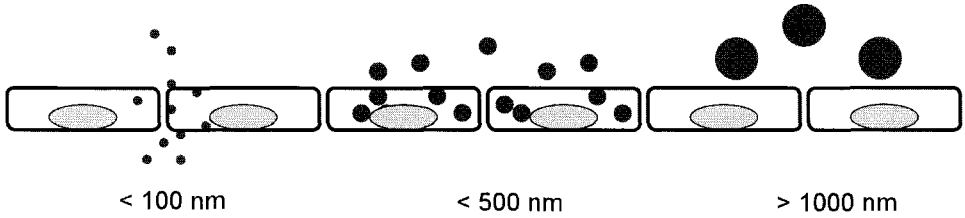


**Fig. 2.** Effect of carrier size on transport through vascular endothelium. Nanocarriers < 100 nm diameter are capable of passing though certain endothelial barrier either between the cells or via transcellular mechanisms involving endocytosis. Nanocarriers < 500 nm poorly transport between endothelial cells in the lungs, yet they are still capable of being internalized by endothelium. Particles larger than 1 $\mu$m, may still be capable of circulating and being targeted, yet they are unlikely to leave vascular lumen in the lung unless pathological factors induce abnormally high vascular permeability (leakiness, not shown).

Such size ranges provide a mechanism for maintaining targeted drug carriers to reside on the luminal side of the endothelium, an ideal situation for drugs that require blood/plasma contact for therapeutic activity.[16]

Size also determines the carrier's fate in the circulation. Despite the fact that sub-micron size range permits unimpeded vascular circulation, nanocarriers are cleared from the bloodstream within minutes via uptake by reticuloendothelial system (i.e. RES, including hepatic and spleenic resident macrophages available to the blood, via openings in the vessels). In mice, this can result in 60–90% clearance of the injected dose in the first instance.[17–19]

Grafting the surface of nanocarriers with large molecular weight hydrophilic polymers, negative or neutral, the primary example being poly(ethylene glycol) (PEG), greatly extends the circulation time.[17,20] PEG modified carriers (stealth) have a hydrophilic molecular brush that repels cellular and protein interactions, thus reducing recognition and uptake by RES.[21] Tissue uptake of PEG-coated carriers depends more on mechanical retention than on active recognition and phagocytosis by RES; hence smaller, carriers circulate for longer duration than large ones.

There is growing evidence that carrier geometry is critical to circulation and cellular localization effects. For instance, worm-like micelles have been reported to align with flow, a feature that has been hypothesized to extend and prolong the circulation of stealth carriers.[22] Also, liposomes containing polymerized micelles possessed both an elongated, ellipsoidal shape, as well as a greatly enhanced circulation profile.[23,24] It is not clear whether these effects are a result of improved fluid dynamics or phagocytic evasiveness. However, this effect allows for additional levels of design/control of circulation, and perhaps other pharmacokinetic features of nanocarrier systems.

## 3.2. *Passive targeting*

### 3.2.1. *Mechanical retention*

Microspheres larger than the pre-capillaries (i.e. >10 micron diameter) injected into the venous system, embolize the downstream capillary bed. Thus, the site of injection dictates the localization site; hence, targeting lung vasculature can be achieved by simply injecting into a pulmonary artery or an upstream femoral vein.

Since embolism occurs at the first bifurcation that is too small for carrier passage, targeting is limited to the arterioles. Delivery to the venous sites occurs only in a form of released drug passage through this downstream vascular compartment. Furthermore, while pulmonary vasculature can tolerate low levels of embolism, it is not a fully benign process, resulting in ischemic vascular pockets losing contact with the blood flow and the nutrient exchange. Yet, mechanical retention of degradable microcarriers in the pulmonary vasculature has medical utility, e.g. for visualization of the lung blood vessels and perfusion patterns using radiolabeled microspheres. Furthermore, newer treatments for massive hemoptysis (the coughing up of blood) have focused on the embolization of the bronchial arteries.[25]

While microspheres embolize vasculature, nanocarriers' size allows for unobstructed flow throughout all vessels. Yet, nanocarriers can also be designed to associate into micron-sized aggregates, prior to or upon injection, which are then delivered and mechanically retained in the capillary bed (Fig. 3). Through the proper selection of nanocarrier size and rate of aggregate breakup, either subcellular or transcellular compartments can be reached, disconnecting embolism and drug delivery, and allowing for shorter durations of ischemia with a longer term drug delivery phase.

Further, $<500$ nm diameter nanocarriers may provide a more favorable degradation pattern, compared with solid microspheres degrading via either surface erosion or bulk degradation. For a more detailed discussion, see the reviews at Refs. 26–28. Since surface erosion results in the overall shrinking of a particle, the remnant microspheres will eventually be washed away from the delivery site, prematurely terminating local effects. Bulk degradation is more suitable for a stable deposition of microspheres, since the overall structure remains intact until the polymer has degraded to the point where structural integrity is completely lost. Yet, under a continuous back pressure in the vasculature, particle disintegration can result in highly disordered debris of various sizes, geometry and surface roughness that can induce local and systemic damage. In the case of aggregated nanocarriers, such hazardous debris is likely to be avoided, since individually released nanocarriers possess designed nano-scale geometry, permitting non-obtrusive behavior in the circulation.

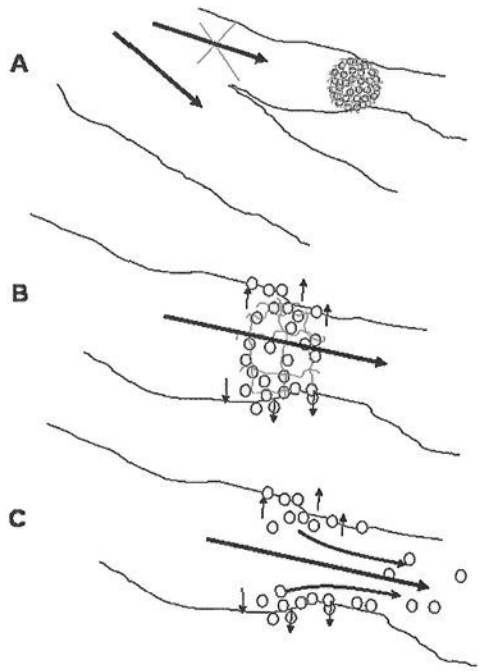


**Fig. 3.** Mechanical retention of nanocarriers in the pulmonary vasculature. (A) In the presence of cross-linking stimuli (e.g. plasma opsonins or circulating ligands in blood), large (~10–50 $\mu$m) aggregates of nanocarriers will form after injection and embolize the pulmonary capillary bed, thus creating a high local concentration of a drug and ceasing blood flow. (B) As the aggregate disintegrates, released individual nanocarriers can diffuse into the surrounding tissue via inter-endothelial gaps or/and transcellular pathways, allowing them to accumulate in the pulmonary interstitium. Disintegration of emboli initiates reperfusion of blood. (C) As disintegration proceeds and blood flow is reestablished, released nanocarriers will be washed away. Drugs delivered by and released from aggregated nanoparticles will be eliminated by the restored flow.

### 3.2.2. *Charge-mediated retention and non-viral gene delivery*

Nanocarriers possessing a positive surface charge accumulate in the first vascular bed, similar to the targeting behavior of mechanical retention, although the mechanism of retention is different. The highly anionic glycocalyx covering the endothelium binds cationic molecules and particles.[29–31] In cell cultures, such binding has resulted in the internalization and enhanced levels of transfection by non-viral DNA delivery means, e.g. cationic liposomes.[32] Yet, many blood components are also negatively charged. Hence, the aggregation of serum components and/or the thrombus formation resulting in embolism may also occur.[33]

High levels of lung targeting due to charge retention in the pulmonary vasculature have been displayed by IV injected cationic liposomes and carriers decorated with either cationic polymers (e.g. polylysine) or peptides (e.g. TAT) sequences.[30,34]

While it is not clear if *in vivo* localization is due to particle-endothelium association or aggregation, it does provide an interesting mechanism for the internalization and cytosolic delivery of DNA for gene delivery. Interestingly, in many instances, charge-mediated retention of the non-viral gene delivery means in the pulmonary vasculature results in transgene expression in cells underlying endothelium (e.g. vascular smooth muscle cells), but not in endothelial cells *per se*.[35]

### 3.2.3. *Pulmonary enhanced permeation-retention (EPR) effect*

The enhanced permeation and retention effect was originally described when long circulating stealth liposomes were found to accumulate into vascularized solid tumors, due to the erratic, highly permeable nature of the tumor vasculature.[36,37] As nanocarriers circulate and encounter this area, characterized also by poor lymphatic drainage, leakage into and retention in the interstitium resulted in gradual accumulation. EPR targeting improved with increased circulation times, and when nanocarrier size is small enough to pass through the pores in the leaky vessels of $< 200$ nm.

A similar mechanism has been found to enhance the delivery into the sites of inflammation, where the vasculature is also highly permeable.[38] Since the pulmonary vascular bed receives the entire venous blood flow and is highly susceptible to enhanced vascular permeability under pathological conditions, it is plausible that EPR-related accumulation in the lungs might occur. This mechanism might permit the visualization of inflammation sites in the lungs and provide a means of treating localized pulmonary inflammation and edema (Fig. 1).

## 3.3. *Active targeting*

Active targeting involves the engagement of specific recognition ligands with surface determinants present in the site of interest. This can be achieved by either using immunoglobulins raised against target antigens, affinity peptides or using a native ligand receptor pair. For a review of endothelial determinants used as targets and antibodies, and other affinity ligands used as vectors for active drug targeting into the pulmonary vasculature, please see reviews at Refs. 8 and 39. A brief list of the key guidelines in pulmonary target selection includes the following factors:

(1) The target should be present on the luminal surface of pulmonary endothelium, accessible spatially and temporally, and should not be down regulated or masked in disease states. For example, adhesion of activated blood cells and accelerated shedding inhibit targeting to some constitutive endothelial determinants.[40] On the other hand, determinants exposed on the endothelial

cells under pathological conditions (e.g. selectins) have a distinct transient surface expression profile, which may permit selective drug delivery to pathologically altered endothelium, but require exact timing of administration to match the duration of target availability.

(2) The target should not be present in non-endothelial counterparts that are accessible to the circulating nanocarriers. For example, endothelial cells have transferrin receptors, which are also abundantly exposed in hepatic cells that are accessible to the bloodstream. As a result, transferrin-targeted drugs accumulate in the liver with minimal delivery to the lungs. Also, analogues of the target determinants circulating in the blood (e.g. soluble forms of transmembrane glycoproteins or P-selectin on platelets) will compete with endothelial counterparts, compromising targeting.

(3) Targeting should not cause harmful side effects in the vasculature. Binding of targeted drugs may cause shedding, internalization, or inhibition of endothelial determinants, which may be detrimental. For example, thrombomodulin, a surface protein responsible for thrombosis containment, is abundantly expressed in the pulmonary vasculature, providing high pulmonary targeting specificity.[41] Yet, its inhibition by antibodies may provoke incidences of thrombosis that prevents clinical potential for drug delivery. Ideally, engaging of the target should provide therapeutic benefits such as the inhibition of pro-inflammatory molecules.

(4) It is ideal for the docking to a surface determinant to result in optimal sub-cellular addressing of a drug.[15] Thus, depending on the therapeutic goal, a targeted nanocarrier should either be retained on the cell surface (blood therapies) or undergo trafficking to a proper sub-cellular compartment (e.g. nucleus in the case of DNA,[41] or lysosomes in the case of enzyme replacement therapies[42]).

No single targeting suits all therapeutic needs. Specific therapeutic goals require different secondary effects mediated by binding to the endothelium, drug targeting to different sub-populations of endothelial cells, and diversifying the cellular compartments. A plethora of affinity carriers, sometimes directed to relatively similar endothelial targets (e.g. cell adhesion molecules) or even binding to different domains of the same target molecule, are currently explored to capitalize more fully on unique opportunities offered by vascular targeting.[8,39,43]

Strategies for defining molecular determinants (targets) for affinity delivery of nanocarriers to endothelial cells, include both high-throughput analyses (e.g. *in vivo* selection of phage display libraries,[43] subtractive proteomics of endothelial plasma membrane[39]) and low-throughput analysis of affinity ligands to identify endothelial molecules with known functions.[44] Some of the most

promising endothelial determinants for such ligands include constitutive antigens such as angiotensin-converting enzyme (ACE),[44–46] cell adhesion molecules of Ig-superfamily (PECAM and ICAM),[16,47,48] inducible adhesion molecules (E- and P-selectins, VCAM-1),[49–53] aminopeptidases and caveoli-associated glycoproteins.[54–56]

## 4. Carrier Design

As a whole, nanocarriers require a "ground up" design approach for each application. Depending on the particular needs of a given strategy, material selection can vary greatly. This section will outline the general considerations of the design of nanocarriers for pulmonary drug delivery.

### 4.1. *Biocompatibility*

The initial material constraint is biocompatibility, a term that might be misleading, without considering the context of a given application. The materials used for nanocarriers should induce no deleterious (e.g. thrombogenic, mutagenic or carcinogenic) effects in the body. These effects (like with any medicines) depend on dose, location, structure, and residence time of nanocarriers. For this reason, while pre-labeling a material as "biocompatible" has been used in many papers, it provides rather limited information to specific situations and applications. A rigorous re-evaluation of carriers' biocompatibility for each new indication in a given pathological context (likely, even in given patients cohorts), does not seem to be an excessive precaution in a post-Vioxx era.

For instance, titanium and titanium oxide coated implants has long been considered a highly inert, biocompatible material in bone prosthetics and dental implants.[57,58] Yet, sub 100 nm nanoparticle forms of titanium oxide have highly active surface sites capable of catalyzing the formation of oxygen radicals, which can result in cell and tissue injury.[59–61] As such, the "biocompatible" label must not simply be given to titanium oxide nanoparticles, although this does not mean that there is no potential therapeutic use of this carrier. However, there are settings in which its use is unadvisable, e.g. drug delivery into the pulmonary tissue which is prone to oxidative stress, due to high level of oxygen and reactive oxygen species produced by leukocytes and pulmonary endothelial cells.[10,62]

On the other hand, some materials that have been previously labeled as non-biocompatible may be revisited for use in nanocarriers, having to undergo degradation and excretion pathways unsuitable for larger carriers. However, the primary requirement of nanocarrier compatibility is the ability to break down into non-toxic, plasma soluble components that can be eliminated via renal filtration or hepatic bile

excretion. For this reason, most carriers under development are composed of either degradable polymers, or possess MWs lower than 40 KDa.[63,64]

## 4.2. *Material selection (by application)*

### 4.2.1. *Imaging*

The lungs are a classically difficult organ for imaging due to low-signal to noise ratio, multiple air-tissue interfaces, and physiological motion such as cardiac and ventilating.[65,66] Of all imaging technologies available, the most commonly used technology for pulmonary imaging (except routine chest X-rays) is computer tomography (CT). Yet, it is still difficult to properly identify many pulmonary disease pathologies. The use of targeted contrast agents may allow for the improved identification of these disease states.[66] In the case of CT, high density materials (e.g. metals, crystalline polymers and high atomic weights) are ideal candidates. Indeed, early studies using iodinated nanoparticles have been used for the imaging of lymph nodes.[67,68]

In spite of its utility, CT resolution is limited to $\sim 1$ mm. NMR, a higher resolution imaging technology, has been classically limited to the use of pulmonary imaging. Yet, current advancement in imaging algorithms and contrast targeting may improve NMR imaging of diseases such as acute pulmonary embolism and chronic infiltrative disease.[66,69,70]

### 4.2.2. *Gene delivery*

Initial success with gene delivery to the pulmonary tissue was obtained using adenoviral carriers. Indeed, heat shock protein HSP70, nitric oxide synthase (NOS), and interleukin-10 have all been adenovirally transfected into pulmonary endothelial cells, for the attenuation of ischemia-reperfusion injury.[71–73] However, systemic adenoviral transfection is greatly limited due to an associated cytokine release and immune response. In this context, enhancement of local transfection by re-targeting viral gene delivery is a highly promising strategy[74,75] to pulmonary endothelium (e.g. using ACE antibody coupled to viral particles).

Non-viral gene delivery poses an interesting set of material requirements, allowing for the effective delivery of DNA into a target cell and the subsequent trafficking of the DNA into the nucleus. These carriers must be able to load high levels of DNA into a single particle, and be able to target endothelial cells with the subsequent internalization and endosomal escape mechanism to allow for the DNA to reach the nucleus. Most of these processes have focused on charge coupling to condense DNA into a nanoscale aggregate. The most common of these have been the use of cationic polyplexes.[76,77] For example, polycationic electrolytes such as

poly(ethylenimine) (PEI) and poly(l-lysine) (PLL) have been used to condense the negatively charged DNA. PEI (of small chain length) has been shown to reverse charge at endosomal pH and release DNA.[78,79]

Pulmonary vascular delivery of DNA was possible with cationic surface charge alone,[80] yet lung specificity can be greatly improved upon application of immunotargeting toward endothelial markers such as thrombomodulin,[41] PECAM-1[81] or ACE.[82,83]

While highly cationic vectors also display a significant inflammatory response,[84] this immune reaction can be greatly attenuated without a reduction in degree of transfection by lowering the overall carrier charge.[29,32,85]

### 4.2.3. *Delivery of therapeutic enzymes*

Examples of enzymatic therapies amenable pulmonary targeting using nanocarriers, include delivery of: (i) lysosomal enzymes (enzyme replacement therapy, ERT), for the treatment of non-neuronal lysosomal storage diseases that affect pulmonary endothelium (e.g. Niemann–Pick disease),[42] (ii) anti-thrombotic enzymes (e.g. plasminogen activators) for the dissolution of blood clots formed or lodged in the pulmonary vessels, and (iii) antioxidant enzymes, for the containment of vascular oxidative stress in the lungs, which is a highly morbid pathological condition.

Targeting can be achieved by the chemical coupling of enzymes with affinity carriers, producing nano-scale protein conjugates. For example, catalase conjugated with antibodies to endothelial antigens ACE, PECAM or ICAM, accumulates in the lungs of laboratory animals after IV injection and protects against oxidative injury in the models of human diseases such as lung transplantation ischemia/reperfusion injury[86] and acute edematous vascular oxidant stress.[87] On the other hand, targeting of plasminogen activators to endothelial cell adhesion molecules boosts antithrombotic capacity of the pulmonary vasculature.[16] Targeting enzymes clearly illustrates the importance of proper sub-cellular addressing of drugs, namely, luminal surface for fibrinolytics, non-degrading intracellular compartments for antioxidants, and lysosomes for ERT.[42]

Loading into nanocarriers might optimize some of the enzyme therapies. For example, antioxidant catalase loaded into $H_2O_2$-permeable, protease-resistant polymer nanocarriers[88] might retain its protective activity even within lysosomes. Yet, loading into highly amphiphilic carriers (i.e. micelle form, vesicle form) may cause undue folding and inactivation of enzyme. Optimally, the carrier material would stabilize protein in an anhydrous state to avoid inactivation. This can theoretically be achieved via the hydrophobic sequestering of solid protein into a polymer core.

### 4.2.4. *Small molecule drugs*

Liposomes have already seen FDA approval for the delivery of small molecule delivery.[89] Doxorubicin, an amphiphilic anticancer agent, has a great therapeutic potential, yet it is complicated by questionable low solubility, high toxicity and poor circulation. By loading in aggregates in the liposome core, it has been able to target tumors via the previously mentioned EPR effect with greater doses than previously possible. As illustrated by this example, the key advantage is the ability to enhance serum solubility of the small molecule drugs and achieve longer release profiles. In pulmonary settings, this has been used for the enhancement of free radical scavengers,[90] enzyme inhibitors,[91] and in anticancer treatments.[92,93]

## 4.3. *Types of nanocarriers*

Nanocarriers utilizing natural biomaterials or structures (e.g. liposomes consisting of natural phospholipids found in cellular plasma membranes) were the first to be explored for drug delivery.[94] Since then, designs have included solid nanoparticles, double emulsion nanoparticles, polymeric micelles, polymersomes and worm-like micelles. Synthetic materials, especially polymeric materials, offer great freedom in that they can be designed to enhance circulation, reduce immunogenicity, provide environmentally responsive elements and possess biologically derived properties, also known as biomimetic properties, such as adhesion response elements and receptor ligands. All these carriers are amenable for pulmonary delivery. For a detailed review of the formation mechanisms and technical aspects of nanocarrier formulation, please refer to the reviews at Refs. 28, 95–98.

## 4.4. *Mechanisms of drug loading*

The main mechanisms for loading drugs into nanoparticles include surface absorption, aqueous inclusion, solid-phase immobilization, and complexation aggregates (Fig. 4).

Surface absorption occurs via either hydrophobic interactions between the particle surface and hydrophobic interactions (e.g. tryptophan, tyrosine, phenylalanine for proteins) or electric charge interactions.[99] This method is not effective for coating stealth nanocarriers, due to the nature of stealth mechanism, but can be used for the coupling of targeting moieties (see below) and therapeutic agents to non-stealth nanocarriers.

In the context of pulmonary vascular targeting via IV route, stealth characteristics are not critically important due to the option of first pass delivery

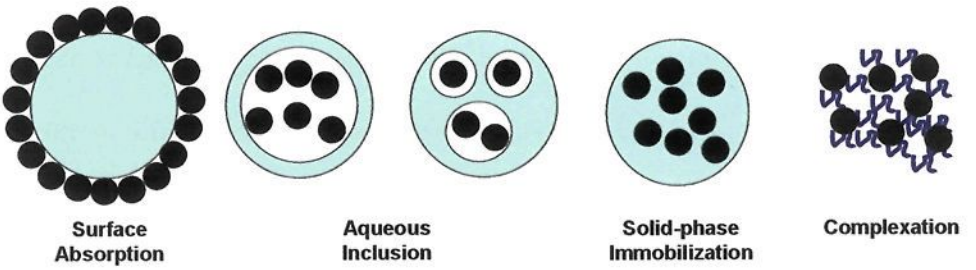


**Fig. 4.** Methods of nanocarriers loading with therapeutic agents. In the nano-scale range, surface absorption offers the greatest drug/particle loading, and most likely accounts for a fraction of loading in all reported nanocarriers, including those loaded by the alternative approaches. However, isolation of a cargo en route to target is most effective with inclusion mechanisms of loading. Currently, aqueous inclusion methods are most extensively explored for the loading of hydrophilic agents into polymer nanocarriers. Therapeutic effect may be achieved via either release of cargoes or diffusion of their substrates via polymer. Solid-phase immobilization is mostly used for loading of hydrophobic solutes, yet some proteins may also be amendable to this mechanism. Complexation relies upon the interaction of drug and polymer for particle formation, which permits formation of size-controled loaded vehicles. However, homogeneity of nanocarriers and drug release from these carriers are difficult to control. Carrier materials (e.g. polymers) are shown in a light grey color, drug loads are shown as dark spheres.

mechanism. Indeed, latex poly(styrene) beads used as model prototype non-stealth nanocarriers (100 nm diameter) coated with surface-absorbed anti-ICAM, but not control IgG, showed very high pulmonary uptake after IV injection in mice.[48]

Surface absorption does not protect a cargo from inactivation en route or in aggressive intracellular compartments (e.g. lysosomes), nor does it limit systemic side effects of circulating drugs. However, it may prolong circulation time, alter tissue targeting, and subsequently alter sub-cellular addressing of the drugs.[15] It is the easiest method for nanocarrier loading with large MW drugs (e.g. therapeutic proteins).[48,99–104] Latex beads surface coated with anti-ICAM and a therapeutic enzyme (catalase) provide a useful tool to the study of binding, internalization and degradation pathways for nanocarriers targeted to endothelial cells, the main cellular target in the pulmonary vasculature.[48,102,105]

Liposomes can be loaded by aqueous core inclusion and by hydrophobic association within the lipid bilayer.[19,106] Liposomes provide a large internal aqueous cargo compartment separated from milieu by the bilayer membrane. Since the cargo remains in an aqueous environment, its molecular mobility and enzymatic activity are not compromised. Liposomes afford effective loading and delivery of small hydrophobic agents (e.g. doxorubicin in Doxil®). In polymer nanocarriers, a polymer layer can provide even more protective barrier

via either self-assembly mechanisms employed in synthesis of polymersomes,[107] double emulsion formation mechanism,[88] or in nanoscale hydrogel synthesis techniques.[108,109]

Solid-phase immobilization is an alternative strategy in which crystallized or lyophilized protein and small MW drugs are loaded as a suspension within the solid core of an organic, hydrophobic nanoparticle. High loadings of certain hydrophobic drugs have been reported. For instance, irinotecan, an anticancer therapeutic, was capable of being loaded at 4.5 wt% into 120 nm nanoparticles, composed of diblock PEG-poly(lactic-co-glycolic acid).[110] This method may provide an added benefit in the delivery of bioactive drugs. The organic environment restricts mobility for some therapeutic protein resistant to unfolding, that may paradoxically yet simultaneously reduce activity and extend functional use.[98,111,112] Moreover, since the protein is not in a soluble state, loading is not constrained by aqueous solubility limits and the entire particle core could support inclusion; hence, this mechanism may provide highly effective loading.

The fourth mechanism for loading employs the complexation of a drug with the carrier material. Common approaches to complexation include inter-ionic associating mechanisms, the biotin-streptavidin cross-linking system, or covalent bonding. For instance, regular polymeric micelles of poly(ethylene glycol)-b-poly(aspartic acid) were formed in the presence of the positively charged lysozyme.[113] Complexes can also take the form of polyplexes (e.g. poly(ethylimide) (PEI) and DNA), or in a single polymer chain coupling multiple proteins.[64,114] This latter form has been popularized by the use of hydrophilic polymers such as poly(n(2-hydroxypropyl)methacrylamide) (HPMA), which uses amide linkages to covalently attach proteins and small molecules onto the polymer backbone.[115] This also includes the polymer prodrugs that utilize degradable bonds to limit/control the therapeutic release rate.[116,117]

Yet, even hydrophobic associations, disulfide linkages, streptavidin-biotin or antibody-antigen pairs can be used to form drug-polymer complexes. By controling the extent of modification of a therapeutic cargo and the affinity carrier by cross-linking agents and feed conditions, the complexation mechanism can result in nano-sized aggregates with a relatively high degree of drug inclusion.[118] However, these conjugates (polyplexes) are characterized by significant heterogeneity, both in molecular composition and in size. Due to the nature of the conjugation mechanism, release from these systems is typically poor and mainly controled by degradation of the components. Thus, in the case of enzyme therapies (see Sec. 4.2.3), conjugates of this type, function effectively, typically only if enzymes substrates are small and diffusible enough to be accessible within the aggregate core, such as $H_2O_2$ in the case of catalase delivery.[88]

## 4.5. *Drug release mechanisms*

Nanocarriers can provide 3 main mechanisms of release for its drug cargo (Fig. 5). The most commonly considered release profile is that of continuous release (for a more detailed review, see Dziubla and Lowman[119]). Under this regimen, the drug slowly diffuses out of carrier particles over time, allowing for sustained high local concentrations of the drug. However, current nanocarrier formulations typically release ~ 40–70% of the total drug loaded within the first 6 hrs. This does not permit long-term therapy, but is rather suitable for therapies that require a burst release (e.g. gene and cancer treatment).

Ideally, the cargo remains isolated from the systemic circulation and tissues until the intended target cells are reached and the release is triggered.[120] This pattern allows for both the minimization of deleterious side effects, loss of activity, and

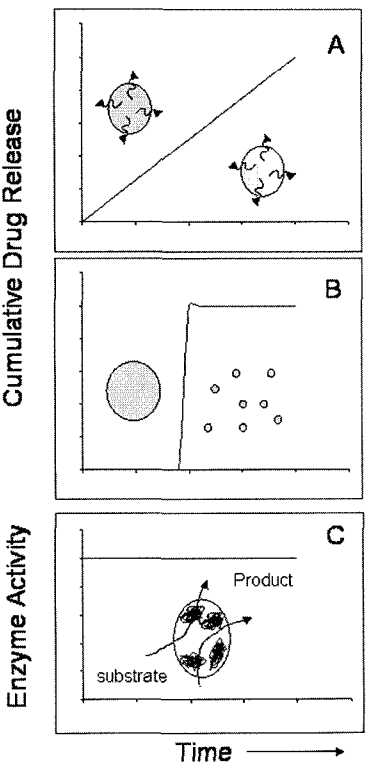


**Fig. 5.** Modes of drug release. (A) Controled release allows for a therapeutic level of drug to be maintained for the greatest amount of time. (B) Delayed burst release is ideal for gene and cancer therapy, where immediate, local high concentrations are desired. (C) Sequestered enzyme delivery allows for a continuous activity of enzyme, even when the nanocarriers reside in compartments typically hostile to protein activity (e.g. lysosomes). In this scenario, carrier must be permeable for enzyme substrates or/and products.

the minimization of the necessary effective dose. Finally, the nanocarrier may also be designed not to release the drug at all. For most instances, this prevents pharmacological activity. However, in the case of enzyme delivery where the substrate is diffusible (e.g. hydrogen peroxide, NO, oxygen, glucose, NAD) across the carrier wall, therapeutic activity may be achievable. This is especially suitable if the final targeting destination is lysosomes, which is likely to degrade the enzyme, thereby resulting in a loss of activity.[88]

## 4.6. *Nanocarriers for active targeting*

In order to achieve active targeting, affinity ligands are coupled to the surface of nanocarriers. Affinity and specificity of these ligands govern targeting. Yet, targeting of multivalent antibody-carrying nanoparticles differs from that of individual maternal antibodies in several important aspects. Firstly, high affinity of such complexes results in highly significant, in some instances, order of magnitude, enhancement of the pulmonary targeting of IV injected nanocarriers *vs* maternal antibodies.[44,47] Secondly, multivalent nanocarriers cross-link endothelial determinants, thus inducing highly effective endocytotic uptake, even though maternal antibodies are non internalizable.[15,47,102,121]

Surface absorption, protein conjugation chemistries or biotin-streptavidin cross-linking can be utilized for the coupling of targeting entities, mainly monoclonal antibodies and their fragments to nanocarriers.[9,105,122] Yet, the most important consideration is that of antibody presentation onto the carrier surface. For example, the antibodies attached covalently directly to the phospholipid head group of PEGylated liposomes, providing rather poor targeting due to the fact that extended PEG chains masked antibodies. This shortcoming can be solved by coupling the antibodies to the distal end of PEG chains. In fact, targeting of such stealth immunoliposomes exceeds that of standard liposomes, due to suppression of clearance mechanisms, and target group mobility and accessibility.[19,122]

One of the most commonly employed conjugation strategies is that of maleimide sulfhydryl chemistry.[123] Maleimide group is more hydrolytically stable than other protein conjugation means, such as the amine directed n-hydroxysuccinate esters. Maleimide reacts with free thiol to create a non-reducible sulfide linkage. Since most proteins do not contain a free thiol group, competition between the drug (e.g. therapeutic protein) and the targeting moiety for available binding sites can be eliminated.

Maleimide can be included onto the distal end of a PEG group in a PEG diblock copolymer.[124,125] Upon nanoparticle synthesis, the PEG chain will extend out into the hydrophilic solution, ensuring the exposure of the maleimide group for subsequent conjugation. This allows for the separation of drug loading and nanocarrier

formation from the conjugation of the targeting group. However, while maleimide hydrolysis is relatively slow at typical nanoparticle synthesis temperatures, it may still occur to a significant extent, thereby limiting the overall capacity for target group addition.

## 5. Conclusion: Safety Issues, Limitations and Perspectives

Results of *in vitro* and animal studies accumulated within the last decade strongly suggest that nanocarriers, especially those utilizing active targeting principles, will eventually provide a versatile and powerful technology platform for optimized drug delivery to the pulmonary vasculature. Extended surface of the pulmonary endothelium represents arguably the best target for drug delivery in the body, hence higher chances for sufficiently specific and effective drug delivery.

On the other hand, in contrast with drug delivery to tumors, in which local toxic side effects can be considered as secondary benefits, safety of drug delivery to pulmonary vasculature is of greater concern. Thus, acute and delayed effects of targeting and endothelial uptake of nanocarriers on health and functions of the lung must be tested extremely rigorously.

For example, pulmonary circulation is sensitive to subtle pro-inflammatory changes, often leading to edema and proliferation of sub-endothelial and interstitial components, pulmonary fibrosis and hypertension. In this context, an important question is how will nano-scale structures residing in a given pulmonary compartment, i.e. vascular lumen, lysosomes, be tolerated? How rapidly bloodstream and pulmonary lymphatic drainage can eliminate products of nanoparticles degradation?

General safety concerns add to these specific issues that are pertinent to pulmonary targeting. Strictly speaking, the actual biocompatibility of materials for carriers remains unknown, until it is carefully tested in adequate clinical settings using carriers of adequate size. For example, nanocarriers based on poly(lactic glycolic acid) polymer, accepted for human use for macro-implants, may degrade into lactic acid and glycolic acid within the target cells, potentially exceeding its metabolic potential. Potentially harmful effects of activation systemic defense systems (i.e. complement, cytokines), overload of clearance systems (e.g. liver, kidneys) and immune reactions, represent general concerns of advanced delivery systems. However, despite these concerns, the most exciting prospect of nanocarriers are the near limitless possibilities for treatment strategies. Nanocarriers may be designed to contain multiple drugs, allowing for complex dosing regimes through just a single injection.

Translational, industrial and commercial issues have to be addressed. For example, dosing (e.g. which drug load and particles dose afford therapeutic effects) and

the timing of treatments have to be tested. Synthesis schemes and reagents readily adaptable to cGMP practices should be explored. Batch to batch variations and processing choices must be minimized, whereas the synthesis yield and drug loading effectiveness must be boosted to warrant practical utility.

Targeting of nanocarriers to endothelial determinants in the pulmonary vasculature promises unprecedented levels of specificity and subcellular precision of drug delivery. Many endothelial determinants potentially useful for drug delivery including ecto-enzymes, cell adhesion molecules and caveolar antigens have been identified by methods including proteomics of endothelial plasma membrane, phage display libraries selections *in vivo* and the tracing of labeled antibodies. High-throughput, discovery-driven approaches such as phage display, map vascular lumen and identify novel targets enriched in defined areas of the lung or endothelial domains. Due to a limited insight into functions of these targets, some of them are unlikely to have a utility for drug delivery (e.g. due to safety concerns), yet all could be used as molecular probes in animal studies.

Careful selection of targets and modulation of valency and size of the antibody-directed nanocarriers help to control intracellular uptake and traffic of cargoes. These parameters can be further fine-tuned, capitalizing on specific features of carriers including relatively labile protein conjugates, liposomes or polymer carriers with built-in rates of degradation and release, and membrane permeating moieties. It is tempting to speculate that the treatment of pathologies, including but not limited to acute lung injury, lung transplantation, pulmonary edema, thrombosis, hypertension and inflammation, will eventually benefit from targeting the delivery of drug nanocarriers to the pulmonary vasculature.

## Acknowledgments

This work was supported by NHLBI RO1 grants HL71175, HL078785 and HL73940, Department of Defense Grant (PR 012262) and Pennsylvania NTI core project. The authors thank Drs. S. Muro, M. Koval and V. Shuvaev (University of Pennsylvania) for the exciting and stimulating discussions and advice.

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# 24

# Nanoparticulate Carriers for Drug Delivery to the Brain

*Jörg Kreuter*

## 1. Introduction

The following chapter deals with a subject that according to the journals *Science* or *Nature*, is of no general interest, namely the brain or to be more specific, drug delivery to the brain (personal communication). The brain is one of the best protected organs of the body, to the outside by the skull and towards the blood circulation by the blood-brain barrier (BBB). The purpose of the BBB is to maintain the homeostasis of the brain, and to allow the creation of a unique extracellular fluid environment within the central nervous system (CNS), whose composition can as a consequence be precisely controlled.[1] The extracellular fluid compartments of the CNS comprise of the brain, the spinal cord parenchymal interstitial fluid and the cerebrospinal fluid contained within the ventricles of the brain, as well as the cerebral and spinal subarachnoid spaces. The structural BBB is created by the endothelial cells forming the capillaries of the brain and the spinal cord.[1] These endothelial cells are characterized by having tight continuous circumferential junctions between them, thus abolishing any aqueous paracellular pathways between these cells.[2] The presence of the tight junctions and the lack of aqueous pathways between cells greatly restricts the movement of polar solutes across the cerebral endothelium.[3] Certain substances may diffuse passively across the brain endothelial cells. This diffusion is dependent on lipophilicity and molecular weight. Drugs with a molecular weight above 500 Da are normally excluded from a passive diffusional transport across the BBB.

However, a large number of drugs that would possess a favorable lipophilicity and molecular weight, which should normally enable an easy transport across the BBB, are rapidly pumped back into the blood stream by extremely effective efflux pumps.[3–5] These pump systems include among others, P-glycoprotein (Pgp), also referred to as multidrug resistance protein (MDR), as well as MOAT (multiple organic anion transporter). Since the brain is dependent on the blood to deliver substrates as well as to remove metabolic waste, the endothelial cells are also required to maintain a high level of carrier-mediated transport systems that enable the entry or the elimination of a variety of endogenous compounds including hydrophilic substances such as hexoses, amino acids, purine compounds, and mono-carbonic substances,[6] as well as lipoproteins including LDL (low density lipoprotein).[7,8] Some of these transporters are unidirectional and some bi-directional in their transport of solutes across the cell membrane. This polarization means that some solutes can be preferentially transported into or out of the brain.[1]

As a consequence, the BBB presents a huge challenge for the effective delivery of a large number of therapeutics to the brain, and, therefore, many attempts have been made to overcome this barrier. For instance, these attempts include the osmotic opening of the tight junctions,[9,10] use of prodrugs or carrier systems such as antibodies,[11,12] liposomes[13,14] and nanoparticles. Opening of the tight junctions by osmotic pressure, however, is a very invasive procedure that also enables the entry of unwanted substances into the brain. The employment of prodrugs may yield a higher lipophilicity, enabling a better permeation and transport into and across the lipophilic endothelial barrier, and/or these prodrugs may use the membrane associated carrier systems. In many cases, however, a suitable prodrug cannot be synthesized, or the resulting molecular weight is too large. Colloidal carriers also can take advantage of these carrier systems present in the BBB. These systems, for instance, include the lipoprotein receptors and the transferrin transcytosis systems, and may be employed in the delivery of drugs by the above particulate colloidal drug delivery systems.

## 2. Nanoparticles

Nanoparticles for pharmaceutical purposes as defined by the Encyclopedia of Pharmaceutical Technology[15] are solid colloidal particles ranging in size from 1 to 1000 nm (1 $\mu$m), consisting of macromolecular materials in which the active principle (drug or biologically active material) is dissolved, entrapped, or encapsulated, or to which the active principle is adsorbed or attached. The use of nanoparticles for the transport of drugs across the BBB was already suggested in the early 1980s by Prof Speiser at the ETH (Swiss Federal Institute of Technology) in Zürich (personal

communication), who was also the first to systematically develop nanoparticles for drug delivery purposes in the late 1960s and early 1970s.[15–17]

The possibility to use nanoparticles for the transport of drugs into the brain across the BBB was first shown with the hexapeptide dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg), a Leu-enkephalin analogue with opioid activity.[18,19] This drug was bound to nanoparticles of a size of about 250 nm, made of the very rapidly biodegradable polymer poly(butyl cyanoacrylate). This material is one of the most rapidly biodegradable nanoparticle materials.[20] The nanoparticles were incubated with this drug for 4 hrs, yielding the sorptive binding of 40% of the initial amount of dalargin. Overcoating of these particles with the surfactant polysorbate 80 (Tween® 80) was then achieved by further incubation for another 30 min with this surfactant, resulting in an equilibrium between surface-bound polysorbate and polysorbate in solution. A dose-dependent antinociceptive (analgesic) effect was observed using the tail-flick test, after intravenous injection to mice (Fig. 1) which was later repeated by other research groups with the hot plate test.[21,22] The antinociceptive effect was accompanied by a pronounced Straub effect and could be totally inhibited by injection of the $\mu$-opiate receptor antagonist naloxone 10 min before the injection of the nanoparticle preparation. Both results indicate a central action of dalargin on the CNS, demonstrating that it was indeed transported across the BBB and that the observed antinociceptive effects were not due to peripheral activity. In contrast to the polysorbate 80-coated nanoparticles, none of the controls including

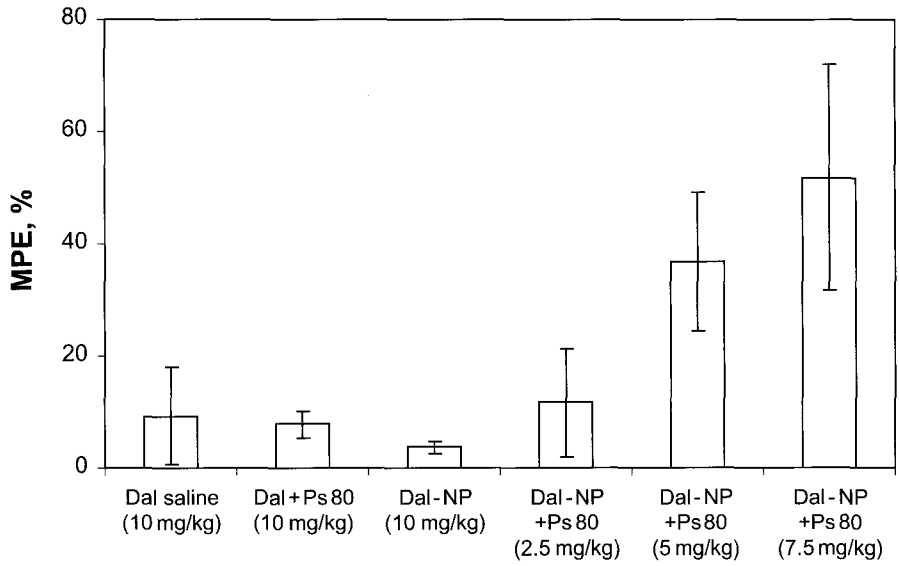


**Fig. 1.** Antinociceptive effects after intravenous injection of different dalargin (Dal) formulations into mice. MPE = maximal possible effect; NP = nanoparticles; Ps 80 = polysorbate 80.

a solution of dalargin, a solution of polysorbate 80, a suspension of poly (butyl cyanoacrylate) nanoparticles, a mixture of dalargin with polysorbate 80, dalargin with nanoparticles or a mixture of all three components, dalargin, polysorbate 80, and nanoparticles, mixed immediately before injection, as well as dalargin bound to nanoparticles without polysorbate 80 coating, were able to exhibit any antinociceptive action (Fig. 1). The antinociceptive effects also showed circadian phase-(daytime)-dependency, as well as a shift of the minima and maxima of the nociceptive reactions of the mice of almost 12 hrs compared with the controls and the dalargin solution.[22]

# 3. Biodistribution

## 3.1. *Influence of surfactants on the biodistribution of nanoparticles*

Fundamental biodistribution studies of Tröster *et al.*[23] with $^{14}$C-labelled poly(methyl methacrylate) nanoparticles demonstrated that the coating of these nanoparticles with certain surfactants increased the whole brain concentrations of the nanoparticles in rats after intravenous injection. However, at that time, the authors were convinced that the nanoparticles were not taken up by any brain-associated cells, including the brain capillary endothelial cells, nor were transported across the BBB, but rather remained in the blood lumen adhering to the luminal surface of the endothelial cells.[23] In addition, it has to be noted that some surfactants in Tröster's experiments led to high [$^{14}$C] brain concentrations, which were unable to achieve any antinociceptive effects with dalargin bound to the nanoparticles.[24]

These important antinociceptive effects in the CNS with the polysorbate 80-coated dalargin nanoparticles reported above (Sec. 24.2) led to the investigation of the biodistribution of this drug after intravenous injection to mice, using $^{3}$H-labelled dalargin in the form of [Leucyl-4,5-$^{3}$H]-dalargin[25] as well as of [$^{3}$H-Tyr]-dalargin.[26] Up to 3-fold higher concentrations in brain homogenates were found with the polysorbate 80-coated nanoparticles than with dalargin solution. These concentration differences were statistically different at most time points, although smaller than expected from the huge difference in the pharmacological responses. However, it has to be considered that the determination of the $^{3}$H-radioactivity in brain homogenates cannot discriminate between drug that has and drug that has not actually crossed the BBB. In addition, the observed concentration differences between different brain homogenate fractions[25] may be the reason for the lack of efficient BBB crossing of dalargin in solution form.

Much larger and important brain concentration differences were obtained after intravenous injection of poly(butyl cyanoacrylate) nanoparticles loaded with doxorubicin.[27] In this case, the drug was added during polymerization. Four

doxorubicin formulations, (1) doxorubicin solution in saline, (2) doxorubicin solution plus 1% polysorbate 80 in saline, (3) doxorubicin bound to nanoparticles, or (4) doxorubicin bound to nanoparticles coated with polysorbate 80, were injected into the tail vein of rats at a doxorubicin dosage of 5 mg/kg. In the brain, the polysorbate 80-coated nanoparticles yielded high doxorubicin concentrations of 6 $\mu$g/g tissue between 2 and 4 hrs after injection. The brain concentrations were still at a level of about 1 $\mu$g/g after 8 hrs, while the three other preparations remained below the detection limit of 0.1 $\mu$g/g at all times. In contrast, very low concentration differences appeared between all four preparations in the blood only. Interestingly, the heart concentrations of both nanoparticle formulations remained very low, confirming earlier results of Couvreur *et al.*,[28] whereas the heart concentrations with the two solutions were about 17 times higher than with the nanoparticles. Since the use of doxorubicin is limited by its cumulative high heart toxicity, this observation is of major significance.

Solid lipid nanoparticles (SLN) were also able to achieve significant brain concentrations after intravenous, and even after duodenal administration. SLNs consisting of stearic acid, the surfactant Epicuron® 200, and taurocholate sodium loaded with doxorubicin,[29] tobramycin,[30] or idarubicin[31] were prepared by dispersing a microemulsion containing the above components in water. At a dose of 6 mg/kg doxorubicin, brain concentrations of about 2 $\mu$g/g were obtained after 180 min only with the SLNs, and no doxorubin was detectable in the brain of rats after i.v. administration of the solution through the jugular vein.[29] With tobramycin (5 mg/kg), the intravenous route was compared with duodenal administration through a surgically implanted cannula. No tobramycin was detectable in the brain after administration of tobramycin solution to the rats. However, with the solid lipid nanoparticles, the amount of tobramycin in the brain 4 hrs after duodenal administration (4.8 $\mu$g/g) was comparable to that after i.v. administration (4.5 $\mu$g/g). The tobramycin brain concentration was decreased 24 hrs after duodenal dosing, while the levels after i.v. administration remained fairly high (5.1 $\mu$g/g). In all other tissues except the brain, the tobramycin levels were higher after i.v. administration of the solution than those obtained with the solid lipid nanoparticles.[30] Duodenal administration of idarubicin, at a dose of 1mg/kg bound to solid lipid nanoparticles, yielded brain concentrations of about 11.2 ng/g after 24 hrs. This concentration was similar to that in the heart (11.5 ng/g) and about half of that in the liver. No detectable idarubicin nor the metabolit idarubicinol was found in the brain after administration of the solution.[31]

Solid lipid nanoparticles consisting of stearic acid, soybean lecithin, and the surfactant poloxamer 188 (Pluronic® F68) loaded with the anticancer drug camptothecin were produced by high pressure homogenization.[32] The *in vitro* release of the drug lasted for one week. After intravenous injections of 1.3 mg/kg camptothecin to mice, the drug residence time in the body was significantly prolonged by

the nanoparticles compared with the solution, and the plasma AUC was increased by a factor of 5, the brain AUC even by a factor of 10, and the AUC in other organs by a factor of between 2 (lungs) and 8.7 (heart). An increase of the dose to 3.3 mg/kg camptothecin (factor 2.5) in the nanoparticle formulation further increased the plasma AUC by 2.7, the brain AUC by 2.6, and the AUC in the other organs by 2.9 times on the average.

Incorporation of 3′,5′-dioctanoyl-5-fluoro-2′-deoxyuridine into solid lipid nanoparticles also increased its brain uptake.[33] After i.v. injection of the SLNs, its brain AUC was increased two-fold over the solution of this compound.

Different types of solid lipid nanoparticles with a size of about 100 nm, consisting of emulsifying wax/Brij® 78 and out of Brij® 72/polysorbate 80, were made by Lockman *et al.*[34,35] and Koziara *et al.*[36,37] and investigated in rat brain perfusion experiments. For both nanoparticle types, a statistically significant uptake was observed compared with [$^{14}$C]-sucrose in rat brain perfusion experiments, suggesting central nervous system uptake of the nanoparticles.[36] Perfusion of the nanoparticles did not induce any statistically significant changes in barrier integrity, membrane permeability, or facilitated choline transport.[34] [$^{3}$H]-thiamine was then bound to the emulsifying wax/Brij® 78 nanoparticles via a PEG-spacer (distearoylphosphatidyl-ethanolamine (DSPE)-PEG-NHS) to target the particles to the thiamine transporters in the brain.[35] Although an association with the thiamine transporter occurred, no difference in the brain uptake was observed in BALB/c mice after i.v. injection between emulsifying wax/Brij® 78 nanoparticles with protruding PEG chains on the outside and nanoparticles with thiamine bound to the PEG chains. The emulsifying wax/Brij® 78 solid lipid nanoparticles were then loaded with paclitaxel and tested in the U-1118 and HCT-15 cell lines and by rat brain perfusion. Entrapment of paclitaxel in the solid lipid nanoparticles significantly increased its brain uptake and its toxicity towards the P-glycoprotein expressing tumor cells.[37]

## 3.2. *Influence of PEGylation on the biodistribution of nanoparticles*

Besides, by coating with surfactants, the body distribution may also be altered by covalent attachment of polyethylene glycol (PEG) chains to the nanoparticle surface (PEGylation). Like a number of surfactants such as poloxamine 908 and 1508,[23] the nanoparticle-surface-bound PEG chains can prevent the opsonization and rapid capture and removal of the nanoparticulate carriers by the cells of the reticuloendothelial system (RES), and consequently, can significantly prolong the blood circulation times of the particles.[38–44] Calvo *et al.*[41] showed in mice and rats that the $^{14}$C-concentration in different brain tissues was also significantly enhanced

after intravenous injection of PEGylated [$^{14}$C]-poly[methoxy poly (ethylene glycol) cyanoacrylate-co-hexadecyl cyanoacrylate] nanoparticles ([$^{14}$C]-PEG-PHDCA nanoparticles) in comparison to uncoated or poloxamer 908- or polysorbate 80-coated [$^{14}$C]-poly (hexadecyl cyanoacrylate) nanoparticles ([$^{14}$C]-PHDCA nanoparticles). Surprisingly, coating with polysorbate 80 and also with poloxamer 908 led to lower brain concentrations than uncoated particles in both species. In addition, a species-dependent influence of the surfactants on the brain concentrations was observed; in mice, the brain concentrations of the [$^{14}$C]-PHDCA nanoparticles were higher after coating with polysorbate 80 than with poloxamer 908, whereas this order was reversed in rats. It is important to further note that after reduction of the nanoparticle dose, while maintaining the same total polysorbate concentration, higher [$^{14}$C] brain levels were observed with the polysorbate 80-coated nanoparticles than with the PEGylated [$^{14}$C]-PEG-PHDCA particles. The authors suggested that these higher brain concentrations were caused by a higher BBB permeability as a result of higher free blood polysorbate concentrations at the lower nanoparticle dose, and tried to support their assumption by another experiment injecting i.v. 5% [$^{14}$C]-sucrose in a 1% polysorbate solution in saline, which also led to higher [$^{14}$C]-sucrose levels in the brain.[41] However, this assumption that free polysorbate 80 concentrations up to 1% leads to an increased BBB permeability resulting in a larger drug transport, is contradicted by pharmacological studies with drugs.[18,19,21,22,25–27,45–49] In all of these studies, 1% polysorbate 80, containing drug solutions without nanoparticles that were used as controls, were unable to achieve any significant pharmacological effects.

Interestingly, in the body distribution study of Calvo *et al.*[41] the brain concentration pattern was not mirrored in the other organs and tissues. The highest blood concentrations were obtained in mice and rats with the poloxamer 908-coated particles, followed by the PEG-PHDCA particles. The poloxamer 908-coated nanoparticles also yielded the lowest total uptake in the RES organs in rats but not in mice. In the latter, the PEG-PHDCA particles achieved the lowest total RES organ uptake.

Similar results as those of Calvo *et al.*[41] were also obtained with solid lipid nanoparticles, consisting of stearic acid (non-stealth SLN) or stearic acid, i.e. PEG 2000 (stealth SLN), Epicuron® 200, and taurocholate sodium, after intravenous injection to rats[43] and rabbits.[44] Doxorubicin was bound to these particles using hexadecylphosphate as a counterion. In the rabbit study, the amount of the stealth agent stearic acid-PEG 2000 was systematically increased in 0.15% steps from 0% to 0.45%.[44] All nanoparticles achieved much higher and prolonged plasma concentrations than the doxorubicin solution. The increase in the stearic acid-PEG contents was mirrored by an increase and prolongation of the doxorubicin plasma concentrations. A comparable increase was observed in the brain reaching a doxorubicin concentration of 240 ng/g after administration of 1 mg/kg doxorubicin. After only

6 hrs with the PEGylated solid lipid nanoparticles with the highest stearic acid-PEG content, doxorubicin was still detectable. No doxorubicin was found in the brain after administration of the doxorubicin solution. As in the abovementioned studies,[27,28] the nanoparticles decreased the heart concentration, and in addition, the liver and other organ concentrations of the doxorubicin.

The biodistribution of the PEGylated [$^{14}$C]-PEG-PHDCA nanoparticles was also tested by Calvo *et al.*[50] in DA/Rj rats with experimental allergic encephalitis (EAE) and compared with [$^{14}$C]-PHDCA nanoparticles. The PEGylated nanoparticles achieved much higher brain and spinal cord concentrations than the normal particles. The concentration of the PEG-PHDCA nanoparticles was significantly higher in the pathological situation, where the BBB permeability was increased and was especially pronounced in the white matter. An enhanced macrophage infiltration with macrophages containing nanoparticles was observed at the EAE lesions, confirming earlier results of Merodio *et al.*[51] after intraperitoneal injection of albumin nanoparticles. This transport within macrophages could augment the overall nanoparticle transport across the BBB. Coating of the non-PEGylated [$^{14}$C]-PHDCA nanoparticles with poloxamine 908 resulted in very low and insignificant brain and spinal cord concentrations, although this surfactant again achieved very high nanoparticle plasma levels.[50] Consequently, the PEGylated poly cyanoacrylate nanoparticles may represent promising brain drug delivery systems for neuroinflammatory diseases.

## 4. Pharmacology

As mentioned above, dalargin was the first drug that was transported across the BBB using the polysorbate 80-coated nanoparticles. Besides polysorbate 80, coating of the poly(butyl cyanoacrylate) nanoparticles with polysorbate 20, 40, and 60 also enabled a transport of the nanoparticle-bound dalargin across the BBB, whereas coating with other surfactants such as poloxamers 184, 188, 338, 407, poloxamine 908, Cremophor® EZ, Cremophor® RH 40, and polyoxyethylene-(23)-laurylether (Brij® 35) achieved no effects[24] (Table 1), clearly demonstrating the importance of the surface properties of the nanoparticles for brain drug delivery.

Dalargin was then followed by other antinociceptive drugs such as the opioid loperamide[45] and the Met-enkephalin kyotorphin,[46] both showing similar effects. Unlike dalargin and kyotorphin, loperamide is not a peptide and is very lipophilic in contrast to these compounds. However, it is a strong Pgp substrate, and for this reason, it normally cannot cross the blood-brain barrier. In contrast to binding to poly(butyl cyanoacyrylate) nanoparticles, this drug was not able to induce any antinociceptive response after binding to polylactic acid nanoparticles, neither after coating with polysorbate 80 nor after preparation of the polylactic acid

**Table 1** Maximal possible antinociceptive effect (MPE [%]) obtained after intravenous injection of dalargin-loaded surfactant-coated poly (butyl cyanoacrylate) nanoparticles and amount of apolipoprotein E (apo E) adsorbed on the surface of these particles in percent of the total amount of adsorbed plasma proteins. (Adapted from Kreuter *et al.*[24] and from Lück[70]).

| Surfactant | MPE [%] | Apo E adsorbed [%] |
|---|---|---|
| Uncoated | 4.1 ± 1.0 | 0 |
| Polysorbate 20 | 51 ± 19 | 21.6 |
| Polysorbate 40 | 61 ± 41 | 29.7 |
| Polysorbate 60 | 30 ± 36 | 13.9 |
| Polysorbate 80 | 89 ± 22 | 14.6 |
| Poloxamer 338 | 1.4 ± 2.4 | 0 |
| Poloxamer 407 | 8.1 ± 2.9 | 0 |
| Cremophor® EL | 11.7 ± 15.1 | 0 |
| Cremophor® RH40 | 23 ± 17 | 0 |

nanoparticles in the presence of this surfactant (unpublished results), although particles with a large variety of compositions with different release characteristics were manufactured.[52,53] These observations clearly demonstrate that the ability of nanoparticles to enable a delivery of nanoparticles across the BBB, in addition to the surface properties, also depends on the core polymer.

Tubocurarine normally also cannot cross the BBB. It induces epileptic spikes after direct intraventricular injection of tubocurarine into the brain. This drug was bound to the poly(butyl cyanoacrylate) nanoparticles[47] and used in brain perfusion experiments in rats, in which the development of epileptic spikes in the EEC was recorded. Addition of the polysorbate 80-overcoated tubocurarine-loaded nanoparticles to the perfusate induced frequent severe spikes in the EEC that were comparable to direct intraventricular injection of the drug into the brain whereas a normal EEC was obtained after a solution of tubocurarine, the turbocurarine solution combined with polysorbate 80 or uncoated tubocurarine-loaded nanoparticles were added to the perfusate.

The novel NMDA receptor antagonists MRZ 2/576 (8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazino[4,5-b]quinoline-5-oxide choline salt) is a potent but rather short-acting (5–15 min) anticonvulsant after intravenous administration.[48] This short action is most likely caused by the rapid elimination of the drug from the central nervous system by efflux pump-mediated transport processes. Accordingly, these efflux processes can be inhibited by pretreatment with probenecid. Probenecid pretreatment prolongs the anticonvulsive action of MRZ 2/576 from about 15 min to 150 min. Intravenous injection of MRZ 2/576 bound to poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80, led to an even more prolonged duration

of the anticonvulsive activity in mice of up to 210 min, and in combination with probenecid up to 300 min.[48,49]

In contrast to MRZ 2/576, the NMDA receptor antagonist MRZ 2/596 (8-chloro-1,4-dioxo-1,2,3,4-tetrahydropyridazino[4,5-b]quinoline choline salt) is not able to cross the BBB at all. However, again after binding to the polysorbate 80-coated nanoparticles, MRZ 2/596 also yielded similar anticonvulsive effects.[49]

Two other drugs, amitriptyline,[46] a tricyclic antidepressant, and valproic acid, a first line antiepileptic drug,[54] were also bound to polysorbate 80-coated nanoparticles. While the brain AUC of amitriptyline was increased after intravenous injection of the polysorbate 80-coated nanoparticles which was accompanied by a reduction in serum AUC,[46] no brain concentration increase was observable with valproic acid.[54]

As mentioned above under Sec. 3.1, some indications exist that surfactant-coated nanoparticles or solid lipid nanoparticles may also increase the distribution of some drugs into the brain after oral administration,[30,31,55] and may even lead to pharmacological effects.[56] Coating of poly(butyl cyanoacrylate) nanoparticles with polysorbate 80 yielded antinociceptive effects with dalargin via the oral route, although these effects were not as pronounced but rather prolonged as after the i.v. injection.

## 5. Brain Tumors

Brain tumors, especially malignant gliomas, belong to the most aggressive human cancers. Despite numerous advances in neurosurgical operative techniques, adjuvant chemotherapy, and radiotherapy the prognosis for patients remains very unfavorable.[57,58] These tumors are characterized by a rapid proliferation, diffuse growth, and invasion into distant brain areas, in addition to extensive cerebral edema and high levels of angiogenesis. Nevertheless, the disruption of the blood-brain barrier (BBB) remains a local event, which is evident in the tumor core, but absent at its growing margins. For this reason, anticancer drugs can penetrate into necrotic tumor areas, while the drug levels in peritumoral regions were reported to remain low or non-detectable.[59]

For this reason, very efficient anticancer drugs such as doxorubicin cannot cross the intact BBB and reach only the necrotic but not the peritumoral areas. As noted above (Sec. 3.1), however, this drug reached very high brain concentrations of about 6 $\mu$g/g, after binding to poly(butyl cyanoacrylate) nanoparticles. These nanoparticles were then tested in rats with intracranially implanted glioblastoma 101/8.[58] In contrast to many experimental tumors such as RG 2 and 9L which are characterized by a nodular growth, this tumor has a stable monomorphous structure and shows the characteristic histological picture of aggressive glioblastomas with fast

diffuse growth in the brain parenchyma and a rather low tendency towards necrosis. Therefore, it is morphologically very similar to human glioblastomas. Doxorubicin bound to the polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles injected at a dose level of 1.5 mg/kg/day at days 2, 5 and 8 after tumor transplantation, increased the mean survival time by 85% and repeatedly led to the survival of 20 to 40% of the animals for 180 days (from 8 repetitions of unpublished results). After this time, the animals were sacrificed, and the absence of tumors was demonstrated by histology in these animals. In contrast, the controls, empty polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles, doxorubicin solution, doxorubicin solution plus polysorbate 80, and doxorubicin bound to poly(butyl cyanoacrylate) nanoparticles without polysorbate 80, led to no or much shorter increases in survival times or number of long-time survivers (Table 2). No indications of neurotoxicity were observable by histology with the nanoparticles. Also, the toxicity against other organs appeared to be reduced by binding to nanoparticles, in comparison to the doxorubicin solution.

Brigger *et al.*[42] showed an accumulation of $^{14}$C-labelled PEG-PHDCA and PHDCA nanoparticles after intravenous injection to Fischer rats bearing an intracerebrally transplanted 9L glioblastoma. This accumulation was accompanied by a pronounced tumor retention effect. The tumor concentrations of the PEGylated nanoparticles were about 3 times higher than with the normal PHDCA particles, and about 5–6 times higher than in the adjacent brain areas. Interestingly, in the

**Table 2** Increases in survival times (IST [%]) and long-term survivors (survival 100–180 days) of rats with intracranially transplanted glioblastoma 101/8 after 3 intravenous injection of doxorubicin (1.5 mg/kg/day or 2.5 mg/kg/day) on days 2, 5, and 8 after tumor transplantation. (Adapted from Steiniger *et al.*[53]).

| 3 × 1.5 mg/kg | n | IST [%] | survival 100–180 days |
|---|---|---|---|
| Control | 21 | | 0 |
| Empty nanoparticles | 13 | 0 n.s. | 0 |
| Doxorubicin solution | 23 | 54* | 0 |
| Doxorubicin solution + polysorbate 80 | 22 | 65* | 2 |
| Doxorubicin bound to nanoparticles | 23 | 38* | 2 |
| Doxorubicin bound to nanoparticles + polysorbate 80 | 23 | 85* | 5 |
| 3 × 2.5 mg/kg | | | |
| Control | 10 | | 0 |
| Doxorubicin solution | 8 | 88* | 0 |
| Doxorubicin solution + polysorbate 80 | 8 | 108* | 0 |
| Doxorubicin bound to nanoparticles | 7 | 62* | 0 |
| Doxorubicin bound to nanoparticles + polysorbate 80 | 9 | 169* | 2 |

*Statistically difference to controls ($p < 0.05$); n.s. not statistically different from control.

tumor-bearing rats, the brain concentrations in the areas adjacent to the tumor as well as in the controlateral brain hemisphere were also increased significantly compared with normal animals without tumor, indicating a generally higher permeability in the diseased animals. This was supported by co-injection of [$^3$H]-sucrose together with the nanoparticles. The [$^3$H]-sucrose level ratios between tumor, adjacent brain area, and the adjacent brain area obtained with the two types of nanoparticles were similar to the $^{14}$C-nanoparticle level ratios, and much lower levels again resulted without tumors.[42]

Unfortunately, these nanoparticles did not increase the survival of Fisher rats bearing the same tumor, 9L, after intracranial transplantation.[60] Biodistribution studies revealed that the binding of doxorubicin to the nanoparticles decreased the tumor accumulation of the particles by a factor of 2.5, which may be the cause for the lack of efficacy of these particles against 9L.

The loss of wild type tumor suppressor genes like p53 function renders many tumors resistant to the induction of apoptosis by drugs such as doxorubicin.[61] Therefore, the delivery of wild type suppressor genes across the BBB is of enormous importance for the therapy with highly active chemotherapeutic drugs. The possibility of suppressor gene delivery into the brain with nanoparticles was evaluated in rats with an intracranially implanted F98 rat glioblastoma. Five days after tumor implantation, these rats received an intravenous injection of a ß-galactosidase reporter bound to poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80. The animals were sacrificed 24, 48, and 72 hrs after nanoparticle injection, and a time dependent transport of the gene across the endothelial cells and glial cells was obtained, showing the strongest gene expression in the experimental tumors, whereas injection of naked control DNA did not render any expression at all.[61]

## 6. Toxicology

The acute toxicity of empty poly(butyl cyanoacrylate) nanoparticles as well as of the above poly(butyl cyanoacrylate) nanoparticle formulations, doxorubicin solution in saline, doxorubicin solution plus 1% polysorbate 80 in saline, doxorubicin bound to nanoparticles, and doxorubicin bound to nanoparticles coated with polysorbate 80, was assessed by Gelperina *et al.*[62] in normal and glioma 101/8-bearing rats. Doses up to 400 mg/kg of empty nanoparticles did not cause any mortality within the period of observation (30 days), nor did they affect body weight or weight of internal organs after intravenous injection. Higher doses cannot be administered intravenously because of biological[63] and technical limitations. No significant difference in toxicity occurred between the groups obtaining i.v. the four doxorubicin formulations in healthy as well as in the tumor-bearing animals. The results indicated that the toxicity of doxorubicin bound to nanoparticles is similar or may even be lower than that of free doxorubicin.[62]

In the above described chemotherapy study of Steiniger *et al.*[58] with doxorubicin bound to the polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles, similar toxicological results were obtained. A limited dose-dependent systemic toxicity was found in the group treated with doxorubicin in saline. Autopsy of the whole body in healthy animals in this study revealed an empty gastrointestinal tract only in all animals treated with doxorubicin. The healthy animals treated with doxorubicin solution also showed slight signs of lung edema, which was confirmed by histology. These changes were not observed in animals treated with doxorubicin bound to the nanoparticles: indications of short-term neurotoxicity, such as increased apoptosis in areas distant from the tumor, increased expression of GFAP or ezrin on distant astrocytes or degenerative morphological changes of neurons, were entirely absent in treated animals on day 12, as well as in long-term survivors. In addition, there was no indication of chronic glial activation in areas distant from the tumor site in long-term surviving rats. Moreover, long-term survivors did not exhibit any obvious neurological symptoms.[58]

## 7. Mechanism of the Delivery of Drug Across the Blood-Brain Barrier with Nanoparticles

Presently, the mechanism of the delivery of drugs with nanoparticles across the BBB is not totally elucidated. A number of possibilities were suggested for this mechanism[4,5,64]:

1. An increased retention of the nanoparticles in the brain blood capillaries combined with an adsorption to the capillary walls. This could create a higher concentration gradient that would enhance the transport across the endothelial cell layer, and as a result, the delivery to the brain.
2. The polysorbate 80 used as the coating agent could inhibit the efflux system, especially P-glycoprotein (Pgp).
3. A general toxic effect on the brain vascularite, leading to the permeabilization of the brain blood vessel endothelial cells.
4. A general surfactant effect characterized by a solubilization of the endothelial cell membrane lipids that would lead to membrane fluidization and an enhanced drug permeability through the blood-brain barrier.
5. The nanoparticles could lead to an opening of the tight junctions between the endothelial cells. The drug could then permeate through the tight junctions in free, or together with the nanoparticles, in bound form.
6. The nanoparticles may be endocytosed by the endothelial cells, followed by the release of the drugs within these cells and the delivery to the brain.

7. The nanoparticles with bound drugs could be transcytosed through the endothelial cell layer.

All these mechanisms could also work in combinations.[4,5,64]

Mechanisms 1 and 2 appear to be unlikely for the following reasons: if the drug-loaded nanoparticles would have merely created a high drug concentration gradient by adherence to the inner surface of the blood capillary walls (mechanism 1), the diffusing drug would still have been subjected to the highly efficient efflux transporters in the membranes of the endothelial cells. On the other hand, if the polysorbate 80 would have inhibited these efflux transporters (mechanism 2), injection of polysorbate 80-coated empty nanoparticles 5 or 30 min before injection of dalargin, should also have induced antinociceptive effects, which was not observed in this case.[65] The view that mechnisms 1 and 2 are unlikely are additionally supported by the brain perfusion experiments of Koziara *et al.*[36]

Olivier *et al.*[66] postulated that the enhanced drug transport across the BBB was caused by a toxic effect by the polysorbate 80-coated nanoparticles, resulting in the permeabilization or disruption of the blood-brain barrier (mechanisms 3 and/or 5). Pointing in the same direction, Calvo *et al.*[41] tried to explain the higher [$^{14}$C]-sucrose levels that they observed in the brain after i.v. injection of 5% [$^{14}$C]-sucrose in a 1% polysorbate 80 solution in saline with BBB permeabilization caused by unbound free polysorbate 80 present in the nanoparticle formulations (mechanism 4). However, both hypotheses can be refuted by the abovementioned experiment, where no antinociceptive effects were obtained after pre-injection of the polysorbate 80-coated empty nanoparticles.[65] In addition, no antinociceptive responses were observed after the injection of dalargin nanoparticles coated with other surfactants such as poloxamers 184, 188, 338, 407, poloxamine 908, Cremophor® EZ, Cremophor® RH 40, and polyoxyethylene-(23)-laurylether (Brij® 35),[24] further outruling mechanism 4, a general membrane fluidization. This opinion that toxicity is not the mechanism for the nanoparticle-mediated drug transport across the BBB was also substantiated by the experiments of Sun *et al.*[67] and of Koziara *et al.*[36] Partial coverage of the particles by polysorbate 80 was sufficient for brain delivery,[67] and the brain perfusion experiments showed that the nanoparticles did not induce any statistically significant changes in barrier integrity, membrane permeability or facilitated choline transport.[34] Finally, opening of the tight junctions as the underlying mechanism (mechanism 5) can be refuted by the findings that no major increase in the inulin spaces was observable in rat brain perfusion experiments.[26] Additionally, electron microscopical studies also did not find any evidence for an opening of the tight junctions.[65]

Therefore, the most likely mechanism appears to be mechanism 6, endocytotic uptake of the nanoparticles carrying the drug. This mechanism was already shown

*in vitro* in tissue cultures of brain endothelial cells of human, bovine, porcine, mice, and rat origin.[26,68,69] At an incubation temperature of 37°C, a significant and rapid uptake was observed with the polysorbate 80-coated nanoparticles, whereas without coating, this uptake was minimal and it was inhibited at 4°C, a temperature at which phagocytosis does not occur, or after treatment with cytochalasin B, a potent phagocytic uptake inhibitor.[69]

Mechanism 6 is further supported by the observation that, in contrast to the abovementioned surfactants, poloxamer 184 etc., besides polysorbate 80, polysorbates 20, 40, and 60 were also able to induce antinociceptive effects after the coating of dalargin-loaded nanoparticles and injection to mice.[24] In addition, all 4 polysorbates, 20, 40, 60 and 80, and not the other surfactants, were able to adsorb apolipoprotein E (apo E) on the surface of the nanoparticles after their incubation in blood plasma[70] (Table 1). Kreuter *et al.*[64] then showed that the dalargin nanoparticles were also able to induce antinociceptive effects after the adsorption of apolipoproteins E and B. These effects were even much higher after polysorbate 80 pre-incubation. Therefore, the following scenario can be suggested: due to the polysorbate on their surface, the nanoparticles adsorb apolipoproteins E and/or B from the blood after injection. The particles, thus seem to mimic lipoprotein particles, and are taken up by the brain endothelial cells that express numerous lipoprotein receptors via receptor-mediated endocytosis. Since the efflux transporters are mainly located in the luminal membrane, the drug can then be transported into the brain by diffusion, after release from the very rapidly biodegrading[20] nanoparticle polymer. It is also possible that the nanoparticles are transcytosed (mechanism 7), although no concrete evidence for this mechanism exists at present. The nanoparticles, therefore, seem to act as a "Trojan Horse". This hypothesis that drug transport via endocytotic uptake of the nanoparticles represents the underlying pathway was also supported by Sun *et al.*,[67] Koziara *et al.*,[36] and Gessner *et al.*[71] Since the lipoprotein receptors are overexpressed in brain tumors,[72] the above suggested scenario, lipoprotein receptor interaction, would also be an explanation for the good efficacy of the polysorbate 80-coated doxorubicin-loaded poly(butyl cyanoacrylate) nanoparticles.[58]

## 8. Summary

A number of drugs that normally cannot cross the blood-brain barrier (BBB), or only in insufficient amounts, can be transported across this barrier after binding to polysorbate-coated poly(butyl cyanoacrylate) nanoparticles or to solid lipid nanoparticles, and achieve significant brain drug concentrations and pharmacological effects in the brain after intravenous injection. These drugs include the hexapeptide dalargin and the dipeptide kyotorphin, loperamide, tubocurarine, amitriptyline, the NMDA receptor antagonists MRZ 2/576 and MRZ 2/596,

doxorubicin, idarubicin, campthothecin, paclitaxel, as well as tobramycin. Doxorubicin bound to polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles was able to strongly improve the survival time of rats with intracranially transplanted glioblastoma 101/8, an extremely aggressive tumor. The general toxicity of this drug was not increased by binding to the nanoparticles. PEGylation of poly cyanoacrylate nanoparticles prolonged their blood circulation time after intravenous injection and strongly increased their concentration in the intracranially transplanted glioblastoma 9L, but failed to prolong the survival of these rats.

The mechanism of the nanoparticles-mediated drug transport across the BBB after intravenous injection seems to be the adsorption of apolipoproteins from the blood, leading to receptor-mediated endocytotic uptake of the particles into the brain capillary endothelial cells via lipoprotein receptors. The nanoparticles can then release the drugs within these cells, followed by the diffusion into the brain, or the access to the brain by transcytosis.

## 9. Conclusions

Poly cyanoacrylate nanoparticles or solid lipid nanoparticles (SLN) can enable the transport of many essential drugs across the blood-brain barrier (BBB) that normally cannot cross this barrier.[4,5] The nanoparticles may be even useful for the delivery of larger and complex molecules such as proteins,[18,19] nucleic acids and genes[61] across this barrier. They may also improve the treatment of brain tumors, since after binding to nanoparticles coated with polysorbates, anti-tumor drugs are also transported across the intact BBB,[27,58] thereby accessing sites that cannot be reached by most anti-cancer drugs.

Although the mechanism for the transport of nanoparticle-bound drugs across the BBB is not fully elucidated presently, binding of apolipoproteins after their injection into the blood stream, followed by receptor-mediated endocytotic uptake of the particles into the brain capillary endothelial cells, seems to be the most likely mechanism. Thus, the nanoparticles would act as a "Trojan Horse" which can then release the drugs within these cells, or after transcytosis into the brain.

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# 25

# Nanoparticles for Targeting Lymphatics

*William Phillips*

## 1. Introduction

Nanoparticles have received increasing attention as lymph node drug delivery agents.[1–6] The desire to develop of new methods of lymph node drug delivery stems from the recent awareness of the importance of lymph nodes in cancer prognosis, their importance for vaccine immune stimulation and the realization that the lymph nodes harbor human immunodeficiency virus (HIV) as well as other infectious diseases. New methods of delivering drugs and antigens to lymph nodes are currently under investigation.

The lymphatic system consists of a network of lymphatic vessels and lymph nodes that serve as a secondary vascular system to return fluid that has leaked from the blood vessels in the extremities and other organs back to the vasculature.[7] The lymphatic system also moves substantial volumes of fluid from the peritoneal cavity and pleural cavity back into the blood circulation. In addition to this critical role in the regulation of tissue fluid balance, the lymphatic system also plays an important role in intestinal absorption of fats and in the maintenance of an effective immune defense.[7] Lymphatic vessels serve as a major transport route for the dissemination of antigens, microorganisms and tumor cells as well as interstitial molecules that have gained entry to the interstitial space.[8] These lymphatic vessels are also traversed by immune cells such as dendritic cells, macrophages and as their name reveals, lymphocytes. As a part of the lymphatic system that recycles fluid from the interstitial spaces and the body's cavity back to the arteriovenous

vascular system, the lymph nodes are ideally positioned to serve as surveillance organs to monitor microbial invasion and to defend the body against these invading microorganisms. The importance of the lymphatic system for the development of an effective immune response has led one author to describe the lymphatic vessels and lymph nodes as the body's "information superhighway".[9]

## 1.1. *The lymphatic vessels*

Lymphatic vessels are composed of thin, endothelial cell lined lymphatic capillaries located in spaces between cells and tissues. These lymphatic vessels are distributed throughout the body with the exception of cartilage, optic cornea and lens, and the central nervous system. Lymph fluid originating from the interstitial spaces between tissue cells and from within the body's cavities moves into lymphatic capillaries through lymph nodes and back into the blood circulation. The overlapping nature of the lymphatic endothelial cells and loose attachment of intercellular junctions allows for the absorption of interstitial fluid into the lymphatic capillaries. This mechanism also explains how macromolecules, infectious organisms and subcutaneously injected nanoparticles gain entrance into the lymphatic circulation. These lymphatic capillaries carry lymph fluid into collecting lymphatic vessels and channels which slowly flow in the afferent lymphatic vessels into the lymph node. After passing through the lymph node, the lymph fluid then exits the lymph node through the efferent lymphatic vessels. The lymph fluid flows slower and under much lower pressure than blood in the artery and veins. Its flow rate can be greatly accelerated by body movement. The efferent vessels combine to form lymphatic vessels that branch either to the next set of lymph nodes or to larger lymphatic trunks as illustrated in Fig. 1. In this way, lymph fluid of different organs and the body's extremities in addition to body cavities is collected by large lymphatic trunks which feed into one of the two lymphatic ducts: the thoracic duct and right lymphatic duct. From these ducts, the lymph fluid then returns to the blood stream through veins in the neck region (i.e. internal jugular and subclavian veins).[10,11]

The lymphatic system also returns fluid from the body's cavities back to the blood stream. This includes fluid from the pleural space surrounding the lungs, the peritoneal space surrounding the intestines, the articular cavity of the joints, and the central spinal fluid surrounding the brain. The rate of movement of fluid from these body cavities varies between different cavities. However, the volume of fluid moving through the lymphatic vessels coming from the body's cavities is substantial. Studies in conscious sheep with tracers have found that clearance of fluid from the peritoneal cavity averaged 2.4 ml/hr per kg of body weight, which was more than twice as high as when the sheep were anesthetized.[12] This rate of

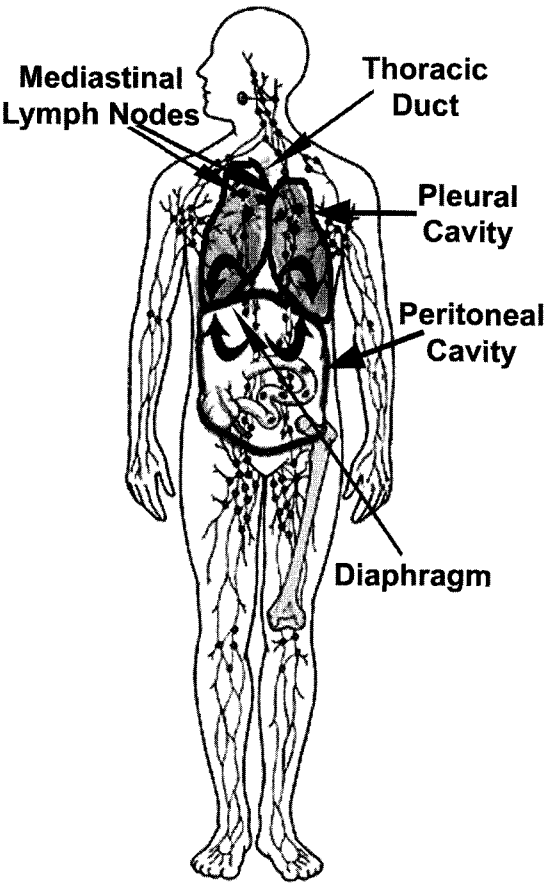


**Fig. 1.** The lymphatic system includes lymphatic vessels draining from the extremities and head and neck region as well as the fluid moving from the cavities of the body. It is estimated that 400–600 lymph nodes that filter drainage from the lymph vessels are in the average human body.

fluid movement in a 70-kg human would be more than 165 ml per hr or nearly 4 liters per day.

## 1.2. *Lymph nodes*

The lymphatic system is also interspersed with lymph nodes placed at intervals along this lymphatic vessel network as shown in Fig. 1. Lymph nodes are encapsulated dense masses of lymphoreticular tissue situated along the pathway of drainage of the lymph. There are estimated to be 400–600 lymph nodes in the human

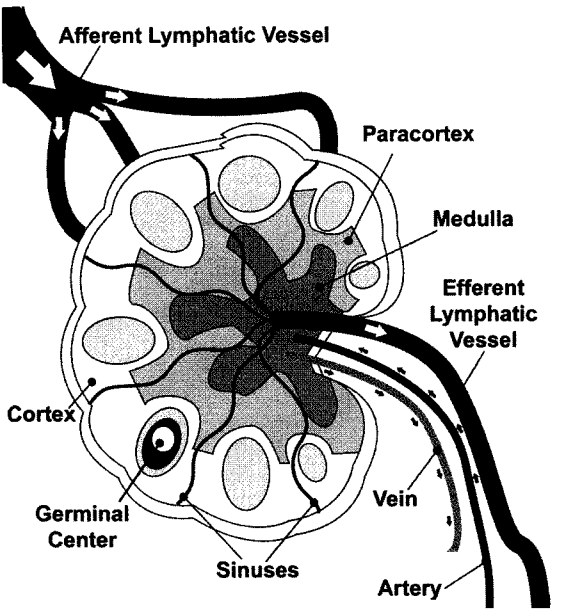


**Fig. 2.** This diagram illustrate the structure of the lymph node. Efferent lymphatic vessels delivery lymph fluid to the lymph node and afferent lymphatic vessels take the lymphatic fluid from the lymph node. Each lymph node is supplied by an artery and a vein. Lymphatic fluid is filtered through the sinuses of the lymph nodes that are lined with macrophages to phagocytized foreign particulate agents. Lymph nodes also contain cortical, paracortical and medullary regions which contain different immune system cells.

body. The outer capsule of the lymph node is composed of dense collagenous fibers and smooth muscle fibers. The interior of the lymph nodes is organized into different functional zones populated by different sorts of lymphocytes, as well as accessory and stromal cells.[11,13] These lymph node zones as shown in Fig. 2 are:

- The cortex, which includes the lymphoid follicles with their germinal centers. This is the B-cell area of the lymph node which is associated with humoral immune mechanisms.
- The paracortex, which is the densely cellular area that extends between the lymphoid follicles. This is the T-cell area which is the main site of cellular immunity.
- The sinuses, a complex system of channels where macrophages belonging to the mononuclear phagocytic system (MPS) reside.
- The medulla, rich in sinuses where the main site of plasma cell proliferation and production of antibodies (the medullary cords) are located.

The three main functions of the lymph nodes are the formation of lymphocytes known as lymphopoiesis, lymph filtration, and antigen processing.[9,14] In terms of

lymph fluid filtration, the lymph nodes provide two main types of filtration: a simple mechanical type through the reticular meshwork which traverses the sinuses; and a phagocytic filtration by macrophages and reticular cells, which is aided by the slow passage of the lymph fluid through the channels of the sinuses.

One of the major functions of the lymph nodes is to help defend the body against diseases by filtering bacteria and viruses from the lymph fluid, and to support the activities of the lymphocytes, which furnish resistance to specific disease-causing agents. However, in abnormal conditions, as in the case of cancer and some infections, it is well known that lymph nodes can act as holding reservoirs from where tumor cells, bacteria or viruses can spread to other organs and regions of the body.[7,11] For example, in the case of cancer, disseminating tumor cells can take root in lymph nodes and form residual metastatic tumors that are difficult to detect and treat. In anthrax infection, endospores from Bacillus anthracis that gain entrance into the body are phagocytozed by macrophages and carried to regional lymph nodes, where the endospores germinate within the macrophages and become vegetative bacteria. The vegetative bacteria are then released from the lymph nodes, multiply in the lymphatic system and invade the blood stream causing massive septicemia.[15]

Adequate therapy of lymph nodes affected by disease runs a considerable risk of side effects. For example, current methods of treating or preventing metastasis in lymph nodes are characterized by serious drawbacks including: (1) radical surgical excision of lymph nodes is a burdensome procedure, and the risk of postoperative lymph node cancer recurrence is often high; (2) external radiation therapy can damage sensitive organs unnecessarily, while delivering a small percentage of radiation to the targeted lymph nodes; and (3) intravenous chemotherapy to patients with advanced disease is associated with significant toxicity, even though adequate therapeutic concentrations in the targeted lymph nodes are rarely achieved.[1] For these reasons, nanoparticle targeted drug carriers offer a potential solution to the challenge of adequate lymph node therapy.

## 2. Potential for Nanoparticles for Drug Delivery to Lymphatics

Nanoparticles are ideal structures for delivering therapeutic agents to the lymph nodes. Their ideal features are based on their size, which prevents their direct absorption into the blood, the large amount of drugs and other therapeutic agents that nanoparticles can carry, and their ability to be retained in the lymph nodes. In comparison, small molecules will be directly absorbed into the blood at the site of injection and will not move into the lymphatics. Although larger molecules such as dextran and albumin will move into the lymphatics, they rapidly pass through the draining lymph nodes and are not well retained in individual lymph nodes.[16,17]

Although nanoparticles are too large to be directly absorbed into the blood stream, they are small enough to enter the lymph vessels and lymph nodes, following either subcutaneous injection, intradermal injection, intramuscular injection or injection directly into organs or tumors and injection into the body's cavities. Following subcutaneous injection or injection directly into the tissue of a body organ, it appears that a certain portion of nanoparticles are taken up locally and retained for a prolonged time, while another portion of the nanoparticles are cleared from this local site and move into the lymphatic vessels, where they can be trapped in lymph nodes or else move completely through the lymphatic system and return to the blood at the thoracic duct. Nanoparticles that are injected directly into the body's cavities appear to have much less local retention at the site of injection, as they disperse freely throughout the whole cavity and then drain almost completely into lymphatic vessels, where they can be trapped in lymph nodes or return to the blood circulation. These intracavitary sites whose fluid is cleared through the lymphatics include the pleural space surrounding the lungs, the peritoneal space surrounding the intestines, the articular cavity of the joints, and the central spinal fluid surrounding the brain. If the particular particle that is injected into the intracavitary space is only minimally retained in all of the draining lymph nodes, then the ultimate fate of the nanoparticles injected into a cavity can appear very similar to the same distribution that it would have had, following intravenous administration.[18] This similar distribution has been demonstrated with radiolabeled nanoparticles that are injected into the peritoneal space. By 24 hrs following intraperitoneal administration, these nanoparticles have a high liver and spleen uptake and minimal retention in the peritoneum, as if they had been injected intravenously.[18] This is the normal distribution of neutral and anionic liposomes unless there is special modification of the liposomes to increase their uptake in the lymph nodes.[3,18]

Considering the importance of the lymphatics in relationship to many disease processes, the number of studies investigating drug delivery or targeting of other therapeutic agents to the lymphatics has been relatively modest.[19] The recent development of an increasing number of different types of nanoparticles that can facilitate the lymphatic transport of therapeutic agents provides many new approaches to lymphatic drug delivery and the basic investigation of lymphatic transport.

## 3. Importance of Lymph Nodes for Disease Spread and Potential Applications of Lymph Node Drug Delivery

### 3.1. *Cancer*

The majority of solid cancers spread primarily by lymph node dissemination.[20] The status of the lymph node in regard to cancer metastasis is a major determinant of the

patient's prognosis. This includes very small metastasis detected by histopathologic analysis of removed lymph nodes, as well as by magnetic resonance imaging (MRI) imaging using magnetic nanoparticle MRI contrast agents.[21] Accurate lymph node staging is the most important factor that determines the appropriate care of the patient.[22] Therapeutic interventions that treat metastatic cancer in lymph nodes with either surgery or local radiation therapy have been shown to improve patient survival.[23]

## 3.2. *HIV*

Primary infection with human immunodeficiency virus (HIV) is characterized by an early viremia, followed by a specific HIV immune response and a dramatic decline of virus in the plasma.[24] Long after the HIV virus can be found in the blood, HIV can be found in high levels in mononuclear cells located in lymph nodes. Viral replication in these lymph nodes has been reported to be 10–100-fold higher than in the peripheral blood mononuclear cells.[25] Drug delivery to these lymph node mononuclear cells is difficult with standard oral or intravenous drug administration. Although highly active antiretroviral therapy (HAART) reduces plasma viral loads in HIV infected patients by 90%, active virus can still be isolated from lymph nodes even after 30 months of HAART therapy.

## 3.3. *Filaria*

Lymph nodes are an important part of the life cycle of several parasite organisms, including filaria. Adult worms are found in the lymphatic vessels and lymph nodes of infected patients. These adult filaria are responsible for the obstruction of lymphatic drainage that causes swelling of extremities that are distal to the infected lymph node. The condition associated with very swollen limbs often found in patients with filarial disease has been termed elephantiasis. Ultrasound imaging can be used to visualize the adult worms by detecting the classic "filaria dance sign" which is associated with the adult worms.[26] This ultrasound imaging has demonstrated that the worms reside in "nests" located in the lymph nodes and lymphatic vessels. The preferred site for adults worms in males is the intrascrotal juxtatesticular lymphatic vessels.[27] Adult worms are also commonly found in the inguinal nodes[28] and they have even been reported to be found in the mediastinal lymph nodes.[29]

It is very difficult to eradicate the adult worms located in the lymphatic system, although microfilaria that are released into the blood stream from adult worms are very responsive to anti-filarial medications. This difficulty in treatment may be due to the localization of adult worms within "nests" in the lymphatic system, where

drug penetration is very poor. Frequently, eradication of adult worms is not possible and it commonly takes a very extended course of medical therapy to have any effect on the adult worms.[30] Nanoparticle drug delivery has potential for drug delivery in filarial disease, particularly before the lymphatics have become totally obstructed. Many asymptomatic patients have been shown to carry adult worms in their lymph nodes. Thus, early diagnosis may be crucial for the treatment of filaria before adult worms are well established in the lymphatic vessels and nodes.

## 3.4. *Anthrax*

New methods of treating anthrax have become of urgent interest, following the recent outbreak of terrorist caused infections and deaths in the United States as a result of terrorism. Following inhalation of the anthrax spores and their deposition in the lungs, the bacteria spread to the mediastinal lymph nodes where their local invasion and associated toxin production is the cause of death. Patients are frequently found to have a widened mediastinum due to the expansion of mediastinal lymph nodes with anthrax.[31]

Computed tomography of the chest has been performed on 8 recent patients infected with inhalational anthrax. Mediastinal lymphadenopathy was present in 7 of the 8 patients.[32] In a recent case report of one patient, the anthrax bacillus was shown to be rapidly sterilized within the blood stream after initiation of antibiotic therapy. However, viable anthrax was still present in postmortem mediastinal lymph node specimens.[33] This case demonstrates the difficulty that drugs have in penetrating the mediastinal lymph nodes. A potential use of nanoparticles could be for delivery of anti-anthrax drugs to the mediastinal lymph nodes for therapy or prevention of anthrax extension to the lymph nodes.

## 3.5. *Tuberculosis*

The tuberculosis infection is caused by mycobacteria which invade and grow chiefly in phagocytic cells. Tuberculosis is frequently found to spread from the lungs to lymph nodes, so that lymph node tuberculosis is the most comm on form of extrapulmonary tuberculosis. In one study, 71% of the tuberculosis lymph node involvement was located in the intrathoracic lymph nodes, while 26% of the cervical lymph nodes were involved with tuberculosis and 3% in the axillary lymph nodes.[34] The development of methods to target drugs to these lymph nodes could greatly improve the therapy of tuberculosis and potentially decrease the amount of time that drug therapy is required. Currently, patients with tuberculosis are required to take medication for $> 6$ months. One possible reason for this lengthy treatment is the difficulty in deliverying drugs into these tubercular lesions. This requirement of lengthy drug treatment could also be responsible for the development of

resistance to anti-tuberculosis drugs, as the organisms are exposed to relatively low levels of drugs over a very prolonged time. The development of resistance to anti-tuberculosis therapy is a growing health problem and the number of tuberculosis cases has been increasing worldwide. Multidrug-resistant tuberculosis presents an increasing threat to global tuberculosis control.[35] Nanoparticles could be used to specifically carry high levels of drugs to lymph nodes containing tuberculosis. Nanoparticles encapsulating anti-tuberculosis drugs have already been developed as potential intravenous therapeutic agents for the treatment of tuberculosis.[36]

## 3.6. *Importance of lymph node antigen delivery for development of an immune response*

The importance of the lymph nodes in the development of an immune reaction induced by vaccines is gradually becoming recognized. Experimental evidence suggests that the induction of immune reactivity depends on antigen reaching and being available in lymphoid organs in a dose- and time-dependent manner.[37] This concept has been termed the geographical concept of immune reactivity.[37–40] The delivery of antigen to a lymph node in a manner that resembles an actual microbial invasion may be one of the most important functions of a vaccine adjuvant. The adjuvants are considered effective, if they either enhance or prolong expression of antigen components to reactive T cells in lymph nodes.[38] Antigen-presenting cells are thought to be of critical importance in transporting antigen from the periphery to local organized lymphoid tissue. However, delivery of antigen to the lymph node by any means may be more important. Several studies have investigated the immune response following direct injection of antigen into lymph nodes. Instead of injecting peptide-based vaccines subcutaneously or intradermally, researchers injected these agents directly into the lymph nodes.[39] This intralymphatic injection enhanced immunogenicity by as much as $10^6$ times when compared with subcutaneous and intradermal vaccination. Intralymphatic administration induced CD8 T cell responses with strong cytotoxic activity and interferon (IFN)-gamma production that conferred long-term protection against viral infections and tumors. This greatly increased response based on direct delivery to the lymph node has also been reported with naked DNA vaccines. Naked DNA vaccines are usually administered either intramuscularly or intradermally. When naked DNA was injected directly into a peripheral lymph node, immunogenicity was enhanced by 100- to 1000-fold, inducing strong and biologically relevant CD8(+) cytotoxic T lymphocyte responses.[41]

Nanoparticles can be used to greatly increase the delivery of an antigen to the lymph node.[39] For instance, animal experiments have shown that immunization by the intramuscular or the subcutaneous route with liposome-entrapped plasmid DNA encoding the hepatitis B surface antigen leads to much greater humoral (IgG

subclasses) and cell mediated (splenic IFN-gamma) immune responses than with naked DNA.[40] In other experiments with a liposome encapsulated plasmid DNA encoding a model antigen (ovalbumin), a cytotoxic T lymphocyte (CTL) response was also observed. These results could be explained by the ability of liposomes to protect their DNA content from local nucleases and direct it to antigen presenting cells (APCs) in the lymph nodes draining the injected site.

In spite of this awareness of the importance of antigen delivery to lymph nodes, there have been very few studies in which the biodistribution of an injected vaccine antigen has been determined, following either subcutaneous, intradermal or intramuscular administration. Studies of antigen encapsulated within nanoparticles could easily be carried out using scintigraphic tracers and imaging. Scintigraphic imaging can provide quantitative information of the total dose and percentage of the antigen that reaches the lymph node. It appears that the purpose of a vaccine adjuvant is to simulate as closely as possible the delivery of a virus, that enters the body subcutaneously or through a body cavity, to the lymph node. One reason that nanoparticles appear to be useful for vaccine delivery is because their processing resembles that of viruses. Viruses can be considered as naturally occurring nanoparticles, against which the human immune system has evolved a defense mechanism.

In this regard, it is remarkable that there have been very few studies investigating the distribution of nanosized viruses, following their administration subcutaneously or intracavitary in methods in which the first encounter with the immune system is likely to be in the lymph node. These studies could easily be performed in experimental small animal imaging models, by labeling the viruses with a scintigraphic imaging agent and injecting them subcutaneously or into a body cavity. Their distribution in the body could be followed by performing serial imaging studies. There have been, however, several studies of viral sized radiolabeled colloidal particles being injected subcutaneously, in which assumptions were made about the likely distribution of viral particles. In one of these studies, subcutaneously injected, viral-sized particles were found to initially arrive in the blood and later in the lymph.[42] Accumulation in lymph and blood increases for a prolonged time following subcutaneous administration. The results of this study suggested the possibility that strategies could be developed to limit the spread of infectious agents by early aggressive local antiviral treatment.

Nanoparticles also have the potential to be delivered to lymph nodes by means other than subcutaneous injection. Particles as large as 1.1 um in diameter have been found to be translocated from the nasal mucosa to lymph nodes following intranasal administration. 24 hrs after intranasal administration of relatively large 1.1 um diameter fluorescent microspheres, significant fluorescence was visualized in the posterior cervical lymph nodes and in the mediastinal lymph nodes.[43]

# 4. Factors Influencing Nanoparticle Delivery to Lymph Nodes

## 4.1. *Nanoparticle size*

Many factors appear to influence the fraction of the nanoparticles that are retained at the initial site of subcutaneous injection. Nanoparticle size appears to be one of the most important factors affecting the clearance of nanoparticles from the subcutaneous site of injection.[5] The larger the size of the nanoparticles that are injected subcutaneously, the greater the fraction of the nanoparticles that will be retained locally and the lesser that will enter the lymphatic vessels and have a chance to target the lymph nodes.[5,44]

Much work has been performed evaluating the effect of particle size of liposomal subcutaneously injected nanoparticles on lymph node targeting. Liposomes are nanoparticles composed of naturally occurring phospholipids that form spontaneously in an aqueous environment. Much recent research has investigated the potential of liposomes as carriers of drugs and other therapeutic agents to lymph nodes. When small neutral liposomes are injected subcutaneously, more than 60–70% of the liposomes will be cleared from the injection site by 24 hrs,[16,45] with only 30–40% of the injected dose remaining at the site of injection. Liposomes larger than 500 nm will have 60–80% remaining at the injection site.[16,45] The dose of lipid administered does not appear to have an effect on the percentage of liposomes retained in the lymph node. Lymph node uptake did not appear to become saturated over a large of lipid dose administered ranging from 10 nmol lipid to 10,000 nmol of lipid.[45]

Factors that enhance clearance of liposomes from a local site of subcutaneous injection also appear to decrease liposome uptake in the lymph node. For instance, larger liposomes are not cleared from the subcutaneous site of injection as readily as smaller liposomes; however, they are better retained in the lymph node. Even though larger liposomes are less well cleared from the injection site, their total retention in the lymph node is similar to other liposomes due to their improved lymph node retention. This improved lymph node retention by liposomes that are poorly cleared from the injection site results in liposome retention doses that are approximately equal to liposomes that have improved clearance from the local subcutaneous injection site.[16]

## 4.2. *Nanoparticle surface*

Several studies have been carried out to determine the ideal nanoparticle surface characteristics for the delivery of drugs to the draining lymph nodes following subcutaneous injection. Moghimi *et al.* have performed studies of 45 nm polystyrene nanospheres which have been coated with poloxamers.[4,46] The effect of a variety of

different polyoxamers with varying lengths of ethylene oxide (EO) units has been studied. If the nanospheres are coated with polyoxamers with a large number of EO units per chain, the nanospheres will clear rapidly from the site of injection, but they will also escape removal by macrophages in the lymph nodes, so that lymph node uptake will be minimal. If nanospheres are not coated with any polyoxamer surface, they will remain largely retained at their site of subcutaneous injection and only a small percentage (< 3% of the injected dose) will accumulate in the lymph node. The polystyrene nanospheres with the most effective delivery and retention in the draining lymph nodes have a polyoxamer coating of 4–15 EO units per chain and a coating thickness of less than 3 nm.[4] Nanospheres of this type have both rapid clearance from the subcutaneous site of injection, as well as significant retention in the draining lymph nodes. By 6 hrs, these ideal nanospheres have less than 50% located in the rat footpad and approximately 20% retained in the primary lymph node and 14% retained in the secondary node.[4] Based on findings with the model particles, Moghimi suggested that it should be possible to develop liposomes or other nanoparticles with ideal properties for lymph node delivery. A suggestion was made to test liposomes composed of 5–7 mol% of 2000 MW polyethylene glycol lipids.[4]

Surface modification of liposomes with polyethylene glycol (PEG) did not appear to have a very large effect of lymph node uptake. Ousseren *et al.* found that the amount of liposomes that cleared from the injection site was slightly greater with the PEG-coated liposomes[47]; however, this improved clearance did not result in improved lymph node retention, because the fraction of PEG-liposomes retained by the lymph node is decreased. The slightly improved clearance of PEG-coated liposomes from the subcutaneous site of injection was also found by our research group.[16]

## 4.3. *Effect of massage on lymphatic clearance of subcutaneously injected liposomes*

The rate of clearance of nanoparticles from a subcutaneous injection site can be greatly accelerated with local manual massage.[48] Without any mechanical stimulation, subcutaneously injected 200 mm liposomes are usually trapped in the interstitial subcutaneous space for a prolonged time. However, 5 min of manual massage over the subcutaneous injection site can clear up to 40% of the injected liposomes from the subcutaneous site into the blood via the lymphatic pathway. Investigators were able to use this effect to control the rate of drug delivery of the vasoconstricting hormone angiotensin II encapsulated in a liposome. They demonstrated that a physiological response to encapsulated drug (average blood pressure increase) can also be induced and modulated by massage.[48]

### 4.4. *Macrophage phagocytosis*

It is generally accepted that nanoparticles are retained in the lymph node by macrophage phagocytosis. Several research findings using nanoparticle liposomes appear to support this contention. For example, inclusion of phosphatidylserine (PS) in the liposome lipid formulation moderately increased lymph node uptake.[45] PS is a strong signal for stimulating macrophage uptake because it is present on the outer surface of cells undergoing apoptosis' instead of its usual location on the inner surface of the cell membrane.[49]

The strong supporting evidence of the role of macrophages in lymph node uptake was provided by a study in which macrophages were temporarily depleted from lymph nodes, by prior administration of liposomes containing dichloromethylene diphosphonate (clodronate). Clodronate is toxic to macrophages and much previous work has been performed using clodronate to temporarily deplete macrophages in the liver.[50,51] Six days after injection of the clodronate liposomes, small and large sized liposomes were also injected subcutaneously. There was a drastic reduction in the uptake of both large and small liposomes in the lymph node.[52] This reduction in liposome uptake supports the hypothesis that macrophages play the most important role in nanoparticle uptake in lymph nodes.

### 4.5. *Fate of nanoparticles in lymph nodes*

Only a few studies have looked at the fate of nanoparticles once they arrive at the lymph node.[5,53] In one study, subcutaneously injected liposomes were found to have accumulated in the subcapsular sinus. Subsequently, these liposomes were dispersed throughout the lymph node either by permeation along the sinus or within cells involved in liposome uptake such as macrophages. Once they were in the macrophages, the liposomes were observed to be digested by lysosomes.[53]

## 5. Nanoparticle Diagnostic Imaging Agents for Determining Cancer Status of Lymph Nodes

### 5.1. *Subcutaneous injection of iodinated nanoparticles for computed tomography imaging*

Nanoparticles have been developed for the delivery of image contrast agents to lymph nodes. Lessons learned in the development of these nanoparticles as lymph node contrast agents can be applied to lymph node drug delivery. Subcutaneously injected iodinated nanoparticles were found to target the lymphatics and have been investigated as computed tomography imaging (CT) contrast agents.[54–57] These nanoparticles were composed of ethyl ester of diatrizoioc acid, stabilized with

3.5% Tetronic 908 with an average particle size of 250 nm. Nanoparticle accumulation in the draining lymph node was found to consistently enhance the contrast by at least 100 Hounsfield units in rabbits. A Hounsfield unit is a description of relative attenuation of X-rays in CT imaging. In this system, water density attenuation is assigned 0 and air density attenuation is −1000 and compact bone attenuation is 1000. A contrast change of 100 Hounsfield units is easily recognized visually on the image. The contrast enhancement by the iodinated nanoparticles provided excellent detailed images of the intranodal lymph node architecture which would permit the diagnosis of cancer metastasis.

Different size iodinated nanoparticles have been compared for lymph node targeting characteristics. When a comparison was made between the lymph node contrast of smaller 116 nm iodinated nanoparticles and larger 250 nm particles, there was minimal difference in the total lymph node contrast eventually obtained with the iodinated nanoparticles. However, the smaller nanoparticles were found to have somewhat faster kinetics for lymph node accumulation and they also clear more rapidly from the lymph node.[58]

This iodinated contrast agent has been shown to aid in the discrimination of lymph nodes with cancer. In this study, perilesional subcutaneous injections (2 ml per lesion) of a 15% wt/vol iodinated nanoparticle suspension were made in pigs with cutaneous melanomas.[56] The average X-ray attenuation by the iodine and average iodine concentration in the lymph nodes with cancer was higher than in normal nodes. The presence of cancer within the node did not block uptake of the iodinated nanoparticles, as total iodine uptake was higher in cancerous nodes with greater than 25% cancer replacement ($p < 0.05$). The lymph nodes with cancer were larger in size, but the uptake of the iodinated contrast agent in the lymph nodes was lower. This suggests that the uptake in the region of the cancer was lower, but the normal areas became larger and compensated for the portion of the lymph node that contained cancer. The architectural alterations in opacified cancerous nodes included medullary lymph node filling defects, expansile cortical lesions, and disruption of corticomedullary junctions. The authors of this study concluded that both quantitative and qualitative differences in iodinated nanoparticle enhancement are characteristics that are useful in distinguishing between normal and cancerous lymph nodes with CT imaging, following subcutaneous injection of iodinated nanoparticles.[56]

In an interesting study, iodinated nanoparticles were administered into the thorax of dogs using bronchoscopy.[59] With this technique, a tube was inserted into the lung bronchi under visual guidance with the flexible scope. The nanoparticles were injected in the bronchi of the right diaphragmatic lobe of the lung. Two days after this procedure, CT images were obtained that did not show any uptake in the tracheobronchial lymph nodes. However, images that were obtained after 1 week

had excellent contrast enhancement of the tracheobronchial lymph nodes. This contrast enhancement remained fixed for 3 weeks following administration.[59] The slow delivery and prolonged retention of nanoparticles in the tracheobronchial lymph nodes has interesting implications for drug delivery. The uptake in the tracheobronchial lymph nodes appeared to be secondary to phagocytosis of nanoparticles by macrophages. The lymph nodes were enlarged and the histologic specimens showed macrophage hyperplasia. Did the macrophages phagocytize the particles in the lungs and carry them to the lymph nodes or did the nanoparticles somehow get to the lymph nodes, where they became phagocytized?

## 5.2. *Subcutaneous and intraorgan injection of magnetic resonance (MRI) contrast agents*

A limited amount of research has been performed, investigating subcutaneously injected gadolinium bound albumin, MS-325, for lymph node diagnosis. In a study of normal as well as tumor-bearing hindlegs of rabbits, the subcutaneous administration of MS-325 resulted in rapid delineation of popliteal, inguinal, iliac, and paraaortic lymph nodes.[60] Tumor invasion into lymph nodes presented as magnetic resonance imaging (MRI) signal voids in the areas infiltrated by tumor, whereas the surrounding residual lymphatic tissue showed enhancement identical to that of normal nodes. In addition to providing a safe means of displaying the normal lymphatic system, 3D image reconstruction of the MRI image was able to depict direct tumor invasion in lymph nodes.[60]

Direct injection of magnetic particles into the brain has also been shown to be a method for tracking local lymphatic drainage. When small magnetic nanoparticles were injected into the brain of rats, up to 50% of the particles were found to drain from the CNS via perivascular, perineural and primitive lymphatic drainage to the cervical lymph nodes. Central nervous system (CNS) lymphatic drainage may occur via connections to the vasculature, but in animal models, up to 50% occurs via perivascular, perineural and primitive lymphatic vessels to cervical lymph nodes. The trafficking of the superparamagnetic iron particles from the CNS in the rat could be visualized both by magnetic resonance imaging (MRI) and histology. These magnetic particles appear to provide a tool to rapidly assess drainage of virus-sized particles from the CNS using MRI.[61]

## 5.3. *Intravenous injection of magnetic nanoparticles for MRI imaging*

Ultrasmall nanoparticles can target all of the body's lymph nodes when injected intravenously. These ultra-small super-paramagnetic iron oxide particles (USPIO),

also known as ferumoxtran-10, and commercially as Sinerem® in the Netherlands (Laboratoire Guerbet, Aulnay sous Bois, France), and as Combidex® in the U.S. (Advanced Magnetics, Cambridge, MA), have been developed for improved lymph node metastasis detection.[62] These particles are composed of 4 to 5 nm iron oxide cores surrounded by a dextran coating. After the dextran coating, these particles are 20–25 nm in size. These particles produce MRI contrast by shortening the T2 weighted signal.

When injected intravenously, these small particles accumulate in the lymph node in a high enough concentration to cause significant MRI contrast.[63] The percent injected dose that ends up in these lymph nodes has not been well studied. In spite of lack of quantitation, the uptake in the lymph nodes is sufficient to result in effective lymph node contrast in all the lymph nodes of the body. Their half-life in circulation is approximately 24 hrs. These intravenously injected nanoparticles were associated with a low incidence of adverse reactions. The adverse events most frequently seen with USPIO were dyspnea (3.8%), chest pain (2.9%), and rash (2.9%). These effects are most likely due to the known complement activation associated with intravenous administration of nanoparticles.[64] No serious adverse events were reported during the 48-hr observation period. There were no clinically significant effects on vital signs, physical examination, and laboratory results. Slow infusion of a relatively low dose avoids previously reported adverse reactions associated with USPIO.

Nodal accumulation of intravenously injected USPIO is thought to occur, following movement of the USPIO through permeable vascular endothelium in lymph node vessels of the post-capillary venules and the adjoining capillaries. In one study, USPIO were regularly observed at the periphery of the lymph nodes, but not in the center of the lymph nodes.[65] Isolated iron particles were observed extracellularly within lymph vessels in the first hr after injection and by 3 hrs after injection, as small dots within macrophages. Numerous dense clusters appeared within the cells at later times (i.e. 6 and 12 hrs after injection). These results suggest that the contrast agent moves rapidly across the capillary wall to the lymph and is then taken up by macrophages.[65]

In an initial evaluation of safety and effectiveness of ultrasmall superparamagnetic iron oxide particles, 30 adults with suspected lymph node metastasis were evaluated with MR imaging before and 22–26 hrs after an intravenous dose of USPIO nanoparticles. The sensitivity for metastatic lymph node diagnosis was found to be 100% with a specificity of 80%.[66]

Another study evaluated USPIO for sensitivity and specificity for differentiating metastatic from benign lymph nodes. The study was carried out in 18 patients with lung cancer. Each patient was evaluated for the homogeneity of the lymph node image and change in the post contrast MR signal. All the patients

underwent resection of the lymph nodes and histopathologic correlation was performed. USPIO was found to have a sensitivity of 92% and a specificity of 80%.[62]

Studies have also shown that USPIO are effective in diagnosing metastasis in mesorectal lymph nodes. Uniform and central low-signal-intensity patterns in lymph nodes are features of nonmalignant nodes. Reactive nodes frequently show central low signal with T2-weighted imaging.[67]

Recently, a semi-automated technique was developed to detect lymph nodes with cancer, following injection of USPIO. Using computer assisted quantitative analysis, accurate discrimination between metastatic and normal lymph nodes was achieved with a sensitivity of 98% and a specificity of 92%.[68]

## 5.4. *Nanoparticle diagnostic agents for localizing the sentinel lymph node*

In the last decade, cancer surgeons have become very interested in methods to definitively localize the sentinel lymph node. The sentinel lymph node is the first lymph node that receives lymphatic drainage from the site of a primary tumor. The sentinel node is much more likely to contain metastatic tumor cells than other lymph nodes in the same region. It is believed that the initial draining lymph node (i.e. sentinel node) of a tumor may reflect the status of the tumor's spread to the remaining lymphatic bed. Localization of the sentinel lymph node and its close histological assessment, following its removal from the body was initially developed as prognostic indicator in patients with malignant melanoma.[69] If no cancer cells are found in the sentinel node on pathologic examination, the prognosis for the patient is greatly improved. After many detailed studies validating the effectiveness of this approach for patient prognosis and as a method to guide future therapy of melanoma patients, this technique has begun to be applied in other cancers, particularly breast cancer. Total lymphadenectomy procedures are being replaced by intraoperative lymphatic mapping and sentinel lymph node biopsy.[70]

This particular lymph node is now being studied in greater detail, since it is able to accurately identify the sentinel node. Close pathological examination of the sentinel node with reverse transcriptase-polymerase chain reaction (RT-PCR) has shown that the traditional procedures of hematoxylin and eosin (H&E) staining and immunohistochemistry underestimate the true incidence of cancer micrometastasis. Use of RT-PCR has been shown to be a more powerful predictor of disease relapse than traditional H&E staining and immunohistochemical methods.[71] The advent of the use of sentinel node localization studies has stimulated a general interest in lymph node therapy, including both the surgical removal of specific lymph nodes as well as lymph node drug delivery.

## 5.5. *Radiolabeled nanoparticles for sentinel lymph node identification*

Nanoparticles have played a crucial role in helping to identify and localize the sentinel lymph node. This is because many types of nanoparticles have significant retention in the first lymph node that they encounter. As much as 40% of the nanoparticles that move from the injected site can be retained in the first lymph node encountered.[16,46] The retention of nanoparticles in the first lymph node is due to phagocytosis of the nanoparticles by macrophages.[52] The total amount of retention in the sentinel node is higher than retention in the subsequent draining lymph nodes, because less of the initial dose reaches these secondary nodes. This characteristic led to the introduction of radioactive technetium-99m-sulfur colloid particles ($^{99m}$Tc-SC) particles as a second method to localize the sentinel lymph node in addition to blue dye which was also being used by surgeons.[69,72] The use of $^{99m}$Tc-SC in addition to blue dye was a significant improvement over blue dye alone. The blue dye was not well retained in the sentinel lymph node and it moved so rapidly that the surgeons frequently had difficulty in distinguishing the sentinel node from secondary lymph nodes. This problem was solved by the additional use of $^{99m}$Tc-SC which provided a better mark of the sentinel node. Although the blue dye provides a desired visual guide for the surgeon, the $^{99m}$Tc-SC provided verification that the correct lymph node had been biopsied. As the $^{99m}$Tc-SC could be imaged and also localized in the operating room prior to and during the surgery, it frequently led to a smaller operative incision and a decrease in the time required to find the sentinel node.[73] The use of radiolabeled nanocolloids, in addition to blue dye has been shown to be complementary techniques that are best used simultaneously.[74]

Studies to localize the sentinel lymph node in malignant melanoma and breast cancer are now considered the standard of care in patient management. Investigations are now being conducted to research the feasibility of using this same methodology in many other types of cancer including colon, stomach, head and neck, prostate, rectal, lung, uterine, vulvar, and penile cancer.

## 5.6. *$^{99m}$Tc-Colloidal nanoparticles for sentinel node identification*

The most common colloidal particle used in the United States is technetium-99m-sulfur colloid ($^{99m}$Tc-SC). $^{99m}$Tc is readily available, inexpensive and has ideal imaging and dosimetry characteristics. Its energy of 140 keV is high enough for emitted photons to escape the body without absorption of overlying body tissues, but low enough to be readily collimated by lead and absorbed by sodium iodide scintillation crystal. Standard SC particles range in size from 10 nm to 1000 nm and they are clinically approved for use as liver imaging agents. In this application, the particles

are injected intravenously, after which they are rapidly removed by macrophages of the liver, spleen and bone marrow. $^{99m}$Tc-SC makes a physiologic image of the liver and prior to the advent of CT scans, it was commonly used to assess the liver for tumors. Following the advent of CT scans, it has been used to assess the physiology of the liver due to the fact that diseases that damage the liver for any reason, such as alcoholic liver disease or infectious hepatitis, also decrease the uptake of nanoparticles by the macrophages the are located in the liver, and shift the liver uptake to the spleen and the bone marrow. This effect provides a physiologic indicator of the health of the liver.

$^{99m}$Tc-SC has been the nanoparticle used for lymph node identification in the United States, because these particles were already widely available and approved for clinical use as liver and spleen imaging agents. In the United States, no agent has been specifically approved for lymph node detection. These $^{99m}$Tc-SC particles range in size from 10 nm to 1000 nm. However, these particles are not ideal agents for the detection of the sentinel lymph node, due to the retention of the majority of the injected dose at the peritumoral site of the injection. Studies in animals have demonstrated that $< 5\%$ of the injected dose (ID) is cleared from the site of injection within 60 min after injection. With this low clearance, $< 2\%$ of the injected dose accumulates in the sentinel lymph node at 60 min. The intensity of the $^{99m}$Tc activity that is retained at the site of injection frequently makes it hard to locate the sentinel lymph node, either by imaging or by the use of a handheld detector probe used in surgery. As a result of this problem of retention at the injection site, $^{99m}$Tc-SC particles have been filtered through a 200 nm filter, so that only particles smaller than 200 nm would be administered.[75] These filtered $^{99m}$Tc-SC nanoparticles have greater movement from the injection site, although there is still a debate as to whether filtered $^{99m}$Tc-SC is better than standard $^{99m}$Tc-SC for sentinel node identification.[76]

In Europe, several other colloidal nanoparticles, $^{99m}$Tc-nanocolloid and $^{99m}$Tc-rhenium colloid, are available for use as sentinel node detection agents.[77–79] $^{99m}$Tc-rhenium colloid has an average particle size of less than 100 nm and filtration is not required. This $^{99m}$Tc-rhenium colloid has been shown to be highly effective for the identification of the sentinel lymph node.[79]

Several other techniques have been investigated that use other nanoparticle-based systems to enhance the ability of the surgeon to detect the sentinel node. One investigated technique has utilized liposomes containing blue dye to localize the sentinel lymph node. Hirnle *et al.* encapsulated patent blue dye within liposomes for potential use in localizing the sentinel lymph node during surgery.[80] When this technique was studied following injection of the blue liposomes into the lymphatic vessels, the lymph nodes were stained blue. Most notably, retroperitoneal lymph nodes in rabbits remained dark blue up to 28 days after hindlimb endolymphatic instillation of liposomal patent blue dye. This group has also investigated blue

liposome for sentinel node detection in the pig. The blue liposomes were found to provide greater intensity blue staining which lasted for a longer duration than free unencapsulated blue dye.[81] This group later performed a study in humans, in which blue liposomes were injected directly into the lymphatic vessels of the foot of a patient prior to retroperitoneal staging-lymphadenectomy.[82] The lymph nodes were well stained with blue dye and were readily visualized at the time of the surgery performed 24 hrs following the intralymphatic injection of the blue liposomes.

Plut *et al.* have also developed a liposome formulation containing blue dye that can be radiolabeled with $^{99m}$Tc.[83] The use of the liposome nanoparticle to provide a visual identification and the tracking of the liposomes through the lymphatic channels, along with the ability to trace the preparation using standard radiation detection instrumentation, provides the surgeon with an improved radiolabeled compound for lymphoscintigraphy and intraoperative sentinel lymph node identification. This method also demonstrates the versatility of nanoparticles to carry multiple diagnostic tracers on the same nanoparticle.

Our group has developed special formulation of liposome encapsulated blue dye that can be radiolabeled for imaging and probe detection.[44] With this system, liposomes trap in the lymph nodes for a prolonged time and they have a high efficiency of retention in the lymph nodes. This blue liposome formulation will be discussed in detail, in relation to lymph node drug delivery in Sec. 9.

## 5.7. *Optical*

In a very interesting and recently developed technique, fluorescent quantum dots have been investigated as agents to determine the location of the sentinel lymph node.[84,85] These quantum dots are particles that are 15 nm in diameter. They have ideal properties as a non-quenching fluorescent light emitter, following stimulation with near infrared light. Animal studies of these quantum dots have shown trapping in the sentinel node, following injection in normal animals. They move rapidly to the sentinel node so that they can potentially be used during surgery. In one study in pigs, the quantum dots were introduced into the lungs as a method of finding the sentinel lymph node draining from a lung cancer. The lymph node draining from the lung was rapidly identified. This rapid identification of the sentinel lymph node using a non-radioactive method was thought to provide a significant advantage for the method.

An important consideration in the future will be the possibility of toxicity from the heavy metal, cadmium, which is a major component of these quantum dots. The recent synthesis of quantum dots from other agents such as silver may eventually lead to quantum dots with improved biological toxicity profiles.[86]

### 5.8. *Ultrasound nanobubbles*

Another type of nanoparticle under investigation for the localization of the sentinel lymph node is the nanobubble. Nanobubbles consist of a bilayered shell of albumin and an inner layer of a biodegradable polymer known as polycaprolactone. This shell encapsulates the gas, nitrogen. Following subcutaneous injection, these nanobubbles are tracked using ultrasound imaging. In a study comparing microbubbles to sub-micron nanobubbles performed in dogs, the nanobubbles were more effective in detecting the sentinel nodes, with 94% or 30 of the 32 sentinel nodes being detected. This nanobubble technology could serve as an alternative method for detecting the sentinel lymph node. This approach is also unique in that the use of ultrasound to detect the bubbles also causes the bubbles to break apart and form smaller bubbles.[2] It has been proposed that jets released from these nanobubbles following exposure to high-frequency ultrasound could be used to nanoinject drugs into cells.[87] As these nanobubbles can also carry drugs, they could be used to deliver high levels of drugs to lymph nodes which could be rapidly released following insonation.[88] Concerns have been expressed that the energy released during insonation of these nanobubbles could produce harmful bioeffects due to thermal damage, and further investigation will be required to examine these issues in detail.[89]

## 6. Recently Introduced Medical Imaging Devices for Monitoring Lymph Node Delivery and Therapeutic Response

For studies of nanoparticle localization of lymph nodes and the assessment of drug delivery to lymph nodes for the treatment of cancer metastasis and other lymphatic disease processes, the development of clinical imaging systems to direct and verify therapy will continue to gain importance. In this regard, the recent advent of clinically available single photon emission computed tomography (SPECT)/computed X-ray tomography (CT) systems will be important. These systems permit the simultaneous image acquisition of a 3D scintigraphic image with a 3D CT image, which allows the perfect superimposition of the two types of images. With this new SPECT/CT camera, scintigraphic imaging of the location of the nanoparticle can be superimposed upon the high resolution CT images which clearly demonstrate the anatomy of the body. These systems are already proving to be useful for the diagnosis of lymph node metastasis in prostate cancer using targeting radiolabeled antibodies.[90] The ability to perform a CT image to verify the location of the activity visualized on the 3D SPECT scan will be important. Without anatomic verification by CT, it is difficult to determine whether the "hot spot" visualized on the image is a

lymph node. It is also difficult to accurately diagnosis the occurrence of a lymphatic metastasis.

By having an imaging machine that can simultaneously co-register a CT image, which accurately displays the image, with high resolution and superimposing the location of the delivered activity, it will be possible to accurately determine the distribution of lymph node directed nanoparticle therapy. In a recent study, the performance of SPECT/CT was particularly useful in identifying lymph nodes adjacent to the primary lesion. Such nodes are easily overlooked by planar lymphoscintigraphy and intraoperative gamma probes, as the high activity at the injection site can obscure their detection.[91]

Another study also found that SPECT/CT was highly effective for precise preoperative localization of the cervical sentinel node in early non-metastatic oral squamous cell carcinoma. The authors of this recent study concluded that they believe the use of SPECT/CT will become extremely useful, once a consensus has been reached on the exclusive excision of the cervical sentinel node in oral cancers, as is the case for melanoma or breast cancer.[92]

Positron Emission Computed Tomography (PET) has recently entered clinical practice for the diagnosis of cancer. Cancer is detected using F-18-fluorodeoxyglucose ($^{18}$F-FDG) due to the greatly increased glucose metabolism of cancer cells. This technique is very powerful at detecting cancer in lymph nodes containing metastasis.[93] It frequently demonstrates cancer in the lymph nodes that are not detectable with routine CT imaging. This technique could be very useful in monitoring lymph node response, secondary to nanoparticle delivered therapeutic agents. Monitoring nanoparticle drug delivery with PET could be particular useful in situations where the lymph node if not easily surgically accessible.

A recent report of a new use of $^{18}$F-FDG PET scanning in patients with HIV appears to clearly demonstrate specific lymph nodes that are harboring HIV.[94] $^{18}$F-FDG is a marker of glucose metabolism which is increased in inflammation as well as tumors. In this study using PET imaging, the total quantified summed activity in lymph nodes was found to correlate with the level of viremia across a 4-log range. Cervical and axillary lymph nodes were found to have significantly more activity than inguinal and ileal nodes ($p < 0.0001$). The lymph nodes were thought to have increased $^{18}$F-FDG uptake due to increased glucose metabolism in lymph nodes, with lymphocyte turnover secondary to viral infection in these infected lymph nodes. This work suggests that the level of virus encountered in the lymph nodes is directly related to the blood levels of HIV virus. These PET studies also clearly indicated that particular lymph nodes were involved, suggesting the possibility that these nodes could be specifically targeted with nanoparticles for drug delivery. A second article investigating PET scanning in patients with HIV has even suggested that surgical intervention to remove the specific nodes should be considered. An alternative strategy would be to use nanoparticles to target antiviral

medications to specific lymph nodes, based on the PET imaging data. Imaging with SPECT/CT could be used to determine that the nanoparticle associated drugs were specifically delivered to the targeted lymph node, and repeat PET imaging could be used to monitor the effectiveness of the delivered therapy for the treatment of the lymph node.

Analogues of these human imaging systems have been developed for small animal imaging research. These commercially available imaging systems combine microSPECT and microCT into the same unit for image superimposition. A new commercially available imaging system has become available, combining microSPECT, microCT and microPET into the same unit. These small animal imaging systems will prove to be valuable in the investigation of nanoparticle lymph node delivery. They should facilitate the translation of basic research to imaging studies in the clinical environment.

## 7. Nanoparticle Lymph Node Drug Delivery

Several authors have recommended that in the case of diseases with lymphatic involvement, it is desirable to develop treatment approaches to deliver diagnostic and therapeutic agents to lymph nodes that could help prevent diseases from spreading.[8,10,13,17] This situation has motivated interest in developing methods for specific drug delivery to lymph nodes and lymphatic vessels, with the hope of improving cancer and infection control and treatment.

Whatever the carrier system chosen, the following basic requirements need to be met in order to develop an effective drug delivery system: (a) the carrier system must concentrate selectively in the target organ or tissue; (b) the carrier system must release the drug in such a way as to achieve its chemotherapeutic or pharmacological effect, over the required time frame; (c) the carrier system must be stable before administration and during its journey to the target, but able to release the drug when it is at the target; and (d) the carrier must also be compatible with the body in terms of toxicity, biodegradation and immunogenicity.[11] Nanoparticles of different types can meet the above criteria as effective carriers for drug delivery to lymph nodes. Targeting of lymph nodes for drug delivery has been attempted by the use of emulsions, non-lipid macromolecules, antibodies, and nanoparticles. Nanoparticles appear to hold the most promise for lymphatic drug delivery, in comparison with other types of carriers. In the present review, we will focus on the variety of nanoparticle systems that have been used for lymph node drug delivery.

### 7.1. *Confusion in reporting lymph node delivery*

The research literature reporting nanoparticle uptake in lymph nodes can be very confusing. This is due to the tendency of many investigators to report the percent

uptake in the lymph node as a percent of the injected dose per gram of tissue. Although reporting uptake as a percent of the injected dose per gram is sensible from a tissue treatment standpoint, it does not easily allow the readers to determine the percent of the total administered dose accumulated in the lymph node. For instance, in animals such as mice with very small lymph nodes that weigh only a fraction of a gram, the accumulated doses can be as high as 100% of the injected dose per gram, but considering that a mouse lymph node weighs only 0.01 gram this is only 1% of the total dose that was administered (1% ID). Had this same fraction of the injected dose accumulated in a rat lymph node that weighs approximately 0.1 gram, this dose would have been only 10% of the injected dose per gram; and in a human with a lymph node with a weight of 1 gram, it would have been only 1% ID per gram. It is very important to keep these species differences in mind when interpreting the previous literature and it would be best if all investigators would report their research not only in terms of dose per gram, but also in terms of the percentage of the total injected dose delivered to the lymph node. From a pharmacologic standpoint, percent dose per gram may be considered correct, but it is highly unlikely that these percent dose per gram results in mice would translate into humans. Based on our experience, it is much more likely that the percent that clears from the injected site will always be approximately the same in each species and the percent that accumulates in the lymph nodes, no matter what its weight in grams, will also be approximately the same.

For example, in one study, researchers reported that subcutaneously injected liposomes without a lymph node targeting mechanism had much higher concentration in the lymph nodes on the side of the subcutaneous injection, compared with the lymph nodes of the opposite side that did not receive the injection.[95] 24 hrs after subcutaneous injection, 57.9% of the ID/gram of tissue was found in the inguinal node on the side of the injection (ipsilateral side) versus only 0.48% ID/gram of tissue in the lymph node of the opposite side of the injection (contralateral side) at 24 hrs. Again, this study was carried out in mice and the % ID per gram is somewhat deceptive due to the fact that mice have very small lymph nodes. This probably represents no more than 1–3% of the total injected dose accumulating in the lymph node that drained from the subcutaneous site. The important point is unchanged. There was more than a 100-fold increased amount of liposomes deposited on the side of subcutaneous injection, compared with the lymph node on the other side that did not receive the subcutaneous injection.[95]

It is important that readers of the lymph node drug delivery literature take lymph node weight into consideration. For instance, if 20% of the total subcutaneous injected dose were to accumulate in the lymph node of a mouse with a lymph node that weighs just 0.01 gram, the % ID/gram would be 20 divided by 0.01 or 2000% of the injected dose per gram.

### 7.2. *Calculation of lymph node retention efficiency*

Using scintigraphic imaging, our group has developed a method to calculate lymph node retention efficiency.[3] The calculation describes the fraction of the nanoparticles that are cleared from the initial subcutaneous site of injection that become trapped and retained in the lymph node. This estimated lymph node retention calculation describes how efficiently the lymph node can retain a particular subcutaneously injected nanoparticle. It also describes the portion of the dose that enters a lymph node and then leaves that primary lymph node and moves to the next lymph node. The calculation requires that the nanoparticle be labeled with a radiotracer and imaged scintigraphically. It is determined by drawing a region of activity around the injection site and determining the total percentage of the baseline injected activity that has cleared the injection site at various times post injection. This total cleared activity is then assumed to be the amount that enters the first lymph node. The activity retained in the lymph node is divided by the total activity cleared from the injection site, so that a lymph node retention efficiency can be calculated. With most unmodified liposome preparations, lymph nodes only retain approximately 4% of the liposomes that enter the lymph node. Retention efficiency is higher with other particles, such as unfiltered $^{99m}$Tc-SC that have a 40% lymph node retention efficiency, but these particles are also associated with a very poor clearance from the initial injection site.[16]

## 8. Specific Types Nanoparticles for Lymph Node Targeting

### 8.1. *PLGA nanoparticles*

Poly(lactide-co-glycolide)(PLGA) nanoparticles have been investigated for lymph node drug delivery agents. By modifying the surface of the PLGA nanoparticles with block co-polymers, PLGA nanoparticles that deliver up to 17% of a subcutaneously injected dose to regional lymph nodes have been developed.[96] Lymphatic uptake was studied by labeling these PLGA nanoparticles with $^{111}$In-oxime. Lymphatic uptake of all coated PLGA nanoparticles was enhanced, compared with the uncoated PLGA nanoparticles. This research suggests that the nanospheres are suitable for diagnostic and therapeutic lymph node delivery applications in clinical and experimental medicine.[96]

In another study, the *in vivo* trafficking of PLGA particles, encapsulating a diphtheria antigen, was possible using a fluorescent marker following subcutaneous administration for the immunization of mice,[97] these fluorescent PLGA microspheres were observed in cells of lymphoid tissues such as mesenteric lymph nodes and spleen. However, particle fluorescence in lymphoid tissues decreased rapidly, as they were degraded inside the cells, thereby enabling the presentation of the

antigen to specific cells of the immune system. This is one of the few studies in which a nanoparticle carrier for vaccine delivery was tracked *in vivo*.[97]

## 8.2. *Micelles*

Particles known as micelles can be formed from amphiphilic biocompatible polyoxyethylene (PEO)-based polymers. These nanoparticles (micelles) are 10–50 nm in diameter. Researchers have labeled micelles with amphiphilic indium-111 ($^{111}$In) and gadolinium chelates and used them as nanoparticulate contrast media for imaging lymph nodes, following subcutaneous injection into the rabbit's paw.[98] Corresponding images of local lymphatics were acquired using a gamma camera and a magnetic resonance (MR) imager. The entire lymphatic vessel chain from the paw to the thoracic duct could be visualized using $^{111}$In labeled micelles. After injection site massage, 40% of the injected dose cleared from the injection site into the lymphatics, 30 min after massage was performed over the site of injection. Although approximately 8% of the subcutaneously injected dose was in the popliteal lymph node at 30 min, this amount decreased significantly after massage over the popliteal lymph node, with less than 2% of micelles remaining in the lymph node. When gadolinium containing micelles were administered, T1-weighted MR images of the primary lymph node had significant contrast enhancement within 4 min following massage.[98] Prior to this imaging research, Torchilin had previously shown that micelles have much promise for the delivery of poorly soluble drugs following intravenous delivery.[99] Micelles also appear to be promising agents for lymphatic drug delivery, particularly if methods can be developed to increase their retention in lymph nodes.

## 8.3. *Liposomes*

The investigation of liposomes as carriers for lymph node drug delivery was first performed by Segal *et al.*[100] Following the intratesticular injection of liposomes encapsulating the anti-cancer drug actinomycin D, high concentrations of the drug were found in the local lymph nodes. Lymph node imaging using $^{99m}$Tc-labeled liposomes was first performed by Osborne *et al.*[101] Liposome distributions were determined in rats following injection of the $^{99m}$Tc liposomes in the rat hind footpads. In these studies, 1 to 2% of the injected dose of neutral and cationic liposomes were found to localize in the draining lymph nodes. Negatively charged liposomes did not show good accumulation in the lymph nodes.[101] Soon after these studies were performed, the reliability of these previous studies for representing the actual distribution of liposomes was questioned and it was suggested that much of the activity localized in the lymph nodes was not associated with liposomes, due to

instability of the $^{99m}$Tc label.[102] This article recommended that new methods of labeling liposomes with $^{99m}$Tc be developed. Follow-up studies demonstrated that the type of labeling used in the prior studies, in which $^{99m}$Tc was labeled to the outer surface of liposomes following reduction of the $^{99m}$Tc with stannous chloride, was not stable.[103] It was particularly unstable for labeling liposomes with a cationic and neutral surface charge, which possibly explained the low accumulation in lymph nodes found with anionic liposomes. These studies demonstrate the importance of label stability in the tracking of liposomes for quantitation of targeting to the lymph nodes.

Since those early studies, more stable methods of labeling liposomes with $^{99m}$Tc have been developed and applied to lymph node imaging. A method developed by our group uses hexamethylpropyleneamine oxime (HMPAO), a clinically approved and commercially available chelator of $^{99m}$Tc used for brain imaging.[104] In this method, $^{99m}$Tc-pertechnetate, which is readily available from a generator, is incubated for 5 min with HMPAO, which chelates the $^{99m}$Tc into lipophilic $^{99m}$Tc-HMPAO.[105] The $^{99m}$Tc-HMPAO is then added to previously manufactured liposomes that encapsulate glutathione. It is generally believed that lipophilic HMPAO carries the $^{99m}$Tc across the lipid bilayer of liposome into the aqueous interior of the liposome where it interacts with the encapsulated glutathione, resulting in its conversion to hydrophilic $^{99m}$Tc-HMPAO. The hydrophilic $^{99m}$Tc-HMPAO is irreversibly trapped in the aqueous phase of the liposome because it is unable to cross the lipid membrane. A similar mechanism has been proposed to explain the process whereby $^{99m}$Tc-HMPAO becomes trapped in brain cells for use as a brain imaging agent. This liposome label is very stable with minimal dissociation of the $^{99m}$Tc from the liposomes. It has been used to study the distribution of intravenously administered liposomes.[106]

Using the above liposome labeling methodology, detailed studies were carried out to assess the effect of liposome size and surface modifications on movement from the subcutaneous site of injection, as well as the retention of the liposomes in the lymph node.[16] The fraction of liposomes that are cleared from the subcutaneous injection site depends on the size and surface characteristics of a particular liposome formulation. Their large size of liposomes (> 80 nm) precludes their direct absorption into the blood stream. In studying a wide range of liposome sizes from 86 nm liposomes to 520 nm liposome, there was little difference in the ultimate accumulation of liposomes in the first or sentinel lymph node at 24 hrs post administration. This lymph node retention ranged from 1.3% to 2.4% of the total administered dose.[16] This retention in the lymph node is relatively low, considering that most subcutaneously injected liposome preparations more than 50% of the injected liposomes are cleared from the injection site. The lymph node retention for unmodified liposomes is low, compared with other colloidal particles such as $^{99m}$Tc-SC. There

were differences between the different liposomes sizes and surface characteristics in regard to the percent of activity that cleared from the subcutaneous site of injection. Small liposomes and those coated with PEG had the greatest clearance from the subcutaneous site of injection, with small 86 nm liposomes having < 40% of the injected dose remaining at the injection site at 24 hrs. Larger neutral and negatively charged liposomes had > 60% of the injected dose remaining at the initial site of subcutaneous injection. However, this smaller amount of large liposomes that were cleared from the injection site was compensated for by better retention in the lymph node.[16]

It appears that the properties of liposomes which enhance their clearance from the injection site, also decrease their retention in the lymph nodes. The generally low overall lymph node retention of most standard liposome formulations is likely to be due to their natural lipid composition, which probably allows a large percentage of liposomes that enter a lymph node to escape recognition and phagocytosis by macrophages that line the endothelium of the lymph node. This relatively low retention of liposomes in lymph nodes has also been reported by Ousseren *et al.*[5,45,47,107]

One of the several liposome surface modifications that have resulted in modestly increased retention of subcutaneously injected liposomes in the lymph node is the use of positively charged lipids in the liposome. Liposomes containing positively charge lipids had approximately 2 to 3 times the lymph node localization (up to 3.6% of the injected dose), as liposomes containing neutral or negatively charged lipids (1.2% of the injected dose).[47] Coating liposomes with the antibody, IgG, has been shown to increase the lymph node localization of liposomes to 4.5% of the injected dose at 1 hr, but this level decreased to 3% by 24 hrs.[108] Attaching mannose to the surface of a liposome has also been reported to modestly increase lymph node uptake by 3-fold, compared with control liposomes.[109] None of these previously mentioned modifications has resulted in large increases in the percentage of liposomes deposited in the draining lymph nodes, while most of the lymphatically absorbed liposome dose passes through the lymph nodes.

This relatively low retention may be due to the fact that the natural lipid composition of liposomes allows them to escape recognition by the macrophages located in the lymph nodes. In comparison with other particles, an abundant fraction of the subcutaneously injected dose of liposomes moves into the lymphatic system, so that greater than 50% clears from the site of injection by 24 hrs. Even though this retention of liposomes is very low in comparison with the injected dose, its uptake can still be quite substantial in terms of drug delivery, when considered on a per gram of tissue basis. For example, 1 to 2% of the injection dose in each lymph node represents a total per gram tissue uptake that is generally greater by 30–40 fold than

the liposome uptake that will eventually reach the liver and spleen following the same subcutaneous injection.

## 9. Avidin Biotin-Liposome Lymph Node Targeting Method

The relatively low retention of liposomes in lymph nodes led our group to search for new ways to improve liposome retention in lymph nodes. This research resulted in development of a new method of increasing liposome retention in lymph nodes following subcutaneous injection.[3] This lymph node targeting method utilizes the high affinity ligands, biotin and avidin. Biotin is a naturally occurring cofactor and avidin is a protein derived from eggs. Avidin and biotin have an extremely high affinity for each other. Avidin has 4 receptor sites for biotin associated with each molecule. These 4 receptor sites permit the binding of multiple biotin molecules which causes aggregation of liposomes that have biotin on their surface. Using this method, liposomes coated with biotin on their surface (biotin-liposomes) are injected subcutaneously, followed by a nearby subcutaneous injection of avidin. Following their subcutaneous injection, the avidin and the biotin-liposomes move into the lymphatic vessels. It was orginally hypothesized that the biotin-liposomes that are migrating through the lymphatic vessels meet with the avidin, resulting in an aggregate that becomes trapped in the lymph nodes. Subsequent research suggests that an alternative possibility may be more likely.[110,111] This alternative hypothesis is that the positively charged avidin becomes bound to negatively charged endothelial cells in the lymph nodes and the biotin-liposomes become bound by these avidin molecules attached to the endothelial surface. It is possible that both processes are occurring, however, research with intracavitary avidin/biotin-liposome systems suggests that the second possibility may be more likely.[110,111]

This *in vivo* nanoassembly of biotin-liposome/avidin aggregates mimics processes that occur naturally in the body such as the aggregation of platelets and the aggregation of infectious agents by antibodies. The biotin-liposome/avidin system has promising potential for therapeutic agent delivery to lymph nodes. It can be applied not only to subcutaneous targeting of lymph nodes, but also to intracavitary lymph node targeting. Although liposomes are the particular nanoparticle in which this methodology has been developed, it should also be possible to apply this methodology to the other types of nanoparticle carriers. Other high affinity ligands pairs as alternatives to avidin and biotin could also be used. Scintigraphic imaging of liposomes labeled with $^{99m}$Tc, labeled in a stable fashion, has greatly aided the determination of the proper concentration of avidin and biotin and could be used to develop similar targeting methodology with other nanocarriers.[3]

As an extension of the avidin/biotin-liposome lymph node targeting system, we have developed a special liposome formulation that contains both encapsulated blue dye as a potential system for localizing the sentinel lymph node, visually as well as scintigraphically, and/or with a gamma probe.[44] Potential advantages of this system over the current methods are that it can be performed any time from 1 hr to 1 day before the surgery is planned, because the lymph nodes are stained blue for a prolonged time and the sentinel lymph has the highest concentration of liposomes. Using this method, a separate blue dye injection just prior to surgery would not be necessary.

Examination of blue lymph nodes by light microscopy reveals that the liposomes tend to be deposited only in the outer cortex of the lymph node, however, this is not always the case as lymph nodes can be completely stained, depending on the concentration and timing of the avidin and biotin-liposome injection. As part of these studies, we have found that lymph nodes remain blue by visual observation *in vivo*, for more than a week following subcutaneous injection. The prolonged retention and slow release observed with blue biotin-liposomes demonstrates the potential of this system for the delivery and sustained release of drugs in the lymph nodes. Clinical studies would be required to determine whether the biotin-liposome/avidin system is effective in targeting the sentinel node in humans.

## 10. Massage and the Avidin-Biotin Liposome Targeting Method

Repeated massage and reinfusion of saline in a subcutaneous site of injection of the biotin-liposomes and avidin can be used to further enhance lymph node accumulation, as well as the rapidity at which the liposomes leave the subcutaneous site. In a study in our laboratory, the effect of this repeated massage on lymph node accumulation with the avidin-biotin-liposomes technique was compared with the same method with filtered $^{99m}$Tc-SC particles. In this study, a 24-gauge angiocatheter was placed subcutaneously in the dorsal foot of an anesthetized rabbit and 0.3 ml $^{99m}$Tc-radiolabeled biotin-liposomes or $^{99m}$Tc-SC were infused through the catheter into the subcutaneous tissue. Avidin (5 mg in 0.3 ml) was injected into the rabbit hind foot, approximately 2 cm proximal to the biotin-liposome injection site, in the same manner as described in the initial biotin-liposome study.[3] The injection site was massaged for 5 min. At serial 5 min intervals, 0.3 ml of saline was infused through the indwelling subcutaneous catheter and massage was performed for 3 min, following each reinfusion of saline for the first hr. During the second hr post injection, this 0.3 ml saline infusion followed by massage was repeated at 10-min intervals until 120 min post injection. Images were acquired at baseline, 30, 60 and 120 min

and at 24 hrs. After acquiring images at 2 hrs, the rabbits recovered from the anesthesia and were returned to the animal facility until they were imaged at 24 hrs.

As shown in Fig. 3, the massage more than doubled the liposome accumulation in the popliteal node of the rabbits. The intensity of $^{99m}$Tc activity in the popliteal node is equal or greater than that of the activity at the injection site. At 2 hrs, an average of 29% of the injected dose of liposomes were trapped in the popliteal node of the foot that received the serial massages and saline infusions versus 13% in the popliteal node on the side that was not massaged. At 2 hrs, more than 2.5 times the injected dose had cleared from the site of injection in the foot on the side with the serial saline infusions and massage, compared with the control side (50% versus 20%), so that massage with serial saline infusions can greatly increase the rate and total movement from the injection site. The liposome uptake in the popliteal node was generally fixed for an extended period. At 24 hrs, 20% of the injected dose was still retained in the lymph node that received the repeated massage and saline infusions. When this same methodology was compared in filtered $^{99m}$Tc-SC particles, the greatly increased accumulation in the lymph nodes was not observed. Although a greater amount of filtered $^{99m}$Tc-SC particles was cleared from the subcutaneous injection site in the foot with the serial saline infusions and massage, (37% vs 20%),

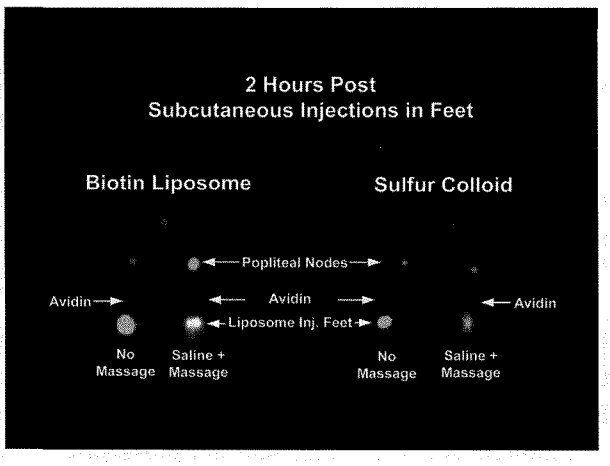


**Fig. 3.** Scintigraphic images of rabbit feet and legs acquired 2 hrs after administration of $^{99m}$Tc-biotin liposomes subcutaneously in the feet of a rabbit demonstrating the effect of repeated massage and saline infusion on lymph node accumulation following injection of radiolabeled biotin liposomes and avidin was compared with the effect of massage on filtered $^{99m}$Tc-SC particles. The massage and repeat saline infusions more than doubled the liposome accumulation in the popliteal node of the rabbits (29% vs 11%). Massage and saline infusion did not greatly increase the lymph node accumulation of filtered $^{99m}$Tc-SC particles (13% vs 9% ). It appears that the extra saline and massage simply pushed most of the $^{99m}$Tc-SC through the lymph node. This was unlike what happened with the biotin-liposomes, where the avidin continued to trap or aggregate the biotin-liposomes in the lymph node.

the percent of the injected dose retained at 2 hrs in the popliteal node was only minimally increased (13.2% vs 9%). It appears that the extra saline and massage simply pushed most of the $^{99m}$Tc-SC through the lymph node. This was very different from the biotin-liposomes, where the avidin continued to trap or aggregate the biotin-liposomes in the lymph node.

## 11. Nanoparticles for Lymph Node Anti-Infectious Agent Delivery

Only a few studies have examined the delivery of nanoparticles to lymph nodes for the treatment of infectious disease. In one study, liposome encapsulated amikacin was injected subcutaneously, intramuscularly, and intravenously. Drug levels in the lymph nodes were studied at various time points following injection. Drug level area under the curve (AUCs) in regional lymph nodes exceeded plasma AUCs by 4-fold, after subcutaneous and intramuscular injection of liposomal amikacin.[112] The authors of the study conclude that liposomes encapsulating amikacin have much potential for drug delivery, and even suggest that these liposomes could potentially be used for local delivery in perioperative prophylaxis, pneumonias and intralesional therapy as well as sustained systemic delivery of encapsulated drugs.[112]

This effectiveness of liposome encapsulated amikacin following subcutaneous injection differs significantly from a previous study, in which 400 nm liposome encapsulated amikacin was administered intravenously. The intravenously administered amikacin encapsulated liposomes were effective against M. avium intracellulare located in the liver and spleen, but they had no effect on the organisms that were located in the lymph nodes.[113] It is unlikely that these intravenously injected liposomes accumulated in the lymph nodes to any degree.

The use of nanoparticles to increase the drug delivery to HIV infected lymph nodes appears promising. When injected intravenously, the anti-HIV drug, indinavir, was found to achieve drugs levels in lymph node mononuclear cells that were only 25–35% of mononuclear cells in the blood. Lipid suspensions of the antiviral HIV drug, indinavir, form particles that are 50–80 nm in size. When these particles were injected subcutaneously in HIV infected macaques, very high drug levels were achieved in the lymph nodes and viral RNA loads in these nodes were greatly reduced. This lowering of HIV viral RNA levels could not be achieved when the same drug was injected intravenously.[114]

Dufresne *et al.* have investigated liposomes coated with anti-HLA-DR Fab' fragments for specifically targeting liposomes to follicular dendritic cells and macrophages within the lymph nodes of mice, with the goal of increasing the delivery of antiviral drugs to these cells infected with HIV.[115] The uptake of anti-HLA-DR

Fab' coated liposomes within lymph nodes was 2 to 3-fold higher when compared with conventional liposomes. However, of more importance is the potential specific delivery of the anti-HLA-DR Fab' liposomes to antigen presenting cells within the lymph node.

More recently, researchers from this same group have investigated the targeting of lymph nodes with indinavir, a protease inhibitor, encapsulated into immunoliposomes coated with the same anti-HLA-DR Fab' antibody fragment. Mice were injected subcutaneously below the neck with either free indinavir or liposome encapsulated indinavir. Animals were sacrificed at various times following injection and tissues collected and analyzed for indinavir drug levels. Drug levels were compared in lymph nodes from the mice receiving the subcutaneously injected free drug and subcutaneously injected liposome encapsulated drug. Drug levels in the brachial and cervical lymph nodes were 126 and 69 times greater with the liposome encapsulated drug, than the free drug.[24]

## 12. Liposomes for Intraperitoneal Lymph Node Drug Delivery

Intraperitoneal drug delivery is currently considered a viable approach for the treatment of ovarian cancer.[116–118] Studies in which free drugs are administered into the peritoneum have shown survival benefits in ovarian cancer patients. Although most intraperitoneally delivered unencapsulated free drugs are rapidly cleared from the peritoneal fluid without entering the lymphatic system, direct intraperitoneal administration of drugs can achieve much higher peak concentrations in the peritoneal fluid, compared with the same drug administered intravenously (20-fold higher for cisplatin and carboplatin, to as high as 1000-fold for taxol).[116–118] Although these drug levels quickly equilibrate with plasma after termination of the peritoneal infusion, transiently elevated peritoneal drug levels provide a significant therapeutic advantage. These have led many investigators to be enthusiastic about this approach for ovarian cancer treatment.[117,119] Unfortunately, rapid clearance of these free drugs from the peritoneum diminishes the advantages derived from the intraperitoneal infusion procedure. One way of improving peritoneal drug delivery could be through the use of nanoparticle drug carriers.[120–122]

Nanoparticles have significant promise as carriers for intraperitoneal drug delivery. Many disease processes spread by dissemination through the peritoneum. For instance, dissemination of cancer cells throughout the peritoneum is a very common manifestation of ovarian and gastric cancer.[123] When the cancer cells spread throughout the peritoneum, they are frequently trapped in lymph nodes that receive peritoneal fluid drainage.[124] The normal pathway of drug clearance from the peritoneum is either through direct absorption across the peritoneal membrane or by drainage into the lymphatic system through absorption by the stomata in the

diaphragm.[125] These diaphragmatic stomata are fairly large and can absorbed red blood cells from the peritoneal fluid.[126,127]

Intraperitoneally administered therapeutic nanoparticles not only increase and prolong the drug delivery in the peritoneum, but they can also increase delivery of therapeutic agents to the lymph nodes that filter lymph fluid drainage from the peritoneum. These lymph nodes frequently contain cancer cells.

## 12.1. *Intraperitoneal liposome encapsulated drugs*

Intraperitoneal administration of a liposome encapsulated drug not only increases the retention of the drug in the peritoneum, it also increases the delivery of the drug to lymph nodes that drain from the peritoneum. This is because liposome encapsulated drugs are mostly cleared through the lymphatic vessels, with at least a portion of the administered drug being deposited in the lymph nodes, where it degrades and is slowly released from the liposome and lymph node macrophages in high concentrations.[121,128]

Other studies demonstrate an improved toxicity profile. For instance, encapsulation of paclitaxel in a liposome has been shown to have decreased toxicity following intraperitoneal administration, while retaining equal efficacy for the treatment of intraperitoneal P388 leukemia.[129] In humans, the dose limiting toxicity from intraperitoneal administration of paclitaxel was severe abdominal pain, which was thought to be due to direct toxicity from either the paclitaxel or the ethanol/polyethoxylated castor oil delivery vehicle.[130]

Intraperitoneal delivery has also shown promise for nanoparticle gene transfection with novel cationic lipid containing liposomes.[131] These cationic liposome contained luciferase and beta-galactosidase genes that served as reporter genes. Intraperitoneal gene delivery for peritoneal disseminated ovarian cancer in nude mice was achieved using a stable chloramphenicol acetyl transferase (CAT)-expressing ovarian cancer cell line (OV-CA-2774/CAT), which permitted quantification of the exact tumor burden of organs. Intraperitoneal gene delivery to these disseminated ovarian cancer cells was excellent, with gene transfection appearing to be specific to intraperitoneal ovarian cancer cells. The O-Chol:DNA lipoplex appears to offer potential advantages over other commercial transfection reagents because of (1) its higher level of gene expression *in vitro* and *in vivo*; (2) its reduced susceptibility to serum inhibition; and (3) its highly selective transfection into tumor cells. These results suggest that the O-Chol:DNA lipoplex is a promising tool in gene therapy for patients with peritoneal disseminated ovarian cancer.[131]

An important potential application of the intraperitoneal delivery of liposomes and other nanoparticles that carry anti cancer agents is in the prophylaxis of peritoneal carcinomatosis. As 50% of patients with malignant gastrointestinal

or gynecological diseases experience peritoneal carcinomatosis shortly after local curative resection, there is a great interest in delivering intraperitoneal chemotherapy during the perioperative period. One study found that the intraperitoneal administration of the chemotherapeutic agents, cisplatin and mitomycin, prevented perioperative peritoneal carcinomatosis in a rat model.[132,133] The rats receiving cisplatin did, however, experience severe, local toxicity with bleeding into the peritoneum and toxic necrotic reactions of the colon. Liposomes encapsulating anticancer agents could potentially be used for this type of perioperative chemotherapy. The potential for the treatment of micrometastasis in lymph nodes with this liposomes is also great. Metastasis to mediastinal and other lymph nodes receiving lymph drainage from the peritoneal fluid are not uncommon findings in ovarian cancer at autopsy.[124]

One important consideration that might influence the effectiveness of intraperitoneal lymph node drug delivery is the possibility that the lymphatics could be completely obstructed with tumor, and therefore not accessible for lymph drainage. Generally, the ascites that develops in patients with intraperitoneal cancer are thought to be due to the obstruction of the lymphatics by the metastatic cancer. In a recent study, only 12.5% of women diagnosed with early stage ovarian cancer presented with ascites.[134] Biodistribution studies with liposome imaging could be performed to determine the effectiveness of lymph node targeting in situations of suspected lymph node obstruction.

## 12.2. *Effect of liposome size on intraperitoneal clearance*

It must be noted that simply making liposomes larger does not increase retention in the peritoneum or lymph nodes that receive drainage from the peritoneum. Hirono and Hunt have performed an extensive study on the effect of liposome size on their subsequent distribution, after intraperitoneal administration of liposomes of different sizes. In their studies, 50–60% of the intraperitoneal dose of liposomes of varying sizes encapsulating carbon-14 ($^{14}C$) labeled-sucrose cleared from the peritoneum by 5 hr in all liposomes studied. These liposomes ranged in size from 48 nm to 720 nm. The greatest amount of $^{14}C$-sucrose ($\sim$ 40%) appeared in the urine after administration of the largest liposomes. The authors speculated that the large 460 nm and 720 nm liposomes were unstable in the peritoneum, so that they rapidly released their encapsulated $^{14}C$-sucrose. It is also unlikely that simply increasing the size of the liposomes, in and of itself, would be sufficient to result in increased peritoneal and lymph node retention, because particles as large as erythrocytes have been demonstrated to readily drain from the peritoneum, by passing through the diaphragmatic stomata and returning to the blood stream. In one study of

chromium-51 labeled red blood cells injected into the peritoneal cavity of sheep, 80% of the red cells returned to the blood circulation by 6 hrs after administration.[126]

## 12.3. *Avidin/Biotin-liposome system for intraperitoneal and lymph node drug delivery*

Few of the above previously described studies with intraperitoneally administered liposome nanoparticles have focused on the fact that liposome nanoparticles clear from the peritoneum by passing through the lymphatic vessels. The liposomes pass through and are only partially trapped to lesser or greater degrees in the lymph nodes that drain from the peritoneum. These lymph nodes frequently contain cancer metastasis.

Our group has applied the previously described avidin/biotin-liposome system to intraperitoneal drug delivery.[18] The intraperitoneal biotin-liposome/avidin delivery method previously described in this paper has potential as a delivery system for the local treatment of intraperitoneal and intralymphatic disease processes, by increasing the retention of drugs in the peritoneum and in the lymph nodes that receive lymphatic drainage from the peritoneum.

The interaction of biotin-liposomes with avidin apparently results in the aggregation of the liposomes in the peritoneum. This aggregation greatly alters the distribution of liposomes and appears to result in a prolonged retention of liposomes in the peritoneum, as well as an increased accumulation and retention of liposomes in lymph nodes receiving drainage from the peritoneum. Rats that received intraperitoneal injection of biotin-liposomes and avidin had only a minimal percentage of the injected dose of liposomes that reach the systemic circulation by 24 hrs and a low % ID was found in the spleen, blood and liver at 24 hrs (< 9% ID). In contrast, control animals, administered only the biotin-liposomes without the avidin, had 23% ID in the spleen, 14% ID in the blood, and 9.8% ID in the liver, for a total of 47% ID in these organs at 24 hrs. Lymph nodes in the abdomen and in the mediastinum in rats receiving avidin had also greatly increased uptake of the biotin-liposomes.

Delivery of liposome encapsulated drugs using this method should provide sustained local release of drug within the peritoneum and the lymph nodes draining the peritoneum, as the liposomes degrade or become phagocytized by macrophages. This delivery system could also attenuate systemic drug toxicities by greatly reducing the rate at which a drug returns to the systemic circulation by either preventing rapid direct absorption through the peritoneal membrane, or by moderately preventing rapid passage through the lymphatic vessels and lymph nodes back to the blood.

Intraperitoneal administration using the avidin/biotin-liposome system has potential as a carrier system for the delivery of anti cancer agents in the peritoneum,

as well as liposome encapsulated radiotherapeutic beta-emitters for the treatment of peritoneally disseminated ovarian and upper gastrointestinal cancers.

## 12.4. *Mediastinal lymph node drug delivery with avidin-biotin system by intrapleural injection*

Mediastinal nodes are involved as centers of incubation and dissemination in several diseases including lung cancer, tuberculosis, and anthrax.[34,135,136] Treatment and control of these diseases is hard to accomplish because of the limited access of drugs to mediastinal nodes, using common pathways of drug delivery. Also, the anatomical location of mediastinal nodes represents a difficult target for external beam irradiation, due to its close proximity to major vessels and the heart. The use of the avidin/biotin-liposome method has been investigated as a carrier system for drug delivery to mediastinal nodes, using intrapleural injection as the pathway of delivery.[137,138] Drug delivery, using the avidin/biotin-liposome system, injected intrapleurally could solve some of these limitations and offer several advantages for the treatment of these diseases. Only minimal investigation has been performed using intrapleural administration as a pathway for drug delivery to mediastinal nodes. Perez-Soler *et al.* have investigated the intrapleural route of administration of liposome encapsulated drugs for the treatment of malignant pleural effusion in human patients.[137]

Our group has performed studies to investigate the use of the avidin/biotin-liposome system for targeting the mediastinal node following intrapleural administration in rats. Studies were performed by injecting biotin-liposomes into the pleural space following by an injection of avidin 2 hrs previously. By 22 hrs after injection, good retention (15.7% ID/mediastinal nodes; 515% ID/g) of liposomes was achieved in the mediastinal nodes with the avidin/biotin-liposome system. The scintigraphic image that visually demonstrates the mediastinal node uptake is shown in the image in the left panel of Fig. 4. An actual photograph of the blue stained mediastinal nodes obtained during necropsy is shown in Fig. 6. The images demonstrate the high uptake of liposomes in the mediastinal nodes. In the absence of avidin, liposomes were minimally retained in the nodes (< 1.0% ID/organ; 36% ID/g). This approach was the reverse of the sequence used in prior subcutaneous and intraperitoneal studies, in which avidin was injected after the biotin-liposomes.[3,18]

The specific targeting of a liposome-encapsulated drug to mediastinal lymph nodes could result in a prolonged targeted sustained depot-like delivery of high drug concentrations to these nodes, while the liposomes are slowly degraded and metabolized by phagocytic cells located within these nodes. Future experiments using intrapleural injection of the avidin/biotin-liposome system to target drugs to mediastinal nodes should be pursued.

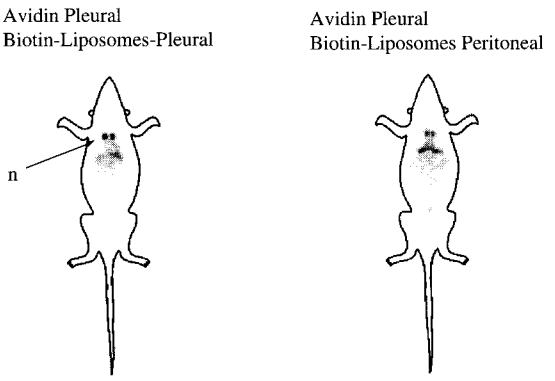


**Fig. 4.** Using the avidin-biotin liposome system in a rat model, high levels of liposomes were trapped in the mediastinal node when the avidin was injected in the pleural space and followed 2 hrs later by injection of the radiolabeled biotin-liposomes as demonstrated on the image on the left hand side. The avidin alone was injected in the pleural space and radiolabeled biotin liposomes were injected in the peritoneal space. Scintigraphic images were performed at 24 hrs. High levels of liposomes accumulated in the diaphragm as well as the mediastinal nodes. The diaphragm is the linear structure with intense uptake at the bottom of the chest region.

## 12.5. *Avidin biotin for diaphragm and mediastinal lymph node targeting*

Using the avidin-biotin liposome system, it was serendipitously discovered that when biotin-liposomes were injected into the peritoneal cavity and avidin was simultaneously injected into the pleural cavity, the liposomes aggregated strongly in the diaphragm as well as in the mediastinal nodes. This accumulation in the diaphragm occurred when the avidin draining from the pleural space into the diaphragmatic lymphatics encountered the biotin-liposomes draining from the peritoneal space, causing the liposomes to aggregate within the diaphragm. The scintigraphic image of this diaphragm and mediastinal node accumulation is shown in the image of the right panel in Fig. 4. The scintigraphic image shows the intense activity of linear uptake in the region of the diaphragm, as well as uptake in the mediastinal nodes. A photographic picture of the diaphragm is shown in Fig. 5. The blue dye containing biotin-liposomes accumulate in the linear lymphatic vessels coursing through the diaphragm. This study confirms the fact that in the rat, the pleural lymphatic drainage pathway and the peritoneal lymphatic drainage pathway share the same lymphatic vessels in the diaphragm. It is not known if there could be some useful applications for diaphragmatic drug delivery, but one potential application is for the treatment of mesothelioma. Methelioma is a cancer of the diaphragm that generally has a very poor prognosis.[139] This prognosis has not changed with any attempted therapies including surgery, chemotherapy and radiation.[139]

**Fig. 5.** A photographic picture of the diaphragm of a rat 24 hrs following injection of the biotin liposomes containing blue dye in the peritoneum and injection of avidin in the peritoneum corresponding to the right hand image on Fig. 4. The blue dye containing biotin-liposomes accumulate in the linear lymphatic vessels coursing through the diaphragm.

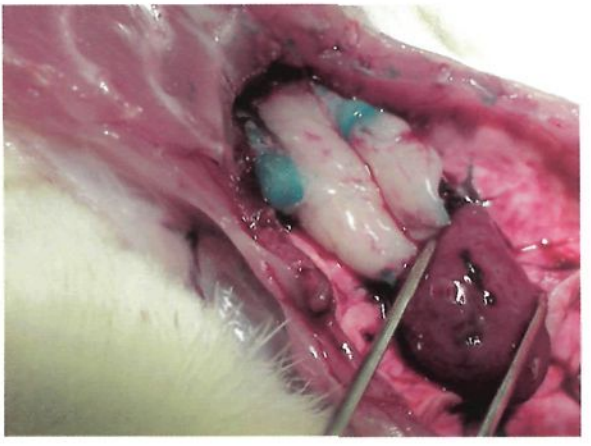

**Fig. 6.** A photographic picture of the biotin-liposome containing blue dye accumulating in the two mediastinal lymph nodes 24 hrs following injection of avidin in the pleural space followed by biotin-liposomes administration in the pleural space as corresponding to the left hand image of Fig. 4. The heart is between and forceps and the mediastinal nodes are just in front of the thymus. The intense blue staining of the mediastinal lymph nodes can clearly be seen at 24 hr post administration of the liposomes.

## 13. Nanoparticles for Cancer Therapy

### 13.1. *Intralymphatic drug delivery to lymph nodes*

One of the first studies to investigate the possible use of drugs delivered intralymphatically was performed by Hirnle.[1] This study investigated the anti cancer drug,

Bleomycin, which was suspended in an oil suspension known as Oil Bleo. This Oil Bleo was injected directly into catheterized lymphatic vessels in dogs. The movement of this agent through the lymph nodes and lymphatic vessels was fairly rapid with peak drug concentrations reached in the blood 15 min after the intralymphatic administration of Oil Bleo. The drug entering the blood was considered to be a spillover from the lymphatic system. Spillover occurred because the drug moved completely through the lymphatic vessels and rejoined the circulation at the thoracic duct. Administering the drug this way required a very tedious catheterization process of the small lymphatic vessels of the extremities. Although drug concentrations were very high in the lymphatic vessels for a fairly short time, the retention of the oil emulsion in the lymphatics was minimal.

Following this work with anticancer bleomycin oil emulsions, Hirnle turned to liposomes as an ideal carrier for intralymphatically delivered drugs.[1] A study in rabbits used liposome-encapsulated Bleomycin in which the liposomes were injected directly into the lymphatic vessels of the hindlegs of rabbits. Lymph nodes were removed and measured for bleomycin content at various times following administration. Three days following intralymphatic administration, the drug concentration in the popliteal lymph nodes was 42 $\mu$gram/gram of node. Drug deposition and apparent release was sustained over a very long period because concentrations of Bleomycin in the lymph nodes of 0.18 $\mu$g/gram were measured in the popliteal nodes at one month following injection.[1]

Further studies were performed by Hirnle with blue dye containing liposomes composed of 80% phosphatidylcholine and 20% cholesterol. The liposomes had a homogeneous size of approximately 170 nm in diameter. The total amount of blue dye injected was 1.6 mg. When the rabbits were sacrificed 28 days later, the retroperitoneal lymph nodes were visually blue and had a concentration of 172 $\mu$g blue dye/gram of lymph node. Unfortunately, when these liposomes were administered by direct intralymphatic injection in the hindleg of a rabbit, a large fraction of the intact liposomes were found to spill over into the circulation.

Several conclusions were derived from this endolymphatic research with liposomes directly infused into the lymphatic vessels. The amount of drug administered intralymphatically should not exceed that which would be administered intravenously, because of the large amount of liposomes that spill over from the lymphatics to the circulation. The limiting factor in administering drugs lymphatically is the amount of the therapeutic agent that moves completely through the lymphatic system and into the circulation through the thoracic duct. The tolerated amount of spillover should be considered in regard to the toxicity of these liposomal agents to the rest of the body. The volume used in humans should remain low, with no more than 4 ml of liposomes being administered into the canulated lymphatic vessels of each leg. It was also suggested that the drug will remain longer

in the lymphatics, if the patient remains in bed for 1 day after endolymphatic liposome administration. Most importantly, the lymph nodes will still be filled with measurable amounts of drug a month after injection. Hirnle also introduced the concept that the prolonged retention of anticancer drugs in the lymphatics might be effective for the prevention of lymphatic metastasis.[1]

## 13.2. *Nanoparticles for treatment of metastatic lymph nodes of upper GI malignacies*

Much work has been performed by investigators in Japan to develop a novel drug delivery system for the treatment of lymph node metastasis from the cancers of the esophagus and stomach.[140] This interest by Japanese researchers are likely to stem from the high incidence of upper gastrointestinal cancer in Japan. This work has also been stimulated by the fact that examinations of surgically resected specimens revealed that the cancer of the upper digestive tract metastasize to regional lymph nodes in 20–30% of patients, even when cancer invasion is limited to the mucosa or submucosa. This has led to the conclusion that even in patients with these superficial cancers, it is important to treat patients with gastric and esophageal cancer for potential lymph node metastasis.

As an attempt to increase drug delivery to lymph nodes that drain from these upper digestive system cancers, a new activated carbon nanoparticle formulation of the anti cancer drug methotrexate was developed for the treatment of lymph node metastasis in patients with cancers of the upper digestive system.[141] Methotrexate was mixed with 20 nm-sized activated carbon nanoparticles in a concentration of 50 mg activated carbon/ml and polyvinylpyrrolidone. This made a suspension of methotrexate loaded carbon particles with an average size of 167 nm, as determined by photon correlation spectroscopy. Methotrexate was shown to be absorbed onto the activated carbon nanoparticles at a concentration of 25 mg/ml.

Mouse leukemia P388 cells were used as an experimental tumor because subcutaneously implanted P388 was known to metastasize to lymph nodes within 7 days.[141] Experiments were carried out on day 7 when the cancer metastasis were known to be in the popliteal lymph nodes. In mice which received an inoculation of P388 leukemia cells and drug treatment using the same procedures, the treatment effects on metastases to the regional lymph nodes were significantly greater in mice treated with the methotrexate loaded activated carbon particles than in those given methotrexate aqueous solution. Methotrexate concentrations in the popliteal lymph nodes were $240 \times 10^{-6}$ mol/kg, with the carbon particles at 1 hr versus $96 \times 10^{-6}$ mol/kg with the free drug. This level rapidly dropped to $18 \times 10^{-6}$ mol/kg by 3 hrs versus $1.8 \times 10^{-6}$ mol/kg for the free drug. By 6 hrs, levels of methotrexate were undetectable in the lymph node. Blood levels of drug in the

serum also increased rapidly, indicating that the association of the drug with the carbon nanoparticles was not very stable. Blood levels at 30 min following injection were only slightly lower than those of subcutaneously injected free drug. These levels peaked at 30 min and were at very low concentration at 3 hrs and were non detectable in the serum at 6 hrs. This rapid drop in drug blood levels would suggest that the carbon particles were not as effective at binding the methotrexate, compared with other liposome-encapsulated drugs, reported to be measured in lymph nodes in detectable quantities for 1 month after subcutaneous injection.[1] No studies were reported describing the stability of this drug attachment to the carbon nanoparticles during incubation in serum. Such studies are essential for the evaluation of the stability of the nanocarrier. Even with this small effect of improved delivery to lymph nodes, survival in this animal model was increased from 12 days in the non-treatment group to 17 days in the carbon particle methotrexate group, 14 days in the free methotrexate group injected subcutaneously and 13 days in the free methotrexate group injected IV. This same group has gone on to perform clinical trials in human patients, in which methotrexate-carbon nanoparticles were injected locally for the treatment of cancer of the upper digestive tract.[141]

This treatment approach has also been applied in small pilot studies by other groups in Japan, who have also reported survival benefit for the treatment of gastric cancer.[142] Another group injected carbon nanoparticles with absorbed bleomycin for the treatment of esophageal cancer. In this study, bleomycin nanoparticles were injected into the esophageal cancer 3 days prior to surgery. Degenerative or inflammatory changes were microscopically observed in 6 of 23 lymph nodes, with metastatic foci indicating to these researchers that bleomycin carbon particles could be a useful tool in targeting chemotherapy for esophageal cancer.[143]

This same activated carbon particle methodology has been applied to the treatment of other cancers. In one study, breast cancer patients were injected intratumorally and peritumorally, with aclarubicin absorbed to activated carbon nanoparticles or in free solution.[144] Following this injection, the patient had surgery and the tumor and peritumoral tissue were removed as well as regional lymph nodes. Drug levels in the lymph nodes were shown to be significantly higher with the carbon nanoparticle associated drug, compared with that of free drug (42 $\mu$g/g tissue versus 20 $\mu$g/g tissue).

In a recent study, local injection of mitomycin C bound to activated carbon (M-CH) combined with intraperitoneal hyperthermic hypo-osmolar infusion (IPHHOI) was intraoperatively administered to prevent lymph node recurrence and peritoneal recurrence of gastric cancer.[145] The 1- and 2-year survival rates for the M-CH1 + IPHHOI group were 91.2 and 72.1%, and those for the control group were 78.9 and 45.5%. The M-CH1I + PHHOI group reaped a significant survival benefit ($p = 0.0352$) compared with the control group. Although this study was

conducted in a small number of randomly selected patients with a short follow-up period, compared with the control group, the M-CH1 + IPHHOI group had a beneficial effect in preventing lymph node recurrence and peritoneal recurrence, after curative gastrectomy for advanced gastric cancer.

## 13.3. *Lessons from endolymphatic radioisotope therapy*

Much of the earlier work has been performed on the lymphatic delivery of the radiotherapeutic lipid emulsion, I-131 ($^{131}$I)-lipiodol, which is a lipid emulsion of iodinated ethylic ester of poppy-seed oil. $^{131}$I emits a therapeutic beta particle which is responsible for its therapeutic effects. This early work provides useful lessons for the potential delivery of radiotherapeutic nanoparticles into the lymphatics for the treatment of cancer. $^{131}$I endolymphatic therapy consists of direct infusion of $^{131}$I-lipiodol into the lymphatic vessels of the cancer affected extremity. Initially, endolymphatic isotope therapy had such promising early clinical results that the M.R.C. (Medical Research Council) in the U.K. set up a clinical trial in 1966. This clinical trial compared patients with lower extremity melanoma who received $^{131}$I-lipiodol endolymphatic therapy to those who were treated with standard methods.[146] Although there was no difference in the 5-year survival rate between the groups, lymph node recurrence was significantly different with only a 2.3% lymph node recurrence rate with the $^{131}$I-lipiodol therapy, versus 19% lymph node recurrence rate with standard therapy. The conclusion from this study was that endolymphatic isotope therapy was justified in specialized centers where good results could be obtained.[146]

Following this initial investigation, many other studies of endolymphatic radiotherapy were performed.[147–149] Studies of radiation dosimetry found that the average radiation dose absorbed by the lymphatic tissues with this therapy was 90 rads. Unfortunately, this method was found to be limited by the hazard of radiation damage to the lungs.[150] Approximately 80% of these patients had detectable concentrations of $^{131}$I radioactivity in the lung fields. The average radiation dose to the lungs was 299 rads. It is very evident that $^{131}$I-lipiodol becomes trapped in the lungs after re-entering the thoracic duct following therapy. This spillover from the lymphatic system that accumulates in the lungs, led to the recommendation that patients receiving this $^{131}$I-lipiodol endolymphatic therapy rest in bed for several days, so that the maximum amount of $^{131}$I would remain in the lymphatic vessels and not be pushed through to the lungs. It was this large lung radiation dose that eventually led to the discontinuation of these $^{131}$I-lipiodol studies, even in the face of promising results for lymphatic therapy and the prevention of local lymphatic metastasis.

Endolymphatic $^{131}$I-lipiodol therapy has been used to treat 426 patients with lymphoma. Traditional X-ray lymphography was performed during the

administration of the therapeutic $^{131}$I-lipiodol. These studies found that endolymphatic therapy was not of value in cases where there was evidence of lymph nodes already involved with cancer at the time of the treatment. However, in cases where the lymphography was apparently negative, the $^{131}$I-lipiodol did produce a statistically significant reduced incidence of relapse in the inguino-retroperitoneal nodes.[151] This suggests that the $^{131}$I-lipiodol therapy was effective in treating micrometastasis in the lymph nodes.

One interesting study carried out with $^{131}$I-lipiodol examined the effect of prior external beam irradiation on lymph node uptake of endolymphatically infused iodinated $^{131}$I-lipiodol.[152,153] In this study, 2 ml of $^{131}$I-lipiodol (76 mg of iodine per ml) was injected subcutaneously into 9 normal adult beagle dogs. Targeted lymph node groups were evaluated with computed tomography (CT). Lymph nodes were irradiated with 50 Gy in 25 fractions of 2 Gy per day, beginning 28–35 days after the CT examination. Contrast media administration and quantitative CT imaging were again performed 3 months after irradiation. Contrast material uptake resulted in a 2-fold increase in node volume before irradiation ($p < 0.0001$). Prior to the external beam irradiation, mean attenuation of contrast-enhanced nodes increased to 230–330 Hounsfield units from a precontrast enhancement value of 36.5 Hounsfield units. After irradiation, opacified node volumes decreased to approximately 25%–50% of their preirradiation volumes ($p < 0.02$), but contrast material uptake in the lymph node only decreased by 10%–15% after irradiation. This uptake in the lymph node was not significantly less than the preirradiation uptake. Qualitatively, no substantial difference was found between irradiated and nonirradiated nodes. The external beam irradiation treatment decreased lymph node size, but the imaging characteristics of opacification were not otherwise appreciably altered 3 months after irradiation. A later study at 12 months showed slightly smaller lymph nodes and a lesser uptake of the subcutaneously injected $^{131}$I-lipiodol,[152] however, the lymph nodes appeared to tolerate the 50 Gy dose without significant alteration in their function.

## 14. Advantages of Nanoparticles for Lymphatic Radiotherapy

Compared with the previously discussed $^{131}$I-lipiodol emulsion, nanoparticles have many potential advantages as carriers of therapeutic radionuclides for endolymphatic therapy. These include the fact that nanoparticles do not accumulate in the lungs to any degree and the ability to control the release and the choice of the particular isotope that is attached or encapsulated in the nanoparticle. The high lung uptake of $^{131}$I-lipiodol is due to the lipophilic nature of its oil component causing it to be absorbed by the lungs, which is the first significant vascular capillary bed encountered after the $^{131}$I-lipiodol rejoins the circulation. It is well known that intravenously administered liposome nanoparticles or liposome nanoparticles,

returning to the blood following drainage from the lymphatic system, do not accumulate in the lungs to any significant degree.[18]

## 15. Intraoperative Radiotherapy for Positive Tumor Margins and for Treatment of Lymph Nodes

One possible use of radiotherapeutic nanoparticles is to target residual tumor in the intraoperative situation. In many cases, the surgeon is unable to remove all of the cancer during surgery, so that the margins of the resected tumor are positive. This generally means that there is cancer remaining at the operative site which severely compromises patient's survival. This positive margin can frequently be determined during the operation. Radiotherapeutic nanoparticles that target residual tumor could be injected in the region of the positive tumor margin to sterilize the surgical margin of tumor cells. Since the radiotherapeutic nanoparticles will drain through the lymph nodes, they would also have the potential to treat micrometastasis in those nodes. Nanoparticles could therefore provide an additional tool for the surgeon, particularly when the margins of the tumor are positive.

Even when the margins of the tumor are negative, there is frequently a reoccurrence of cancer in the local region or in the nodes that drain from the local region. Cancer surgeons spend many hours per surgery uncovering and removing lymph nodes in the region of the tumor carefully, without damaging other critical vessels and nerves. Although these surgeries are very long, it is not always possible to find and remove all of the lymph nodes in the local region of the tumor. Removal of distant lymph nodes that also receive lymph drainage from the tumor is usually not possible. The application of therapeutic nanoparticles intraoperatively could provide an additional tool to treat micrometastasis in lymph nodes, with the goal of decreasing local reoccurrences. Extensive clinical trials would have to be performed to determine the effectiveness of this approach, similar to those that have already been performed with $^{131}$I-lipiodol. Effective treatment of lymph nodes draining from a tumor could decrease the need for tedious surgical removal of lymph nodes. One possible method to ensure good lymph node targeting of nanoparticles in the intraoperative situation would be to use the avidin/biotin lymph node targeting system to ensure trapping of the particles in the lymph nodes that drain from the tumor. This methodology would also limit the spillover of radiotherapeutic nanoparticles from the lymphatic vessels into the bloodstream.

## 16. Potential of Using Radiolabeled Nanoparticles for Intratumoral Radionuclide Therapy

The direct injection of therapeutic agents into solid tumors has been recently investigated.[154–157] These studies using direct injection of nanoparticles into tumor

have used many different therapeutic agents. For instance, direct injection of nanoparticles into solid tumor have been investigated as a method of delivering genes into tumors.[158] This approach has also been applied in combination with external physical modalities. Magnetic nanoparticles have been directly injected into a solid tumor and exposed to alternating current as a new type of thermal ablation of solid tumors.[157]

The particulate nature of nanoparticles appears to offer significant advantages for direct intratumoral administration. Nanoparticles appear to diffuse to some degree through the interstitial space of the tumor along primitive and chaotic lymph vessels within the tumor. The degree of diffusion may depend on the characteristics of the particular nanoparticle injected. Nanoparticle intratumoral diffusion should result in improved solid cancer therapy due to a more homogeneous distribution throughout the tumor. In spite of this potential for intratumoral diffusion, nanoparticles can still be well retained within the tumor. When free unencapsulated drug is injected intratumorally, it appears to be absorbed directly into the blood supply of the tumor with less diffusion through the tumor, so that there is a less homogeneous dose throughout the tumor following the intratumoral injection of a free drug, as compared with intratumoral injection of nanoparticles. In addition, depending on the nature of the free drug, free drug is likely to be cleared from the tumor rapidly by direct absorption into the tumor blood capillaries.

Even with this improved local diffusion associated with nanoparticles compared with free drug, obtaining a homogeneous distribution throughout the solid tumor with intratumoral administration of nanoparticles still remains a challenge. One approach is to use modifications of the injection method such as multiple sites of injections within the solid tumor.[154] This approach has recently been applied in the case of gene delivery with nanoparticles. Another possibility is the use of beta-emitting therapeutic isotopes attached to nanoparticles. The beta-emissions penetrate millimeter distances away from the nanoparticle, enabling the beta-emitting nanoparticles to deliver therapy to regions of the solid tumor that the nanoparticles cannot reach themselves. Many other approaches to solve the problem of homogenous distribution within a solid tumor. Nanoparticles may be part of, but not likely the complete solution, to obtaining a very homogeneous distribution within a solid tumor.

A significant advantage of nanoparticles for use in intratumoral injection is that they are more likely to move into the lymphatic vessels that drain from the solid tumor, where they have the chance to deliver anti cancer therapy to the sentinel lymph node and other lymphatics that drain from the tumor. Therefore, it is anticipated that this intratumoral injection would not only treat the tumor, but could also potentially treat lymph nodes that receive drainage from the tumor such as the sentinel node. These lymph nodes could possibly contain metastasis.[159]

## 17. Liposome Pharmacokinetics after Intratumoral Administration

Studies of liposome intratumoral pharmacokinetics have been stimulated by attempts to use liposomes as gene carriers. Clinical trials using cationic liposomes, carrying E1A gene, were performed to treat squamous cell carcinoma, using an intratumoral injection technique for intratumoral administration.[160,161] Pharmacokinetic studies have indicated that the size and surface charge of liposomes have a significant effect on their *in vivo* distribution.[162,163]

Increasing the liposome diameter and adding a positive surface charge to the liposomes slowed their clearance from the injection site, compared with smaller-sized and neutral charged liposomes respectively. At 2 hrs after intratumoral injection, ~70% and 90% of injected dose remained in the tumor with a 254.0 ± 5.1 nm neutral liposome and a 125.0 ± 29.4 nm cationic liposome respectively.[163] Based on their observation of intratumorally administered cationic liposomes, Nomura *et al.* stated that there is a need to improve the control of the cationic liposome complexes to ensure a better distribution throughout the tumor.[163] Biodistribution of $^{111}$In-labeled pegylated liposomes following intratumoral administration has also shown that liposomes have excellent potential as vehicles for intratumoral drug delivery.[95]

## 18. Rhenium-Labeled Liposomes for Tumor Therapy

Our group has developed a novel method of labeling liposomes with the radioisotope of rhenium. This method uses N,N-bis(2-mercaptoethyl)-N′,N′-diethyl-ethylenediamine (BMEDA) to post-load either $^{99m}$Tc, rhenium-188 ($^{188}$Re) or rhenium-186 ($^{186}$Re) into liposomes.[159]

One of the significant advantages of rhenium-labeled nanoparticles that carry therapeutic beta particles is the short range field effect that they have, due to the fixed range of beta particle penetration (i.e. 2 mm for rhenium-186 and 4 mm for rhenium-188).[164] This length of penetration is adequate to treat a large number of cancer cells in the region of the nanoparticle, but not so far as to cause extensive damage to normal tissue. The 2–4 mm range of beta emission penetration with the rhenium-186/188 isotopes compares favorably with $^{131}$I, which only has 1 mm average beta particle penetration combined with a high energy gamma photon. The 4 mm treatment field with rhenium-188 is adequate for treating most lymph nodes, while limiting the dose to normal structures. This field effect of the beta particle can compensate, to some degree, for a heterogeneous distribution of the nanoparticles within cancer containing lymph nodes. The nanoparticle simply has to reach within a 4 mm vicinity of the cancer cells.[164]

For every 10 beta emissions, both rhenium isotopes, rhenium-186 and rhenium-188, emit a single gamma photon. This is an ideal ratio of beta to gamma emissions. A higher number of gamma emissions would deliver an excessive dose outside the local region of the tumor, as is the case for $^{131}$I which has a 1:1 ratio of beta particles to gamma photons. The photon emission energy of both rhenium isotopes is in the range of the photon energy of $^{99m}$Tc (140 keV), so that the radiolabeled nanoparticles can be tracked through the body as they move through the lymphatic vessels. Many therapeutic radioisotopes are pure beta emitter, so that it is more difficult to track their distribution in the body. Rhenium has also many other advantages over most heavy metal radiotherapeutic isotopes, such as yittrium-91, because it has almost no affinity for bone uptake. It shares this characteristic with $^{99m}$Tc, as both radioisotopes tend to be cleared through the kidney, while most heavy metal beta-emitting radioisotopes have a high affinity for bone. This high bone accumulation can deliver a high radiation dose to bone marrow cells which are very sensitive to radiation. This occurs when the radioisotope becomes separated from its chelator, following metabolism in the body.

Previous theoretical dosimetry studies have addressed the potential use of radiotherapeutic liposomes for the treatment of tumors via intravenous injection.[165–167] In addition to these intravenous investigations, our group has investigated the potential use of rhenium-liposomes for intratumoral therapy.[159] There are some significant advantages of using the intratumoral delivery route for rhenium-liposomes compared with intravenous injection, such as the much lower radiation dose delivered to liver, spleen, kidney and other normal tissues, and the potential of simultaneous targeting of metastatic lymph nodes that drain from the region of the tumor.[3]

$^{99m}$Tc-liposomes can be used to pre-evaluate the suitability of using $^{186}$Re/$^{188}$Re-liposomes to treat a tumor. This is because the same chemistry is used to label liposomes with the diagnostic isotope, $^{99m}$Tc, as the therapeutic rhenium isotopes. The likely dose distribution from the rhenium-liposomes can be calculated by performing SPECT/CT images of the $^{99m}$Tc-liposome distribution, in order to determine the potential dose distribution of the rhenium-liposomes.[91]

We have performed studies with $^{99m}$Tc to assess intratumoral administration of radiolabeled liposomes. In these studies, prolonged tumor retention and very high tumor-to-normal tissue ratio of $^{99m}$Tc-activity were observed (manuscript submitted for publication). $^{99m}$Tc-liposomes were injected intratumorally into a head and neck tumor in a rat model, using the same methodology for labeling liposomes with radiotherapeutic rhenium. $^{99m}$Tc-liposomes had good tumor retention with 47.6 to 65.7% of injected activity still remaining in the solid tumors at 44 hrs after injection, while unencapsulated $^{99m}$Tc-BMEDA cleared from tumors quickly, with only 37.1% of injected activity remaining in tumors at 2 hrs and 19.4% at 44 hrs.

## 19. Nanoparticles for Immune Modulation

A few very preliminary studies suggest that the delivery of therapeutic beta-emitting radioisotopes to lymph nodes has the potential to modulate the immune system for therapeutic benefit of auto-immune disease and for the induction of tolerance in organ transplantation. These preliminary studies suggest the possibility that beta-emitters delivered to lymph nodes results in a decreased immune response in the organs and regions of the body that drain that lymph node. This decreased immune response has been demonstrated in pilot studies of patients with rheumatoid arthritis, as well as in patients that have received transplanted kidneys.

In one study, a method was developed and tested for the treatment of patients with rheumatoid arthritis, using radiotherapeutic beta-emitting gold-198 colloid particles which were infused into the lower limb lymphatic vessels. More than 50 patients were treated. A positive therapeutic effect was observed in 84% of the treated patients. This endolymphatic radiotherapy with gold colloid particles made it possible to give up cytostatic and glucocorticoid medications and to reduce the dosage of nonsteroid anti-inflammatory drugs.[168] Immune modulation by radioparticle accumulation in the lymph nodes could also explain some of the beneficial effects of radiation synovectomy. In this procedure, radiolabeled particles that emit beta particles are injected into the joints of patients with rheumatoid arthritis.[169]

A second study also provides evidence of tolerance induction by the pre- transplant endolymphatic infusion of $^{131}$I-lipiodol. This procedure was performed as a pre-transplant preparation for patients receiving a kidney transplant. Twenty six years later, the outcome in the patients that received the $^{131}$I-lipiodol was compared with that of another group of patients that did not receive the $^{131}$I-lipiodol therapy, but were treated with a standard maintenance dose of azathioprine. The incidence of rejection crises was greatly reduced in the group that received the $^{131}$I-lipiodol therapy, compared with the standard treatment group (21% versus 74%, $p = 0.003$). The authors of this study concluded that the pre-transplant treatment with $^{131}$I-lipiodol had an extended immunosuppressive effect and could be indicated for cadaveric renal allograft recipients, especially those showing high panel reactivity. It was also relatively innocuous, as there was no compromising of either the thyroid gland or the gonad function and there was no increase in tumor incidence in these patients over the 26-year period.[170,171] Local infusion of nanoparticles carrying therapeutic beta-emitting radioisotopes that targeted the lymph nodes might have potential applications for the prevention of transplanted organ rejection, as well as the treatment of auto-immune disorders.

## 20. Conclusions

The delivery of nanoparticles to lymph nodes for therapeutic purposes is promising. Significant progress has been made in understanding the various processes involved in nanoparticle delivery and in the development of potential systems for targeting nanoparticles to lymph nodes. Lymph node delivery appears promising for improving cancer and infectious disease therapy, treatment of autoimmune disease and for improvement of vaccine systems.

## Acknowledgments

The author is grateful to Dr. Beth Goins for her help and critical reading of the manuscript.

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# 26

# Polymeric Nanoparticles for Delivery in the Gastro-Intestinal Tract

*Mayank D. Bhavsar, Dinesh B. Shenoy and Mansoor M. Amiji*

## 1. Oral Drug Delivery

In the last few decades, there has been a tremendous explosion in the research pertaining to novel (or advanced) drug delivery systems. Majority of the efforts have been directed towards development of "better" formulations of existing and/or off-patent drugs; the betterment being mostly aimed at improving the performance of the drug by altering the disposition and pharmacokinetics. Similarly, the trend is also being extrapolated to novel therapeutic compounds that are still in the pipeline, with the additional objective of positioning the molecule in highly competitive, technology-based intellectual property environment. The outcome has been phenomenal and the market size of advanced drug delivery systems is expected to swell to whopping US$ 40 billion by 2008, from its current size of US$ 20 billion.

Non-invasive therapeutics has been the time-tested and most favored mode of drug administration. Oral route remains the front-runner in this segment. Current market share of the oral dosage forms is approximately 50% of all the formulations marketed and amounts to approximately US$ 40 billion. When a new chemical entity (NCE) is being developed, the first target of a formulation scientist would be to exploit the oral route. Often, a quick test to evaluate the oral bioavailability of the

NCE is to fill the drug into hard gelatin capsules along with lactose, as this constitutes the simplest formulation that could be developed for oral administration. With the majority of novel drugs being highly hydrophobic or being of biotechnology origin, they pose serious and complicated challenges to the formulation scientists. Besides the ease of administration and patient compliance, the variety of excipients available (or being investigated) and the lesser cost involved for developing oral dosage forms, favor developments in this area of formulation science, compared with other delivery systems, especially those that involve invasive administration.

The 21st century is being dedicated to nano-/bio-technological advancements and this has not spared the pharmaceutical product development section. "Nano" is the most widely used keyword that has penetrated almost every walk of life, and nanotechnology has become the key driving force behind the thriving high-technology based pharmaceutical drug delivery industry. This chapter focuses on one of the components of widely explored product development showcase, that of polymeric nanoparticles.

## 2. Anatomical and Physiological Considerations of Gastro-intestinal Tract (GIT) for Delivery

To explore opportunities that are available for the delivery of bioactive compounds throughout the GIT, one has to first understand the anatomical and physiological conditions of the system because the secret of innovative formulation lies in exploiting these conditions as modulators for a well-programmed drug disposition.

The human digestive system is specialized to perform functions such as ingestion, digestion and absorption. The organs of digestion are essentially divided into two main groups: the gastrointestinal tract or the alimentary canal and the auxiliary structures. The gastrointestinal tract is continuous tube-like structure beginning with the mouth (oral cavity) and extending further as pharynx, esophagus, stomach, small intestine, large intestine, rectum and finally culminating into the anal canal.[1–6] The auxiliary structures include teeth, tongue, salivary glands, liver, gall bladder and pancreas. For the purpose of this chapter, our discussion will be limited to the anatomy and physiology of the gastrointestinal tract in its relation to oral drug delivery. Figure 1 provides a quick understanding of the GI targets, principles of formulation development that could be utilized and the application opportunities of the nanoparticles-based drug delivery system throughout the GIT.

Different portions of the gastrointestinal tract serve different functions, but almost all the portions of the digestive tract are made up of four basic layers: (i) Mucosa, which is the mucus membrane, principally consisting of epithelial tissue and forming the inner most lining of the tract. In esophagus and anal canal, the mucus epithelium is specialized for protection of the underlying tissue. In other

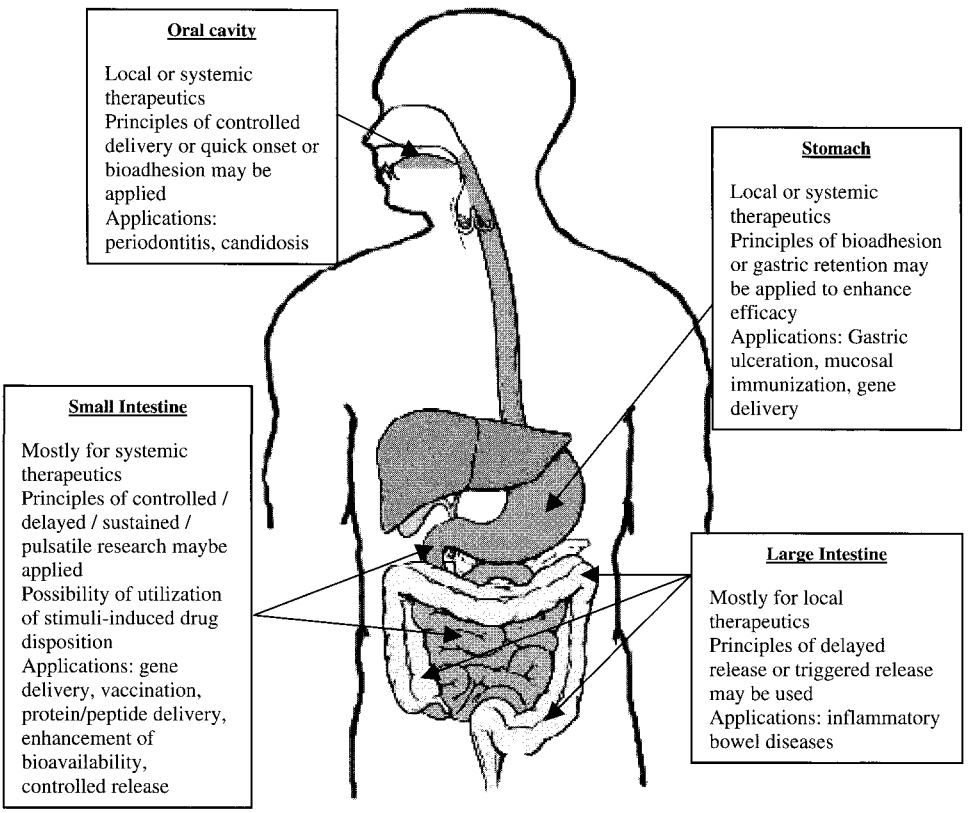


**Fig. 1.** GIT targets, formulation principles, opportunities and applications.

areas of the gastrointestinal tract, the epithelium is specialized for the secretion of mucus or digestive juices or for absorption. (ii) Submucosa, a thick layer of connective tissue containing nerves, blood vessels and glands. (iii) Muscularis, two layers of smooth muscles. The outer muscle layers are arranged longitudinally and the inner layer of muscles encircle the wall of the tract. (iv) Visceral peritoneum, the outermost layer of the tract and is a serous membrane, also known as serosa.[1–6]

The mouth or the oral cavity comprises of the lips, cheeks, tongue, hard palate, soft palate and the floor of the mouth. The oral cavity is lined with the mucous membrane (oral mucosa) and includes the buccal, sublingual, gingival, palatal and labial mucosae.[7–9] The oral mucosal surface has varied thickness with buccal mucosa having a thickness of 500–800 $\mu$m, while the palates, gingivae and floor of the mouth measuring 100–200 $\mu$m.[10,11] The buccal and sublingual tissues are the principal focus for drug delivery via the oral cavity, because of the fact that they are more permeable than the other mucosal regions of the mouth. The oral mucosal surface comprises of less than 1% of the total surface area of the gastrointestinal tract, but is

high vascularized, allowing the drugs to diffuse from the oral mucosa and directly accessing the systemic circulation.[8,9] Thus, the drugs entering the systemic circulation through the oral mucosa can bypass gastrointestinal tract and the first pass metabolism in liver. The permeability of the oral mucosa is greater for sublingual cavity, followed by buccal cavity and than the palatal surface. An enzymatic barrier also exists in the oral mucosa, which causes a rapid degradation of the peptides and proteins. The cells of the oral mucosa are surrounded by an environment of mucus, which is secreted by the mucous membrane, and is believed to play a role in the bioadhesion of mucoadhesive drug delivery systems.[7–10,12] The pharynx and the esophagus also have the same anatomy and physiology as the rest of the gastrointestinal tract, but they are not generally considered as sites for drug delivery, and hence will not be discussed in this chapter.

The esophagus ends into the stomach and is separated from the stomach by a cardiac sphincter muscle which acts as a valve system. It is a J-shaped, bag-like structure and described to have two curvatures, the concave curvature known as the lesser curvature and the convex curvature known as the greater curvature. Stomach is also essentially composed of the same four layers as the rest of gastrointestinal tract, which include the mucosal layer, submucosa, muscularis and serosa. The gastric mucosa contains many deep glands. These glands contain parietal cells which are responsible for the secretion of hydrochloric acid and the chief cells which secrete pepsinogens. Mucus is also secreted by these glands. The major barrier (or alternatively the opportunity) for drug delivery to the stomach is the low pH that exists in the organ because of the secretion of hydrochloric acid. The functions of stomach lie more in the digestion and it has very limited absorptive function. Delivery of proteins via the oral route faces a major hurdle in the stomach because of pepsinogens present in the gastric fluids which are responsible for the breakdown of proteins.

The stomach ends into the small intestine and this region is guarded by a pyloric sphincter. The small intestine is further divided into three major parts which include the duodenum (25 cm in length), jejunum (2 meters long) and the ileum (3 meters long). The walls of the small intestine composed of the four layers previously described. The mucosa of the small intestine contains solitary lymph nodules and aggregated lymph nodules (Peyer's patches). Peyer's patches are found on the side opposite to the mesenteric wall of the intestine. They are usually oval in shape and occur more frequently in the distal areas of the small intestine and also at the terminal end of the colon. The Peyer's patches is comprised of four zones: (i) the germinal center which is in turn made up of three different cell types i.e. lymphocytes, macrophages and the dendritic reticular cells, (ii) small lymphocytic zone which shows the presence of lymphocytes and macrophages, (iii) interfollicular zone that is made up of lymphocytes which are loosely packed with large intercellular spaces,

and (iv) subepithelial zone which shows large accumulation of macrophages and plasma cells. The Peyer's patches are lined above the lymphoid follicles by a membranous layer of epithelial cells called the follicle-associated epithelium (FAE). FAE is composed of absorptive cells, goblet cells, M cells and the enteroendocrine cells. These cells assist the Peyer's patches to transport macromolecules and particulate matter from the GIT into lymphatic/systemic circulation.[13] In addition to the general structure, the small intestine also shows the presence of tiny finger-like structures known as villi, which are made of epithelial tissue overlying the blood and lymph capillary network. The free edges of the cells of the villi are divided into microvilli, which form the brush border. Throughout the length of the small intestine, the mucous membrane is covered by villi. The main function of small intestine is digestion and absorption of the food that is passed down from the stomach. The epithelial cells of the mucosa of the small intestine are specialized for the absorption of nutrients. The process of absorption in the small intestine is also assisted by its length and a very large surface area.[14–16] The delivery of the drugs to the small intestine is preferred because drugs typically exhibit maximal absorption from this site, compared with other regions of the gastrointestinal tract. The absorption of drugs and particulate delivery system from the small intestine is believed to occur through gut associated lymphoid tissue and also from other non-lymphoid tissue.[15] The mucus covers the mucosal layer of the small intestine that controls the absorption of nutrients, electrolytes and fluids, and also forms a physical barrier to the environment and absorption of drugs.[14] The brush border enzymes form an enzymatic barrier for the absorption of proteins from the small intestine. The other major barrier to drug absorption to the small intestine is the action of ATP-dependent efflux protein P-glycoprotein pumps (PGPs), which exists on the cell membrane of the intestinal epithelium. PGPs transport certain drugs actively back into the intestinal lumen. PGPs are a part of the protective barrier of the small intestine that limits absorption of potentially toxic substances.[16]

The small intestine ends into the large intestine. It is called the large intestine because of the larger diameter of the tract compared to the small intestine. It is approximately 1.5 m in length beginning at caecum and ending in rectum and the anal canal. There is a difference between the wall of the large intestine and small intestine. The large intestine shows absence of villi structure and contains simple columnar cells with numerous goblet cells. The goblet cells secrete mucus that lubricates the colonic content as it passes through the colon. The submucous layer of the large intestine consists of more lymphoid tissue than any other part of the alimentary canal to provide non-specific defense against invasion by microbes in the food and the bacterial flora that resides in the gut. Drug delivery to the large intestine via the oral route for local action is a challenging task, as the drug carrier system will have to face the rigors of the preceding sections of the GIT before

reaching the desired site of action. Rectal delivery of drugs is an alternative for local action, but it suffers the disadvantage of patient compliance. The mucus layer of the large intestine can take up particles in a particular size range and this property could be exploited for delivery of the drug to the large intestine.[17,18]

## 3. Introduction to Polymeric Nanoparticles as Carriers

Modern day drugs are very effective in treating disease, but many of these drugs have limitations when it comes to the route of administration. Major advances in the field of biochemistry and biotechnology have led to the findings of a large number of bioactive molecules and vaccines, which are based on peptides, proteins and nucleic acids. Oral route is the most desired for administration for all drugs and bioactive molecules, but some of these drugs and molecules cannot be administered orally due to the fact that they become inactive in the GIT before getting absorbed, mainly due to enzymatic degradation. Hence, the parenteral route of drug administration becomes a very effective route for dosing of such drugs. However, the parenteral route of drug administration has the problem of being inconvenient for self administration by the patient and hence reduces patient compliance.[14,19–23] In the last few years, we have seen rapid development of drug delivery systems for the treatment of human diseases, which is the direct result of the extensive research being done on the applications of materials for medical and pharmaceutical product development. These advanced drug delivery systems include mostly colloidal carriers like liposomes, niosomes, nanoparticles, dendrimers, nanosuspensions, micelles and nano-/micro-emulsions.[24–31] These drug carrier systems offer many advantages like improved efficacy, reduced toxicity and improved patient compliance, and are also cost effective in many cases over conventional drug delivery systems.[32,33] Among the above mentioned colloidal drug delivery systems, nanoparticles represent the most appealing therapeutic nanocarrier systems by comprehensively addressing majority of the issues like stability, scalability, reproducibility, and by offering the best compromise between the efficacy and applicability.[14,26,34–40]

Nanoparticles can be defined as solid colloidal particles, produced by mechanical or chemical means, which are typically in the nanometric size range (1 to 1000 nm).[19,32,33] Nanoparticles, especially those prepared from polymeric materials, enjoy tremendous popularity due to ease of preparation, easy to tune the physicochemical properties (e.g. through an array of polymeric materials), possibility of surface modification, excellent stability, and scalability to industrial production. Since their conception in the mid 70s, nanoparticles have found applicability in almost every section of medicine and biology (besides host of other fields) in general, and also for controlled and/or targeted delivery of drugs and genetic materials in particular.[26,34,37–39,41–52]

The basis in the development of nanoparticles lies in Paul Elrich's idea of designing "magic bullet" carrying active molecules in them and being able to target specific sites in the body for the desired therapeutic effects.[33] Depending on the process by which they are prepared, these systems can be classified as nanospheres (nanoparticles) having a dense and solid polymeric network (monolithic matrix), or as nanocapsules which consist of a hollow core surrounded by a polymeric shell.[32,33] Drug-loaded nanoparticles have been developed for almost every route of administration, i.e. nasal, ocular, mucosal, inhalation, oral, transdermal and parenteral.[14,37,41,53–56] Clinically, they have found applications for diagnosing and treating a wide range of pathological conditions.

Nanoparticles can be prepared from both synthetic and natural. The polymeric materials could be either biodegradable or non-biodegradable, but should be essentially biocompatible. Poly (DL-lactide-co-glycolide) (PLGA), poly ($\varepsilon$-caprolactone) (PCL), poly (alkylcyanoacrylates), poly (styrene-co-maleic anhydride), poly (divinylether-co-maleic anhydride), poly (vinyl alcohol), poly (ethylene glycol) are some examples of synthetic, non-immunogenic polymers extensively used for nanoparticle preparation. Similarly, poly (amino acids), albumin, gelatin, hyaluronic acid, dextran, starch, and chitosan are some of the natural biodegradable polymers. While each of polymers poses its own advantages and nanoparticles can be synthesized with high degree of reproducibility from a majority of them, natural polymers, due to their natural origin, have preference, considering non-toxicity and biodegradability. The striking advantage of synthetic polymers remains the possibility to synthesize them reproducibly with well-defined physico-chemical properties. Advancement in biotechnology is helping the natural polymers to overcome this drawback and we can expect a surge in delivery systems based on them.

Polymeric nanoparticles have been extensively researched for their applicability as oral drug carrier systems. In the following sections, we will discuss how they are being explored in buccal cavity therapeutics, as stomach specific delivery systems, for mucosal targeting in the small intestine, and for the treatment of inflammatory bowel disease.

## 4. Preparation of Polymeric Nanoparticles

There are several methods on the preparation of polymeric nanoparticles and incorporation of bioactive compounds into them. In general, one of the two principles methods is utilized: controlled precipitation or controlled dispersion of the polymer. Few of the popular methods include solvent displacement, salting-out, emulsion-solvent-evaporation, emulsion-solvent-diffusion, polymerization, complexation and supercritical fluid technology. Figure 2 provides an overview of

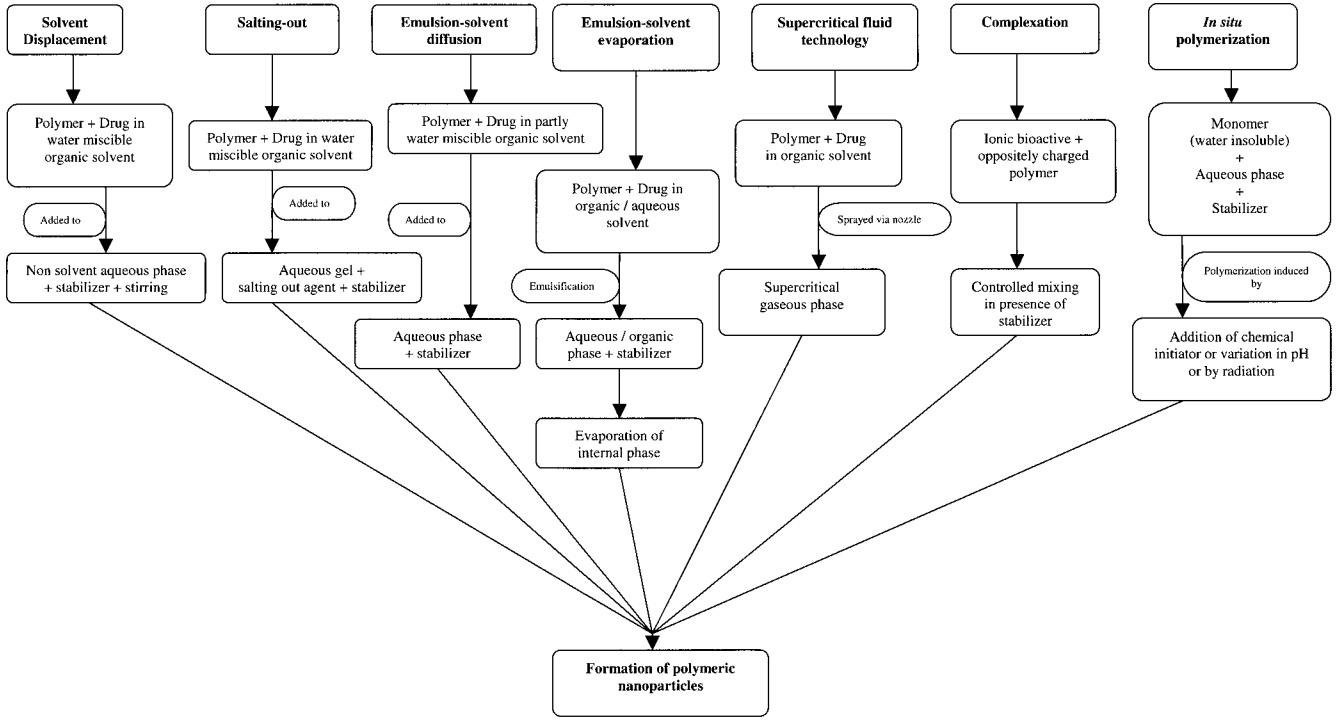


**Fig. 2.** Schematic representation of methods used for preparation of polymeric nanoparticles.

the methodologies and technologies available for the preparation of polymeric nanoparticles.

In the case of solvent displacement method, which is the simplest of all, the polymer is dissolved in a good solvent that maybe partially-polar and water-miscible solvent such as ethanol or acetone.[33] When the drug is to be incorporated into the particles, it can be dissolved in the same phase along with the polymer. This polymer phase is introduced into a non-solvent aqueous phase containing a stabilizer (generally a hydrophilic surfactant) at a controlled rate under continuous mixing. As the partially-polar solvent diffuses rapidly into the aqueous phase (i.e. as the partially-polar phase is displaced by the polar phase), the polymer starts precipitating due to changes in its solubility, resulting in the formation of nanoparticles. The surfactant present in the aqueous phase helps in preventing particle aggregation. Choice of a drug/polymer/solvent/non-solvent system is the major limitation of this method and hence its applicability is confined to hydrophobic drugs and polymers.

Salting out technique is generally used for the preparation of drug-loaded biodegradable nanoparticles. This method was first applied to pseudolatexes.[33] It is based on the separation of water-miscible solvent from aqueous solutions by a salting out effect. An o/w emulsion is formed by adding a solution of the polymer and the drug in a water miscible solvent into an aqueous gel containing a salting-out agent and a colloidal stabilizer. Water is added to dilute this mixture, as a result of which nanoparticles are formed. Solvent and salting-out agents are then removed by cross-flow filtration. The use of this method results in a very high loading efficiency along with high yield and also the scale-up is fairly easy, but this method can only be used for the loading of lipophilic drugs.

Emulsification-solvent-evaporation is based on the formation of a biphasic (o/w or w/o) or triphasic (w/o/w or o/w/o) emulsion.[33] Generally, a preformed polymer is dissolved in an organic solvent which is water immiscible along with the drug, and is emulsified in an aqueous solution (o/w emulsion). The formed emulsion is then exposed to high energy mixers (e.g. high-speed or high-pressure homogenizers, colloidal mills or ultra sonic devices) to reduce globule size. The organic solvent is removed either by using heat or vacuum or even both at times. Nanoparticles are obtained as fine aqueous dispersions which can be collected and purified. The process variables involved in this method are complex and manifold, and the nanoparticles obtained are often polydisperse. However, this method is very popular for preparing polymeric microparticles rather than nanoparticles, as it facilitates industrial applicability and scalability.

Emulsion-solvent-diffusion method is another method which is used for nanoparticles preparation. It is a modified salting-out technique and differs mainly in the organic solvent which is partially miscible with water in this case.[32] This

solvent is pre-saturated with water to achieve initial thermodynamic equilibrium between water and the organic phase. Solvent diffuses out upon addition of water and results in the formation of nanoparticle suspension.

Controlled complexation induced by electrostatic interactions between oppositely charged polymers can yield stable colloidal dispersions. The interacting polymers could be therapeutically active (e.g. oligonucleotides and plasmid DNA) or may have tailored properties (e.g. pH-sensitivity).[57,58] A wide variety of charge-bearing polymers can be utilized to manufacture composite nanoparticles and varying physico-chemical properties.[48,59–63]

Supercritical fluid technology is an emerging science for the production of micro and nanoparticles.[64,65] In this method, an organic liquid solution of the polymer and the active moiety is sprayed through a nozzle into a chamber containing a gas that is miscible with the solvent, but in which the polymer and the active compound are not soluble. The gaseous phase in this case is a super critical fluid (e.g. supercritical $CO_2$). The dispersion of the liquid solution in such a condition generates a high degree of super-saturation, leading to the formation of fine, uniform colloidal particles. The particles can be recovered from the solution by depressurizing the chamber and allowing the gas to escape.[66,67]

While all of the above mentioned procedures employ preformed and well characterized polymers, there are other techniques for obtaining fine nanoparticles from monomers via *in situ* polymerization pathway. The most popular example for this method of synthesis is the nanoparticles made from poly (methylmethacrylates), poly (alkylcyanoacrylates) and poly (methylidenemalonates).[68] Generally, a water insoluble monomer is dispersed in an aqueous medium containing a colloidal stabilizer, and the polymerization is induced and controlled by the addition of a chemical initiator or by variations in physical parameters such as pH or radiation. Both hydrophilic and lipophilic drugs can be entrapped in the polymeric wall when added to the polymerization medium or adsorbed on preformed particles.

While each of the above mentioned nanoparticle preparation method has its advantages and disadvantages, they can all be fine-tuned to encapsulate variety of drugs. The literature evidence shows that the nanoparticles are mostly employed to incorporate hydrophobic drugs, simply because the majority of the techniques facilitate encapsulation of lipophilic compounds with very high loading (approximately up to 40% by weight) and capturing efficiencies (nearly 100%). When hydrophilic drugs are to be incorporated, *in situ* polymerization or complexation remains the most accepted method.

The collective advancements in nanotechnology and engineering sciences are expected to contribute major breakthroughs for bulk manufacturing of polymeric nanoparticles. In the highly competitive pharma/biotech industry, the formulation scientist can concentrate towards development of novel products, irrespective of

complexities involved in the procedures. As in most cases, majority of the scale-up issues can be addressed and solved with the help of parallel advancements in high-technology engineering.

## 5. Design Consideration for Nanoparticle-based Delivery Systems

Polymeric nanoparticles, since their first appearance in the 70s, have been keenly explored as delivery systems for small drug molecules, and also for macromolecules like nucleic acids, proteins, hormones and peptides.[69,70] With the patent protection to a number of blockbuster drugs expiring in this decade, innovative dosage forms, such as polymeric nanoparticles, can form a very powerful drug delivery technology for the pharmaceutical industry. Such technology-based products can be used for the extension of the patent life of the drug, or to prevent/delay the entry of generic versions into the specialized markets. In general, the polymeric drug delivery systems offer advantages such as the reduction in the total dose (hence also the dosing frequency), reduced side effects, delivery with enhanced efficiency (hence better performance), and most importantly, improved patient compliance.[70]

When designing a polymer-based nanoparticulate drug delivery system, the choice of synthetic or biopolymer is the most important consideration. In the following section, we will discuss different characteristics of polymers that play an important role in the final product and hence should be considered during preformulation stages.

### 5.1. *Polymer characteristics*

Polymers have become a vital and integral part for the development of any novel drug delivery system. Factors such as chemical structure of the polymer, composition, molecular weight, morphology of the polymer (amorphous/crystalline/residual stresses), size of the delivery system, process of degradation (enzymatic or non-enzymatic) etc. govern the behavior of polymer within the body.[69] The polymer selection itself is a judicious process and following factors should be considered while making a decision:

*Basic requirements:*

- Non-reactive: Chemical inertness with respect to active compound and the biological environment.
- Biocompatibility: Should be compatible with living cells and tissues that come in contact with the polymer.
- Non-pyrogenic: Should be free of any pyrogenic factors.

- Impurities: All the impurities should be well-established and present in minimal amounts. The impurities, if present, should also be biocompatible or should not pose any toxicity at amounts present.
- By-products: If the polymer undergoes any kind of biotransformation upon introduction into the body, the by-products should also be biocompatible.
- Regulatory issues: The polymer should be available in the cGMP grade and must be approved by the regulatory authorities for human use.

*Specialized requirements:*

- Loading capacity: If complexation or chemical conjugation is the method used for preparation, then the polymer must have sufficient reactive groups to promote respective interactions.
- Permeability: Permeability to molecules and water will govern the diffusivity and the release of the payload.
- Swellability: This could be of relevance when designing a floating or a bioadhesive system
- Viscoelasticity: Could be an important controlling parameter for gel-forming and adhesive systems.
- Sensitivity to environment: The triggering factor could be the pH, specific enzymes, or even the microbial flora prevailing in the GIT.

While the maneuverability around each of the above factors is very limited for a sterile dosage form, it becomes more flexible while developing an oral product. Parallel developments in the field of excipient science have contributed to a range of new high performance polymers, making the choice of an approved polymer easier for the formulation scientist. We recommend that interested readers should visit the websites of major manufacturers of pharmaceutical excipients (e.g. Eastman Chemicals, FMC Biopolymer, Colorcon, Gattefosse, Croda, Lipoid, Noveon, BASF, Roehm Pharma, Degussa, etc.) to build-up a database.

## 5.2. *Drug characteristics*

The performance of the dosage form for any bioactive compound will depend on physico-chemical properties of the drug, as well as the auxiliary factors. Few of the factors belonging to the former are molecular weight, solubility (aqueous or organic), partition coefficient, crystallinity and ionic properties. As these are the inherent properties of the drug, there is less scope for tailoring them to manipulate *in vivo* behavior of the formulation. However, factors such as solubility, for example, can be tuned to a certain extent by altering the particle size or using certain excipients (e.g. cyclodextrins). Some of the auxiliary factors that should be considered while designing a nanoparticle-based oral system are the dose of the drug, site of

action of the drug (e.g. absorption is limited to certain segments of the GIT only), stability of the drug (both under *in vitro* and *in vivo* conditions) and the desired pharmacokinetics/distribution profile.

### 5.3. *Application characteristics*

In the majority of the cases, the formulation parameters are decided based on its intended application. Upon oral administration, the nanoparticles could be expected to exert local action along the GIT, or could be used to deliver drugs to the systemic compartment, or could be meant for uptake by cells lining the GIT. The duration and kind of desired pharmacological action can be used as the guiding principle in designing the delivery module (fast versus sustained; regular versus controlled; unstimulated versus triggered; continuous versus pulsatile). More of these principles of design have been discussed under following section citing appropriate examples.

## 6. Nanoparticles in Experimental and Clinical Medicine

Many of the principles of drug design and delivery are based on naturally occurring phenomena (bio-mimicking approach) and the same principles guide the applicability of nanocarriers in therapeutics. The striking advantage of the nanoparticles is the large surface area that they offer when presented in a biological environment and the flexibility to alter the physico-chemical properties by manipulating the core polymer or by surface nanoengineering. Many clinical situations and conditions demand specialized therapeutics to achieve improved level of healing. In such situations, the requirements are specified by the clinician, which form the basis of product development. Table 1 gives an overview of the applicability of polymeric nanoparticles via oral route.

### 6.1. *Drug delivery in the oral cavity*

Buccal cavity mucosa has been studied for polymeric drug delivery. Bioadhesive or mucoadhesive (used when adhesion is to the mucosal tissue) polymers have been extensively used in buccal drug delivery because of the fact that a major limitation of the buccal cavity is the lack of dosage form retention. Polymers such as poly (methacrylate) derivatives, cyanoacrylates, epoxy resins, polystyrene, polyurethanes, hydroxypropyl methylcellulose, chitosan and poly acrylic acid have been studied for their potential use in buccal cavity therapeutics.[7,8,71–81]

Poly (propylcyanoacrylate) (PPCA) nanoparticles have been studied as a potential carrier for the prophylactic treatment of candidosis.[71] *Candida albicans* is a common organism which is found in the oral cavity. It occurs in the commensal form

**Table 1** Summary of polymeric nanoparticle-based delivery systems for in the GIT.

| Site of action | Incorporated compound | Polymer employed | Size (nm) | Reference |
|---|---|---|---|---|
| Oral cavity | FITC | Poly(propylcyanoacrylate) | 100–900 | 71 |
| | N/A | Lectin-Gliadin | 500–600 | 86 |
| Stomach | Carbazole | Gliadin | 400–500 | 130 |
| | Amoxicillin | Gliadin | 250–400 | 85 |
| | pCMV-lacZ | PLGA | >200 | 87 |
| Small intestine | Streptomycin | Chitosan | 50–500 | 19 |
| | Theophylline in depot tablets | PLGA | 200–260 | 131 |
| | Tetanus toxoid | Poly(ethylene glycol-Poly(lactic acid | 150–170 | 132 |
| | Indomethacin | PLGA | 100–200 | 139 |
| | Carbon-14 | Poly(methyl methacrylate) (PMMA) | 100–160 | 91 |
| | 5-Fluoroudine | Poly(methylvinylether-co-maleic anhydride) | 200–250 | 136 |
| | RBITC | Poly(methylvinylether-co-maleic anhydride) | 200–300 | 138 |
| | Vancomycin | PLGA | 100–200 | 139 |
| | Iodine-125 | Polystyrene | 50–3000 | 90 |
| | Valproic acid | PLGA | 100–200 | 139 |
| | pCMV-lacZ | Poly(ethylene oxide)-poly(propylene oxide) | 150–190 | 113 |
| | N/A | Sulfobutylated-poly(vinyl alcohol)-PLGA | 100–130 | 14 |
| | Phenobarbital | PLGA | 100–200 | 139 |
| | Amifostine | PLGA | 200–300 | 143 |
| | *H. pylori* lysate | PLGA | 300–400 | 116 |
| | DNA | Chitosan | 50–75 | 112 |
| | pCR3Arah2 | Chitosan | 100–1000 | 114 |
| | mEpo gene | Chitosan | 70–150 | 111 |
| | Rifampicin | Lectin-PLGA | 300–400 | 118 |
| | Pyrazinamide | Lectin-PLGA | 300–400 | 118 |
| | Ketoprofen | PLGA | 100–200 | 139 |
| | Isoniazid | Lectin-PLGA | 300–400 | 118 |
| | Calcitonin | Poly(N-isopropylacrylamide) | 148–895 | 11, 95, 134 |
| | | Poly(N-vinylacetamide) | 148–896 | 11, 95, 134 |
| | | Poly(t-butyl methacrylate) | 148–897 | 11, 95, 134 |
| | | PLGA | 200–400 | 135 |
| | Heparin | PCL | 270–300 | 137 |
| | | PLGA | 250–270 | 137 |
| | | Eudragit® RS and RL | 250–280 | 137 |

**Table 1** (*Continued*)

| Site of action | Incorporated compound | Polymer employed | Size (nm) | Reference |
|---|---|---|---|---|
| | Insulin | Poly(iso-butyl cyanoacrylate) | 85 | 142 |
| | | Polyesters | 300–500 | 104 |
| | | Poly(methacrylic acid)-*g*-poly(ethylene glycol) | 200–1000 | 103 |
| | | Poly(alkylcyanoacrylate) | 1000–400 | 100 |
| | | Chitosan | 200–400 | 97 |
| | | Poly(isobutylcyanoacrylate) | 100–500 | 99 |
| | | Poly(acrylicacid)-g-poly(ethylene glycol) | 200–100 | 103 |
| | | PLGA | >1000 | 102 |
| | | Poly(fumaric-co-sebacic) anhydride | >1000 | 102 |
| | | PLGA | 100–200 | 139 |
| | CyA | Poly(methacrylic acid methacrylate) | 30–110 | 22 |
| | | Hydroxypropyl methylcellulose | 50–60 | 108 |
| | | Chitosan | 150 | 106 |
| | | Gelatin | 140 | 106 |
| | | PLGA | 100–200 | 139 |
| | | PCL | 100–130 | 140 |
| | | Eudragit® RS and RL | 170–310 | 141 |
| | Fluorescein | Polystyrene | 50–3000 | 16 |
| | | Polystyrene nanoparticles coated with poloxamer 188 and 407 | 60 | 133 |
| Large intestine | Fluorescent dye | Polystyrene | 100–1000 | 17 |
| | Rolipram | PLGA | 300–500 | 18, 123 |

in healthy individuals, but it can become pathogenic to the body under conditions such as cancer chemotherapy, diabetes mellitus and also during antimicrobial therapy. The first step in candidosis infection is the adherence of the microorganisms to the epithelial cells of the host. The basic motivation of the investigation was to evaluate the ability of PPCA nanoparticles to disrupt the process of adherence of the microorganism onto the host cells. PPCA nanoparticles were prepared by emulsion-polymerization from propylcyanoacrylate monomers. Different kinds of surfactants were used for stabilization of nanoparticles, which resulted in the formation of nanoparticles having different size ranges. Surfactants like Tween® 80, Pluronic® P123, Tetronic® 904, docusate sodium, sodium oleate and sodium laurylsulfate produced particles in the nanometer range, whereas cetrimide, benzalkonium chloride and cetylpyrimidine chloride produced particles in the micrometer

range. Tetronic® 904 produced the smallest particles of the size 90 ± 10 nm. *C. albicans* blastospores were treated with the nanoparticle suspension and these treated blastospores were exposed to the buccal epithelial cells to check for adherence. It was found that nanoparticle treated blastospore adherence per buccal epithelial cells was reduced by up to 73%. The findings of this study may offer the basis for a prophylactic treatment of candidosis in immuno-compromised patients.

Periodontal diseases are one of the major causes of teeth loss and it includes a number of diseases involving the supporting tissue of the teeth. Conventional methods of treatment of periodontitis include periodontal surgery and chemotherapy, but both these treatments cannot prevent the reoccurrence of the disease. Recently, a method for treating periodontitis, by using polymeric nanoparticles loaded with photosensitizer compounds, has been proposed.[82] The nanoparticles exhibit controlled release of the photosensitizer molecule through the matrix polymer. The proposed application uses photosensitizer molecules such as porphyrins, chlorines, pheophorbides, bacteriopheophorbides, phthalocyanines, naphthalocyanines, thiazines, xanthenes, pyrrylium dyes, psoralens, quinones and amenolevulic acids. These compounds are either incorporated or complexed with nanoparticles made from biodegradable or non-biodegradable polymers. The effectiveness of photosensitizers relies on their association with cellular membranes, thereby targeting highly sensitive membranous intracellular organelles that control critical metabolic functions. The hydrophobic character of the photosensitizers means that they cannot be administered directly to a hydrophilic environment due to a tendency to aggregate (by molecular stacking, precipitation or other mechanisms), which can severely curtail photosensitization processes. Thus, they require formulation in carriers which are able to provide a hydrophobic environment to maintain them in a non aggregated form in both the formulation and in aqueous preparations prior to use[83]. These nanoparticles can then be applied by the dentist to the periodontal pockets in the form of gel which hardens on application. This allows for a slow and extended period of release, which can be fine tuned by choosing biodegradable or non-biodegradable polymer of the photosensitizer from the nanoparticles, and hence do not affect the normal cell function. The use of nanoparticles prevents the degradation of the photosensitizer molecule in the presence of saliva, white blood cells and other natural defenses in the mouth. Higher concentrations of the photosensitizer allows for a more effective treatment and this can be achieved by using specialized nanoparticles formed out of dendrimer-photosensitizer complexes. Furthermore, many of the dental diseases are difficult to treat due to a lack of accessibility and quick flushing of the dosage form by the saliva. The nanoparticles can be especially useful in such situations. The size of the carriers enables them not only to reach deeper parts of the infected area, but also to be retained at the site of action.

## 6.2. *Gastric mucosa as a target for oral nanoparticle-mediated therapy*

The major function of the stomach is to digest food and pass down the chyme to the intestine. The principal hurdle to the successful delivery of active compounds to the gastric mucosa using conventional delivery system is the gastric emptying time. These conventional delivery systems do not remain in the stomach for prolonged periods due to their inability to deliver the drug to the desired site in effective concentration and in fully active form. The other barrier to the delivery of drug is the mucus layer of the gastric mucosa. The primary component of mucus is glycoprotein which forms a dense condensed and complex microstructure, by forming numerous covalent and non covalent bonds with other mucin molecules.

*Helicobacter pylori* has been recognized as a major gastric pathogen responsible for a variety of clinical manifestation including the development of gastritis, gastric ulcer and gastric carcinoma.[84] It is a gram negative, spiral, urease producing microorganism isolated by Warren and Marshall in 1982. Umamaheshwari *et al.*, [85] studied the effectiveness of mucoadhesive nanoparticles bearing amoxicillin for the treatment of *H. pylori*. Mucoadhesive nanoparticles prepared from gliadin, having a size range of 285 to 392 nm, were used in the study. Gliadin is a group of polymorphic proteins extracted from gluten and are soluble in ethanolic solutions. They have a very low solubility in water except at extreme pH.[86] *In vitro* stability study of gliadin and amoxicillin was performed in simulated gastric fluid and confirmed by HPLC. *In vivo* mucoadhesion capacity was evaluated by oral administration of fluorescent labeled gliadin nanoparticles. Size dependent mucoadhesive propensity and specificity was exhibited by gliadin nanoparticles with less than 300 nm particles showing 68% mucoadhesion, and more than 300 nm particle showing 75% and above mucoadhesion. Amoxicillin loaded gliadin nanoparticles were administered to Mongolian gerbils previously inoculated with human *H. pylori* to study *in vivo* clearance time (4 hrs, 8 hrs and 12 hrs), and placebo gliadin nanoparticles were also used as a control. Although amoxicillin loaded nanoparticles showed 100% inhibition of *H. pyloi* within 4 hrs of administration, it could not completely eradicate the *H. pyroli in vivo*. This study showed that amoxicillin loaded nanoparticles exhibited a longer gastric residence time than conventional amoxicillin formulation and also that topical action of amoxicillin on the gastric mucosa plays an important role in the clearance of the bacterium.

Gastric mucosa can also be explored for the delivery of genetic material or for vaccination. A recent investigation explored PLGA nanoparticle stabilized with a cationic surfactant (dimethyldioctyldecylammonium bromide) as gene carriers for transport through the gastric mucosal barrier.[87] Composite polymeric nanoparticles having a magnetic element and loaded with anti-metabolites have also been

explored for the treatment of gastric tumors.[88] The magnetic component helps in external guiding and localization of the nanoparticles at the site of action.

## 6.3. *Nanoparticles for delivery of drugs and vaccines in the small intestine*

Gastrointestinal tract provides a variety of barriers, including proteolytic enzymes in gut lumen and on the brush border membrane, mucus layer, gut flora and epithelial cell lining, to the delivery of drugs. Factors which govern the uptake of particles from the gut include particle size, physico-chemical nature of particles, surface charge and attachment of uptake enhancers such as lectins or poloxamer. After oral administration of nanoparticles, they could be (i) directly eliminated in the faeces, (ii) adhering to the cells (bioadhesion) and/or, (iii) undergo oral absorption as a whole. Oral absorption of the nanoparticles results in passage across the gastrointestinal barriers and delivery of the payload into the blood, lymph and other tissues. Before this translocation can occur, the nanoparticles have to adhere to the surface of the intestine. Translocation of particles across the gastrointestinal wall can occur due to intracellular uptake by the absorptive cells of the intestine or paracellular uptake (i.e. between the cells of the intestinal wall), or phagocytic uptake by intestinal macrophages, or uptake by the M cells of the Peyer's patches.[89]

Jani *et al.*[90] have shown that particle size plays a major role in the uptake of particles. They measured uptake by using radiolabeled polystyrene nanoparticles ranging from 50 nm to 3.0 $\mu$m. They have been able to show that lower size particles (50 nm particles showed a 12% uptake by the cells of the small intestine) are taken up at a higher rate by the small intestine when compared to the larger particles (1 $\mu$m particles showed only 1% uptake by the cells of the small intestine). The lower size particles (<500 nm) were detected in blood after intestinal uptake whereas larger size particles (>500 nm) where not detected in blood. Also, these nanoparticles were detected in other tissues such as liver and spleen. A low surface charge on the surface of nanoparticles is desirous for good absorption. While Pluronic® or poloxamer (188 and 407) coating onto the surface of 50 nm polystyrene nanoparticles inhibited uptake in the small intestine, a similar coating on the 500 nm polystyrene nanoparticles showed an increased intestinal uptake.

There has been yet another report to study the effect of surface modification on the uptake of polymeric nanoparticles using $^{14}$C-labeled poly (methylmethacrylate) (PMMA), having a mean size of 130 nm and coated with polysorbate (Tween®) 80 or poloxamine 908.[91] These nanoparticles were administered orally to rats and they were checked for their organ distribution. High radioactivity levels were observed in the stomach contents, below 5% radioactivity was detected in the stomach wall for the coated particles. Highest amount of radioactivity (about 40%) was found

in the small intestine, confirming that these coated particles were absorbed in the small intestine.

Developments in the field of polymer science have made the delivery of proteins and peptide drugs via the oral route possible, by protecting these molecules against pH/enzyme-induced degradation and also by prolonging the time of delivery to the mucosal sites.[23,92–95] The most popular peptide used for oral delivery using polymeric nanoparticles is insulin. The first attempt to deliver insulin via the oral route was made by Couvreur *et al.*[92] in 1980. Insulin was adsorbed on the surface of 200 nm poly (alkylcyanoacrylate) nanoparticles and administered orally to diabetic rats to seek hypoglycemic effects. The investigators did not observe any decrease in glucose level upon oral administration, but good hypoglycemic activity was observed upon subcutaneous administration, suggesting that insulin was getting degraded in the GIT. In another investigation, nanoparticles made out of poly (isobutylcyanoacrylate) (PIBCA) loaded with insulin when administered orally, resulted in a 50–60% reduction in the blood glucose levels of diabetic rats.[96] The onset of action was after 2 days of administration, but was seen for 20 days depending on the insulin dose. These results suggested that PIBCA nanoparticles successfully protected insulin against degradation in the GIT.[93,97,98] A publication by the same group reported the ability of PIBCA nanoparticles to protect insulin from degradation by proteolytic enzymes, and thus providing nanoparticles based formulation for biologically active insulin for oral administration.[99] Insulin labeled with Texas Red® was used for release studies and microcopy observations. The results obtained from fluorescence and confocal microscopy revealed the presence of concentrated fluorescent spots into the mucosa and even in the lamina propria. This suggested that these nanoparticles could cross the barrier presented by the intestinal epithelium.

A patent was issued in 1995 for controlled release of insulin from biodegradable nanoparticles.[100] Insulin was complexed with different polycyanoacrylate monomers at low pH and nanoparticles were prepared from this complex by anionic polymerization process. These nanoparticles were dosed orally to rats and blood glucose levels were monitored over four hours. A considerable decrease in blood glucose levels was observed in a group dosed with insulin loaded nanoparticles, compared with the untreated group. More recently, Pan *et al.*[97] studied the effects of bioadhesive chitosan nanoparticles for improving the intestinal absorption of insulin in diabetic rats. Chitosan was chosen as the polymer for preparing the delivery system, because it exhibits strong electrostatic interaction with insulin, hence improving the loading efficiency of the polymer. It was also used for its bioadhesive properties for prolonged stay in the gastrointestinal tract, which in turn resulted in prolonged release times for insulin.[101] A dose dependent decrease in blood glucose levels was observed after oral administration of these 290 nm particles in diabetic

rats. Chitosan-insulin nanoparticles showed a higher decrease in blood insulin levels when compared with chitosan-insulin solution, suggesting that they could enhance the intestinal absorption of insulin by promoting protection from gastric clearance, and also rendering longer resident time in circulation.

Biodegradable polymers like PLGA, poly lactic acid (PLA), and poly (fumaric anhydride-co-sebacic anhydride) have been explored for the preparation of nanoparticulate formulations of insulin.[102] Although the major finding of the study was intact bioactivity of insulin after intraperitoneal injection, it was also indicated that the nanoparticles prepared in the presence of $Fe_3O_4$ showed the best hypoglycemic results, and were also proved to be orally effective.

Foss *et al.*[103] developed nanospheres from methacrylic acid grafted with poly (ethylene glycol) and also acrylic acid grafted with poly (ethylene glycol) as oral insulin carriers. From the results obtained after oral administration, it can be learned that diabetic animals administered with insulin-loaded nanospheres had a significantly reduced serum glucose levels, with respect to the control animals and this effect lasted over 6 hrs.

Cyclosporine A (CyA) is another peptide which has been studied for transport to the gastrointestinal tract using polymeric nanoparticles via the oral route. CyA is a potent immunosuppressive agent and is widely used for the inhibition of graft rejections in the transplant of organs such as heart, liver, skin, lungs, kidney, etc. It is also prescribed in autoimmune diseases such as rheumatoid arthiritis and Bechet's disease.[104–107] Although various formulations of CyA such as Neoral® (solution), Sandimmune® (microemulsion) and SangCyA® (amorphous nanoparticles) are being marketed, they are faced with the problem of variable bioavailability, and the patient has to be monitored for the blood levels of CyA during the regimen.[108] One of the earlier efforts to improve the bioavailability of CyA was done by preparation of pH sensitive nanoparticles using poly (methacrylic acid and methacrylate) copolymer (Eudragit®).[22] The results were compared with Neoral® (a universal standard for CyA oral bioavailability) formulation in rats. Nanoparticles exhibited drug entrapment of >90% for different formulations prepared from different types of Eudragit® systems. CyA nanoparticles prepared from Eudragit® S100, an anionic polymer, demonstrated the highest relative bioavailability of 132% with respect to Neoral®. Other polymeric nanoparticles also exhibited more than 110% relative bioavailability, except for nanoparticles prepared from Eudragit® E100 (CyA-E100) which is a cationic polymer. *In vitro* release studies of CyA from different nanoparticle preparation illustrated that all nanoparticle preparation showed pH-specific release of CyA at pH 7.4, except for CyA-E100 nanoparticles which released the whole payload at pH 2.0. This proves that major CyA from CyA-E100 was released in the stomach upon oral administration accounting for its low relative bioavailability with respect to other nanoparticle preparations.

In another study, Wang *et al.*,[108] examined hydroxypropyl methylcellulose phthalate (HPMCP) polymer nanoparticles loaded with CyA for oral delivery. HPMCP is a common enteric coating excipient used in the pharmaceutical industry for the enteric coating of the tablets. It dissolves specifically at a pH of 7.4 and releases the contents in the lower intestine. The investigators used two different CyA nanoparticle preparations made from different molecular weight of the same polymer. Again, a high encapsulation efficiency of over $>95\%$ was observed with the nanoparticle preparation, due to hydrophobicity of the drug. CyA nanoparticles made of high molecular weight HPMCP exhibited a relative bioavailability of over $>115\%$, and the ones made from lower molecular weight exhibiting only 82% relative bioavailability against Neoral®. The difference was attributed to the pH-independent property of lower molecular weight polymer which released entire payload within the stomach itself, thus inactivating the peptide drug. The results from the above studies indicate that pH-sensitive nanoparticles loaded with CyA can be designed as new carriers for CyA, which exhibit a better pharmacokinetic profile compared with the currently marketed CyA formulations.

Nanoparticles made from cationic polymers have been explored as surface coatings to improve the oral bioavailability of CyA.[106] Male beagle dogs were orally administered with CyA nanoparticles coated with chitosan as the polycationic surface modifier. From the results obtained, it was observed that chitosan coated drug nanoparticles showed the highest relative bioavailability of 173% with respect to Neoral® oral solution. The results were attributed to two properties of the system: (i) cationic polymer facilitated the electrostatic interaction with the negatively charged mucosa, and (ii) chitosan coated CyA nanoparticles facilitated the opening of the tight junctions of the epithelial cells, thus augmenting the paracellular transport pathway.

A series of investigations have been directed towards preparation and evaluation of bioavailability and toxicity profile of CyA-loaded polycaprolactone nanoparticles.[105,109] The nanoparticles, having a diameter of $\sim 100$ nm were prepared by solvent-evaporation procedure and evaluated for biodistribution, immunosuppressive activity and nephrotoxicity. Sandimmune® was used as the standard for this investigation in rats following oral administration. A significantly higher tissue (especially kidney) concentration of CyA was achieved with nanoparticles formulations, compared with the solution indicating probability of a higher nephrotoxicity. However, further toxicological evaluation with kidney function tests indicated no difference in the profiles of two formulations. *In vitro* lymphocyte proliferative activity (an indication of immunosuppressive potential) also showed better activity for nanoparticle formulations of comparable doses. The conclusion of the investigation was that the nanoparticles formulations can be effective at

lower dose levels, compared with the solution form and thus may help to reduce drug-associated tissue damage.

Cho *et al.*[110] developed several different oral CyA nanoparticle formulations consisting of one alkanol solvent and a polyoxyalkylene surfactant, and tested them in rats for their bioavailability in comparison to Sandimmune® oral solution. Selected formulations based on these pre-clinical investigations were further tested for their pharmacokinetic profile in humans. Forty eight healthy males were chosen and a randomized, double-blinded, three-way crossover study was conducted with Sandimmune® oral solution as standard formulation. From the results obtained, it is observed that CyA nanoparticles exhibited a $C_{max}$ which was twice as high as those achieved by Sandimmune® oral solution and the $T_{max}$ was much shorter for CyA nanoparticles compared with the standard one. Also, the AUC observed for nanoparticle formulations was significantly higher than the standard formulation.

Polymeric nanoparticles, because of their ability to effectively transport active molecules across the gastrointestinal tract have been studied as delivery systems for gene therapy and vaccination.[111–113] Chen *et al.*[111] used DNA-complexed with chitosan for transfection of erythropoietin gene to the intestinal epithelium of mice. Erythropoietin is a glycoprotein, which stimulates production of red blood cells. Erythropoietin is used in patients with anemia associated with chronic renal failure, and in cancer patients for simulation of erythropoieisis. Chitosan nanoparticles, containing plasmid DNA encoding for erythropoietin (mEpo), were administered orally to one group of mice along with other appropriate control dosage forms. Erythropoietin gene expression was registered every two days by measuring the hematocrit of the mice. Mice which were administered with chitosan loaded mEpo showed a 15% increase in hematocrit over other dosage forms, indicating successful transfection of mEpo gene across the intestinal epithelium. These results suggests that chitosan nanoparticles were able to prevent the mEpo from degradation against DNAses and hence the possibility of using them as gene delivery vehicles via the oral route. In another study, nanoparticles prepared from cationic biopolymers (chitin, chitosan and their derivatives) were proposed to be the carriers for oral administration of bioactive compounds for gene therapy.[112] The nanoparticles with encapsulated plasmid DNA encoding for human coagulation factor IX (pFIX) were prepared. The molecular weight of the cationic biopolymers ranged from 5 to 200 kDa. The nanoparticles in the size range of 100–200 nm were generated by the complex coacervation method and were used for oral administration to mice. Human factor IX was detected in the systemic circulation of the mice within 3 days following oral delivery, but declined after 14 days. The investigators also demonstrated the bioactivity of the factor IX transgene product in factor IX knockout mice. Heamophilia B is an X-linked bleeding disorder caused by a mutation in the factor IX gene. After orally feeding Factor IX transgene-loaded nanoparticles to

the knock-out mice, the clotting time was reduced from 3.5 min to 1.3 min, which was comparable with the clotting time of 1 min observed with wild-type mice. The investigators proposed that intestinal epithelium was the site of nanoparticle absorption and transfection.

A range of polycationic polymers including gelatin, chitosan, polylysine, polyarginine, protamine, speramine, spermidine and polysaccharides could be used to prepare the coacervates of the nucleic acids which result in the formation of discrete nanoparticles. Roy *et al.*[114,115] used such coacervates for effective vaccination by the oral route. Chitosan nanoparticles in the size range of 100–200 nm were prepared by salting-out technique with the plasmid DNA (pArah2), which encodes for the peanut allergen Arah2. The nanoparticles were orally fed into the mice and the serum and fecal levels of IgG or IgA were measured periodically. High levels of anti-arah2 IgG were observed in the titer of the group which was fed with low molecular weight chitosan nanoparticles housing the plasmid DNA (pDNA), compared with other groups which were administered with high molecular weight chitosan nanoparticles, with or without booster dose. The mice from all groups were challenged with crude peanut extracts four weeks after the booster dose and positive antibody response were detected in groups immunized by DNA nanospheres. These results suggest that chitosan-pDNA nanoparticles delivered through the oral route can modify the immune system in mice and protect against food allergen induced hypersensitivity.

Kim *et al.*[116] prepared PLGA nanoparticles housing *H. pylori* lysates by solvent-evaporation method. These nanoparticles were administered orally into mice and antibody induction was assayed in serum and gastrointestinal tract. Serum IgG subclasses were determined by ELISA. The mean antibody titers for serum IgG and gut IgA responses were significantly higher than those of the groups immunized with the soluble antigen alone. Cholera toxin (CT — a well-established potent mucosal adjuvant)-*H. pylori* had a higher antibody titer compared with PLGA-*H. pylori* nanoparticles. The results of this study indicates that PLGA-*H. pylori* nanoparticles could stimulate *H. pylori*-specific mucosal and systemic immune responses in mice, and also that nanoparticles can be used for vaccination against *H. pylori*.

Spray-dried PLGA nanoparticles have been investigated for the oral delivery of amifostine.[117] Amifostine is an organic thiophosphate prodrug and is dephosphorylated by alkaline phosphatase in the tissue to the active free thiol metabolite. The major drawback of the drug is that it cannot be administered orally in an active form and when administered systemically, it is rapidly cleared from the body. PLGA nanoparticles containing amifostine were administered to mice orally and tissue distribution was observed for the administered dose. Within 30 min post-oral administration, the drug was detected in almost all the tissues including blood, brain, spleen, kidney, muscle and liver.

Wheat gram agglutinin (WGA) lectin-functionalized PLGA nanoparticles have been successfully prepared and used to encapsulate isoniazid, rifampicin and pyrazinamide, which are the three frontline drugs employed in the treatment of tuberculosis.[118] These PLGA nanoparticles encapsulating antitubecular drugs at therapeutic dosage were administered for their *in vivo* drug disposition studies to guinea pigs which were previously infected with *Mycobacterium tuberculosis* to develop the infection. Results obtained for plasma concentration of different drugs suggested that PLGA-nanoparticles helped to improve the plasma residence time of different drugs after oral/nebulized administration. Rifampicin was detected for 6 to 7 days in the plasma after oral/aerosolized administration of PLGA-NP, when compared with free drug which was detected only for 1 day. Similarly, isoniazid and pyrazinamide were maintained for more than 12 days in plasma, compared with a single day for the free drug. The presence of these drugs in the tissues such as liver, lungs and spleen for a long time favors its application against tuberculosis where infection is largely localized in the tissues. Chemotherapeutic studies revealed that three doses of oral/aerosolized lectin-coated nanoparticles for 15 days could yield undetectable mycobacterial colony forming units, compared with 45 days of oral administration of the free drug to achieve the same results. This study suggests that polymeric nanoparticles could be favorably used for the effective treatment of tuberculosis.

Popescu *et al.*[19] have proposed the use of biodegradable nanoparticles, prepared from naturally occurring polymers such as chitosan, dextran sulfate, dermatan sulfate, chondroitin sulfate, keratin sulfate etc. for oral delivery of highly cationic active compounds which are highly hydrophilic and could be substrates for PGP. Such active compounds include the likes of aminoglycosides, polypeptides, proteins, terefenamate, proglumetacin, tiaramide, apazone, etc. Currently, there are no technologies for delivery of hydrophilic, cationic drugs by oral administration.[19] As an example, we will consider streptomycin, which was loaded to chitosan nanoparticles and tested for *in vivo* efficacy using *M. tuberculosis* infected mice. Streptomycin was successfully loaded with an encapsulation efficiency of 50% or higher, with a minimal drug loading of 30% w/w of polymer. After oral administration of these chitosan nanoparticles in mice a one $\log_{10}$ reduction in colony-forming units of the bacilli was achieved, compared with the control group. These results show that the nanoparticles-based technology can be a break-through for the oral administration of aminoglycoside antibiotics, which are otherwise inactive via oral route.

## 6.4. *Nanoparticles for colon-specific delivery*

The large intestine, which represents the last segment of the gastrointestinal tract can suffer from two major inflammatory bowel diseases which are ulcerative colitis

and Crohn's disease. Ulcerative colitis occurs more in the distal segment of the large intestine and Crohn's disease develops over a very large area of the colon, approximately 40%. Very little is known about the patho-mechanisms involved in both the disease.[119–122] Conventionally, treatment of these diseases involves daily intake of anti inflammatory drugs which include 5-aminosalicylic acid formulations, glucocorticoids and immunosuppressive drugs such as azathioprine, which is taken along with methotrexate.[123] The major draw back with these conventional formulations is that they have to be taken at high doses daily by the oral route, resulting in the absorption of these compounds by the small intestine causing possibly strong and undesirable effects.[17]

Several strategies have been employed for the development of oral delivery system for the transport of drugs to the inflamed sites in the colon. These include sustained release devices such as prodrugs, macroscopic systems such as pH-controlled drug release systems, time-controlled drug release system, enzyme controlled drug release systems and also microsized delivery forms such as microspheres and nanoparticles. The pH-controlled system relies on the physiological difference in the pH of the acidic stomach and that of the distal small intestine, time-controlled drug release occurs after a predetermined time lag which is similar to the transit time of the system in the small intestine and it ensures delivery of the drug into the large intestine. Enzyme-controlled release systems make use of the variety of enzymes that are produces by the colonic mucosa to achieve colon specific drug delivery. These prodrugs and controlled release devices also have the risk of causing adverse side effects, which might result from systemic absorption of drug which might occur due to non-specific delivery of the drug all over the colon.[17,18,123–125]

Polymeric nanoparticles offer an attractive advantage over these systems in that they are preferentially absorbed by the mucosal cells of the colon based on their size. Mucoadhesion is another property of the polymers, which could be used for site specific delivery of the drug to the colon. Polymers such as polysaccharides, which include chondroitin sulfate, pectin, dextran and guar gum, have been researched for their use as colon specific systems. Chitosan, which is one of the most abundant natural polysaccharide, has also been investigated for the development of colon specific delivery system due to its well known mucoadhesive properties. A recent study by Zhang *et al.*[124] on rats also showed that chitosan gets degraded by the cecal and colonic enzymes. Factors which affected the degradation of chitosan in colon include its molecular weight and the degree of acetylation.

A size-dependent bioadhesion of nanoparticles and microparticles in the inflamed colonic mucosa has been demonstrated.[17] Commercially manufactured fluorescent polystyrene particles of different sizes including 100, 1000 and 10,000 nm were used in the study. The experiments were conducted in rats, which were rectally

catheterized and treated with trinitrobenzenesulfonic acid (TNBS), for inducing inflammatory bowel disease. Polystyrene particles were administered orally to the rats and were assessed for localization and deposition of the particles in the GIT. Myeloperoxidase (MPO) activity was determined to ensure and quantify the inflammation in the colonic area. Size-dependent particle deposition was found in the gastrointestinal tract of control group and also in the inflamed tissue. It was found that lower size particles exhibited higher incidence of particle deposition in the inflamed tissue, with the lowest particle size of 100 nm showing a 6.5-fold increase in percentage particle binding, when compared with particle binding of the same size in the healthy control group. The overall distribution of the nanoparticles in the GIT was assessed by confocal laser scanning microscopy and again it was found that 100 nm particles had a higher percentage of localization (38.6%) in the mucus of the inflamed tissue, compared with 31.1% for 1000 nm and only 13.4% for 10,000 nm particles. This study proves that nanoparticles are better localized and deposited by the macrophages of the inflamed tissue, and that size-dependent deposition of particles in the inflamed tissue should be given importance, when designing a nanoparticle carrier system for inflammatory bowel disease.

The same group developed a biocompatible and biodegradable nanoparticle system for targeted oral delivery to the inflamed tissues of the colon for patients suffering from inflammatory bowel disease using PLGA.[18] Two different molecular weights of PLGA (5000 and 20,000) were used to prepare nanoparticles containing rolipram, an anti inflammatory drug. Emulsification-solvent-evaporation method was used for nanoparticles synthesis to yield particle size of less than 500 nm, with an encapsulation efficiency of > 80%. Colonic inflammations were induced into the rats using TNBS and were checked for the severity of colitis by measuring MPO activity. PLGA nanoparticles were orally administered to the rats daily for five days and the control group received only saline. PLGA nanoparticles exhibited a local anti inflammatory effect by controlled drug release and also proved to be as efficient as the free drug in decreasing inflammation of the colitis. Charged interactions of the negatively charged PLGA nanoparticles (MW — 20,000) and the positively charged proteins of the ulcerated tissue showed a further enhancement of the binding of these nanoparticles to the inflamed tissue.

## 7. Integrating Polymeric Nanoparticles and Dosage Forms

If the development of a nanoparticles-based formulation for a drug is a scientifically stimulating job, then development of the means to administer them orally to humans is a challenging art. As the scientific community is currently busy solving the problems associated with the former "scientific" portion, we would like to project a few possible scenarios that could be utilized to develop the "art" of oral

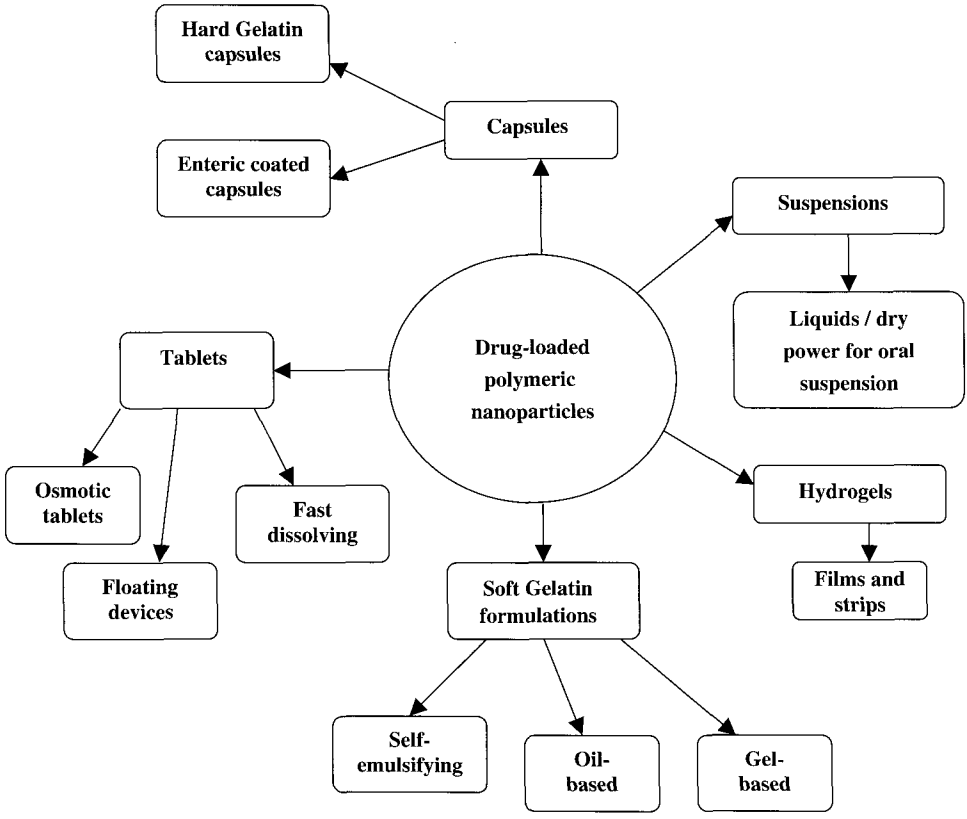


**Fig. 3.** Flowchart showing integration of drug-loaded polymeric nanoparticles and conventional dosage forms.

administration of polymeric nanoparticles. Figure 3 provides an overview of conventional formulations that could be used to pack the drug-loaded nanoparticles for the purpose of oral administration.

Most of the methods used for manufacturing of the nanoparticles yield drug-loaded nanoparticles as suspensions (generally aqueous). If the polymers constituting the nanoparticles remain stable in aqueous environment for the proposed shelf-life period, then they could be directly packaged as oral suspensions, along side suitable additives such as flavors, colors, suspending agents and preservatives. This would constitute the simplest oral formulation.

It may be desirable to freeze dry the original drug-loaded nanoparticles suspension to limit degradation and also to reduce the levels of organic solvents used during their production. In such cases, the drug loaded nanoparticles shall be available as free-flowing powder, with or without added stabilizer (i.e. a secondary polymer that is used to prevent aggregation during synthesis of nanoparticles).

This can be mixed with a standard diluent (e.g. lactose) and directly filled into a hard gelatin capsule. Another appealing strategy is to formulate as a dry power for oral suspension. In this case, the nanoparticles power can be mixed with excipients, including suspending agent, sweetening agent (if necessary), flavors, colors and preservatives. The contents are to be suspended in water before ingestion.

Soft-gelatin capsules are popular among conventional oral dosage forms and with the availability of novel excipients (from companies such as Gattefosse), their application has been extended to meet specialized needs (e.g. sustained release or *in-situ* gelling systems). The drug-loaded nanoparticles can be suspended in a suitable medium (usually oil-based) and filled into soft-gelatin capsules. Special properties can be imparted to the system by including gel-forming components or self-emulsifying components that generate a unique system upon dissolution of the gelatin coat *in vivo*. An *in situ* formed gel can incorporate nanoparticles to extend the dissolution times, or an emulsion may be designed to promote the absorption of drugs from the nanoparticles.

The delivery module can be made in the tablet form as well.[126] However, one has to evaluate the deformations of the drug-loaded nanoparticles at the compression conditions employed. The nanoparticle-tablet can be designed as a floating system to increase gastric resident time, or as a bioadhesive system which would increase the contact time and hence results in a sustained release, or even a fast-dissolving system to expose the nanoparticles quickly to the GIT for further action. Coupling osmotic system for delivery of polymeric nanoparticles could be an interesting option to offer a multi-step control over drug availability.

## 8. Toxicology and Regulatory Aspects

The ultimate mission of the regulatory body governing the approval of pharmaceutical products in the United States (Food and Drug Administration — FDA) is not only to protect, but also to provide improvement of public health by assuring the safety and efficacy of the products for human and veterinary use.

The FDA has taken parallel measures along with the advancements in nanotechnology to meet novel demands and challenges. There are reasons for the FDA to take special steps in promoting the availability of nanotechnology products for public use; it is a rapidly growing area of science and is anticipated to lead in the development of novel and sophisticated (possibly complex) applications in drug delivery systems. As the FDA only regulates to the "claims" made by a sponsor, it may be unaware that nanotechnology is being employed to develop that formulation.

Nanotechnology has been currently evaluated under FDA's Critical Path Initiative to keep in pace with the developments in the pharma/biotech industry. Office of

Combination Products of the FDA coordinates the regulatory framework for nanotechnology products including nanoparticles, and a dedicated FDA Center has been proposed for taking the primary responsibility of the review of applications. Many of the FDA regulated products are expected to be influenced and revamped under nanotechnology, such as drugs, drug/gene/protein delivery systems, vaccines, biotechnology products, medical devices and cosmetics. Historically, the FDA has approved many products and formulations containing solid particulate matter of nano-size range (< 1000 nm). It is also understood that many of the bioactive compounds are reduced to nanosize during the process of bioabsorption and there have been no severe safety concerns relating to particle size that have been reported earlier. To obtain approval for a nanoparticle-based drug delivery system, the industry has to address the following issues[127]:

## 8.1. *Safety*

The nano-formulations should be evaluated with respect to toxicological screening (pharmacology, clinical and histopathological analysis, absorption/disposition/metabolism/excretion (ADME) parameters, genotoxicity, developmental toxicity, irritation studies, immunotoxicology and carcinogenicity) projection of potential novel and unanticipated reactions and evaluation of excipients effects prior to clinical use. An effort should be made to address the following questions[127]:

- With a reduction in the particle size, there could be a change in size-specific effects on the biological activity of the system. Hence, it is important to address the issues such as:
- Will nanoparticles gain access to tissues and cells that normally would be bypassed by larger particles?
- Once nanoparticles enter tissues, how long do they remain intact and how are they cleared?
- If nanoparticles enter cells, what effects do they have on cellular and tissue functions? Would there be different effects in the different cell types?
- What are the differences in the ADME profile of nanoparticles versus larger particles?
- What preclinical screening tests would be useful to identify potential risks (*in vitro* or *in vivo*)?
- Can new technologies such as "omics" help identify potential toxicities and how can these methodologies complement current testing requirements?
- Can nanoparticles gain access to the systemic circulation from the route of exposure? If nanoparticles enter cells, is there an effect on cellular functions?

## 8.2. *Quality of material/characterization*

As new toxicological risks that derive from novel materials and delivery systems are identified, new tests will be required to ascertain safety and efficacy. Industry and academia need to plan and conduct the research to identify potential risks and to develop adequate characterization methodologies.

- What are the forms in which particles are presented to host, tissues, organs, organelles and cells?
- What are the critical physical and chemical properties, including residual solvents, processing variables, impurities and excipients?
- What are the standard tools used for this characterization?
- What are the validated assays to detect and quantify nanoparticles in tissues, medical products, foods and processing equipment?
- How do physical characteristics impact product quality and performance?
- How do we determine long and short-term stability of nanomaterials?

## 8.3. *Environmental considerations*

- Can nanoparticles be released into the environment following human and animal use?
- What methodologies would identify the nature and quantify the extent of nanoparticle release in the environment?
- What might be the environmental impact on other species (e.g. animals, fish, plants, microorganisms)?

As the materials and the techniques used to manufacture the novel formulations may not have prior art to refer to as a standard, there is an additional burden on the pharma/biotech industry to carry out a detailed evaluation of the system to generate sufficient database for successful industrialization of the product. Some of the industrially relevant criteria include understanding the relationship between the physico-chemical properties and product performance, effect of process and formulation variables on product characteristics, development of analytical tools and specifications to regulate product quality, accelerated stability testing as per standard protocols to propose a reliable shelf-life, product scale-up to mass production and establishment of manufacturing standards and development of reference materials/standards as guidelines for quality assurance. Development of validated testing methods/protocols and establishment of reference standards through a thorough and logical process remains to be the major responsibility of the industry for convincing the FDA to get product approval.

While considering the application of a polymeric nanoparticles-based formulation, the FDA may want the industry to include evidence for the parameters listed below:

- Particle size and size distribution
- Surface area, surface chemistry, surface coating and porosity
- Hydrophilicity and surface charge density
- Purity and quality
- Stability (on shelf and upon administration)
- Manufacturing and Controls
- Drug release parameters and bioequivalence testing considerations

## 9. Conclusion and Outlook

Under the light of current literature (i.e. articles, books, patents and information posted on the nanotech company websites) and the product pipelines of leading pharma/biotech companies, it is evident that we would be seeing many nanotechnology-based pharmaceutical products in this century. Table 2 lists few of the important products in the drug delivery pipeline that are based on polymeric nanoparticles. It is likely that the oral formulations would dominate this specialized segment of novel dosage forms. The chemical/polymer industry has been feeding the drug delivery scientists with a variety of biopolymers, having wide range of specialized properties. Nanoparticles made from the biopolymers are likely to dominate the novel drug delivery systems in the oral market because of the cost-to-benefit ratio, excellent stability, flexibility for industrial production and a voluminous database available, with respect to the regulatory issues addressed earlier. Polymeric nanoparticles are also being explored for topical applications and as sterile dosage forms for ophthalmic, nasal, subcutaneous and intravenous applications.

There are several other potential nanoparticles technologies which fall outside the coverage of this chapter, which are based on nanoparticles made from the drugs themselves. They are termed as nanosuspensions, nanocrystals or insoluble drug delivery technologies.[28,128,129] Essentially, all of them are colloidal dispersions of pure drug particles that are stabilized by polymers, surfactants or lipids. They are synthesized either by physical (e.g. size reduction by milling) or chemical (e.g. change in solubility induced by pH or solvent exchange) means in the presence of stabilizing agents. The striking advantage of these technologies is the high drug loading efficiency and the simplicity associated with its production. These have been the first to roll out from the research and development scale to the industrial production scale under nanoparticle category (Rapamune® oral solution and tablets

**Table 2** Product pipeline of polymeric nanoparticles (Source: PharmaProjects).

| Company | Technology | Bioactive compound | Route of delivery |
|---|---|---|---|
| Novavax, USA | Micellar nanoparticles | Testosterone | Subcutaneous |
| Flamel Technologies, France | Medusa® nanoparticles of amino acids | Insulin/Interferon | Subcutaneous |
| BioAlliance, France | Poly(isohexyl cyanoacrylate) nanoparticles | Doxorubicin | Intravenous |
| Munich Biotech, Germany | Drug nanoparticles | Paclitaxel | Intravenous |
| BioSante, USA | Calcium phospahte nanoparticles | Insulin | Oral |
| Targesome, USA | Self-assembling lipid nanospheres | Therapeutic/ Diagnostic | Intravenous |
| American Bioscience, USA | Albumin-Drug nanoparticles | Paclitaxel | Intravenous |
| Advectus Life Sciences, Canada | Poly(butylcyanoacrylate) nanoparticles | Doxorubicin | Intravenous |
| Nanocarrier, Japan | Micellar nanoparticles | Water insoluble drugs | N/A |
| Wyeth Pharmaceuticals, USA | Drug nanoparticles | Rapamycin | Oral |

containing sirolimus from Wyeth and SangCya® oral solution from SangStat Corporation containing CyA). If the science of pharmaceutical product development is undergoing a transformation from a traditional pharmaceutics to a more innovative molecular or nano-pharmaceutics, the major credit would be taken by a combination of polymer based systems and nanoparticles. It is more of a belief than a hope that the polymeric nanoparticles would address many of the therapeutic issues that are posing hurdles to a formulation scientist in this century.

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# 27

# Nanoparticular Carriers for Ocular Drug Delivery

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The major goal in ocular drug delivery is to obtain therapeutic drug concentrations at the intended site of action (i.e. located at the eye surface or in the inner eye), for reasonable periods of time. The strategies explored towards this goal have been (i) the design of topical ocular delivery systems which promote the concentration of the drug on the eye surface, and, if necessary, facilitate the drug transfer from the extraocular tissues to the internal structures of the eye; (ii) the design of injectable controlled release systems which deliver the drug directly to the sclera (subconjunctival injection) or to the internal structures of the eye (intravitreal injection), for extended periods of time. Among the delivery systems designed so far for these purposes, those of a nanoscale size are particularly attractive from the point of view of easiness of administration and patient acceptability, since they can be applied in the form of a non-viscous liquid. This chapter aims to describe the advances and the actual potential of polymer-based nanostructures such as nanoparticles and nanocapsules, for topical ocular drug delivery. Since the complexity of these nanostructures has increased over the time, these nanostructures have been classified into first, second and third-generation nanocarriers. Additionally, the last sections of the chapter were intended to present the possibility to use nanoparticulate drug carriers for injection (i.e. subconjunctival, intravitreal), and to underline the specific advantages of nanosystems over large dimensional devices for intraocular drug delivery. Overall, this review chapter shows the great potential

that nanosystems offer in terms of improving the efficacy of drugs used in ocular therapies. Moreover, it emphasizes that the advances achieved in the understanding of the interaction of nanosystems with the ocular tissues should, logically, result in the design of sophisticated systems specifically tailored for ocular drug delivery.

## 1. Biopharmaceutical Barriers in Ocular Drug Delivery. Classification of Nanoparticulate Carriers for Ocular Drug Delivery

Unlike other routes described in previous chapters of this book, the different modalities of ocular administration (i.e. topical, subconjuctival and intravitreal) are exclusively intended to deliver drugs locally for the treatment of ophthalmic processes, and not as an entry to the systemic circulation. Among these modalities, the topical ocular administration is the easiest and best accepted by the patients. Liquid formulations, solutions and suspensions, are the most commonly applied for topical ocular administration, since they are easy to use and do not interfere with vision. However, these formulations are often quite ineffective due to the defense mechanisms of the ocular apparatus. These mechanisms have been described in detail in several review articles and text-books.[1–3] Firstly, most of the drug applied topically onto the eye is immediately diluted in the precorneal tear film. The excess fluid spills over the lid margin and the remainder is rapidly drained into the nasolachrymal duct. As a consequence, most of the applied drug solution is cleared within 2–4 min.[4,5] In addition, a proportion of the drug will not be available for therapeutic action at the ocular level, but will be absorbed to the systemic circulation through the surrounding extraorbital tissues, mainly the conjunctiva (unproductive drug absorption). On the other hand, in the case of drugs whose target is located in the inner eye, they need to overcome the very important additional barrier represented by the cornea, which is the main entrance to the inner eye. The area of contact of the drug with the cornea is restricted to approximately 2 $cm^2$ This small fraction of drug in contact with the cornea is then confronted with the very restrictive sub-barriers such as the epithelium, the stroma and the endothelium. Both the first and the last barrier, but particularly the first, limit the absorption to water soluble substances, due to the existence of tight junctions between the epithelial cells.[6] The stroma, with high water content, limits the absorption of lipophylic drugs.[7] As a result of the above mentioned processes, typically less than 1–5% of the instilled dose reaches the aqueous humour.[2,8] This extremely low "ocular bioavailability" often implies the necessity of frequent dose administration, a situation that may lead to a significant systemic absorption and the corresponding side effects. In some instances, the required posologic regimen is unviable and hence the intravitreal injection becomes necessary to achieve significant drug levels in the intraocular structures.

These biopharmaceutical constraints clearly evidence the necessity to conceive new ocular drug delivery strategies aimed at overcoming the above indicated barriers. Unfortunately, the requisite to preserve both the specific characteristics of the visual apparatus and the visual acuity, together with the inherent sensitivity of the eye, limit the possibilities of designing optimized ocular drug delivery systems substantially.

Among the delivery strategies aimed at circumventing the above described limitations, the design of polymer nanoparticulate carriers offer unique features, while still benefit from their presentation in a liquid form. Two types of nanoparticulate carries have been described for ocular drug delivery: matrice-type nanoparticles, in which the biologically active molecule is entrapped or simply adsorbed onto their surface; and reservoir-type nanocapsules, which consist of a polymeric wall surrounding a liquid drug-containing core. Within the context of this chapter, the matrice-type and the reservoir-type will be termed nanoparticles and nanocapsules respectively. The fabrication processes of these nanostructures will not be a subject of description in this chapter, since they have already been reviewed.[9]

Despite the above indicated limitations in the design of ocular drug delivery systems, the efforts oriented towards the use of nanotechnologies have been relevant and they have led to significant progress in the field. In this chapter, we review the advances made in the design of nanoparticulate carriers intended for topical ocular drug delivery. These nanocarriers are classified into three categories: first generation of basic nanoparticles and nanocapsules, second generation of nanoparticles and nanocapsules with a hydrophilic polymer coating, and the third generation of functionalized nanoparticles/nanocapsules (Fig. 1). On the other hand, being conscious of the fact that the progress made in this field has not yet resulted in significant improvements in the therapy of inner-eye diseases, the potential of nanoparticles as injectable ocular drug delivery vehicles will also be described in this chapter. Indeed, polymer nanoparticles may circumvent the problem of frequent intravitreal injection by providing a controlled delivery of the encapsulated drug, thus reducing the clinical complications associated with this modality of administration.

## 2. Nanoparticulate Polymer Compositions as Topical Ocular Drug Delivery Systems

As previously mentioned, the eye defense mechanisms represent the main limitation to the use of liquid formulations for ophthalmic therapy. Within this context, nanoparticles offer great possibilities of increasing the amount of drug at the anterior chamber of the eye, while spacing the dose administration. Table 1 and Table 2 summarize the literature reports on the use of nanoparticulate polymer compositions as topical ocular drug delivery systems.

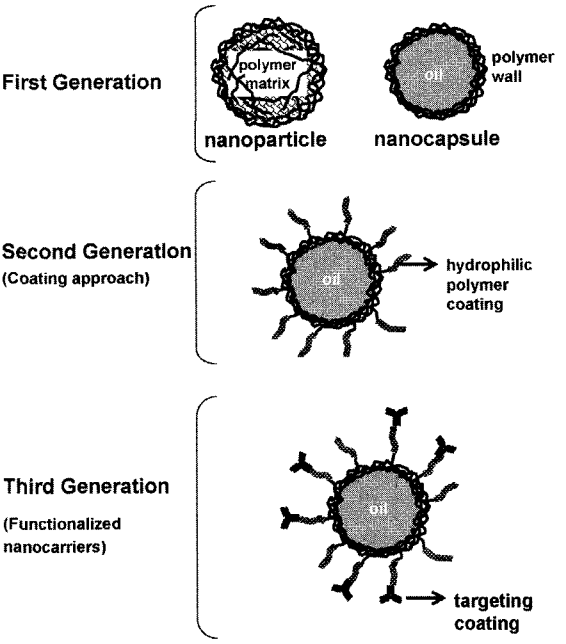


**Fig. 1.** Schematic representation of different nanosystems intended for ocular drug delivery: "first generation" of basic matrice-type nanoparticles (the biologically active molecule is entrapped or simply adsorbed onto their surface) and reservoir-type nanocapsules (the biologically active molecule is dissolved in a liquid core surrounded by a polymeric wall); "second generation" of nanoparticles and nanocapsules (the figure shows a nanocapsule) with a hydrophilic polymer coating; and "third generation" of surface functionalized nanoparticles/nanocapsules (the figure shows a nanocapsule functionalized with antibodies).

## 2.1. *First generation: Polymer nanoparticles and nanocapsules for topical ocular drug delivery*

Nanoparticles, primarily developed for i.v. administration, were first proposed for ophthalmic drug delivery in 1981. Indeed, it was Gurny and co-workers who first indicated the potential advantages of nanoparticles (named pseudo-latexes) over aqueous polymer solutions. More specifically, these authors found that pilocarpine adsorbed onto nanoparticles (0.3 $\mu$m) made of cellulose acetate phthalate (CAP) were able to maintain a constant miosis in the rabbit for up to 10 hours, compared with a 4-hour response attained for pilocarpine eye drops.[10] This initial report was followed by a number of studies aimed at evaluating the potential of different types of polymers including acrylic polymers, and, especially, poly(alkyl cyanoacrylates) (PACA), polyesters, i.e. poly-$\varepsilon$-caprolactone, and polysaccharides, such as hyaluronic acid and chitosan, for ocular drug delivery. A summary of the results obtained with these different nanoparticulate formulations is presented in Table 1.

**Table 1** Nanoparticulate compositions used in ocular drug delivery (topical administration).

| **Polymer type**[a] | **Drug (System type)** | ***In vivo* results**[b]**(references)** |
|---|---|---|
| CAP | Pilocarpine (Nanoparticles) | Prolonged miosis[10] |
| Eudragit® | Ibuprofen/ Flurbiprofen (Nanoparticles) | Improved "ocular bioavailability" (aqueous humour drug levels) and inhibition of the miosis induced by a surgical trauma[21–22] |
| | Cloricromene (Nanoparticles) | Improved "ocular bioavailability" (aqueous humour drug levels)[23] |
| PIPAA | Epinephrine (Nanoparticles) | Prolonged IOP lowering effect[24] |
| PACA | $^{3}$H-Progesterone (Nanoparticles) | Reduced drug concentrations in cornea, conjunctiva and aqueous humor[19] |
| | Pilocarpine (Nanoparticles) | Prolonged miosis[11]<br>Prolonged miosis and improved reduction of IOP[12]<br>Improved "ocular bioavailability" (aqueous humour drug levels), prolonged miosis and improved reduction of IOP for pilocarpine-loaded nanoparticles[13] |
| | Betaxolol (Nanoparticles) | Prolonged intraocular pressure (IOP) lowering effect[14] |
| | Amikacine (Nanoparticles) | Improved "ocular bioavailability" (corneal and aqueous humour drug levels)[16] |
| | $^{3}$H-Cyclosporin (Nanocapsules) | Prolonged therapeutic levels in cornea, sclera, uvea and retina as compared to those provided by an oily ciclosporin solution[17] |
| PECL | Metipranolol (Nanocapsules) | Reduction of cardiovascular side effects and enhanced IOP lowering effect[27,28] |
| | Betaxolol (Nanocapsules/ Nanoparticles) | Enhanced IOP lowering effect in a greater extent than PACA or PLGA. Effect more important for PECL nanocapsules than for nanoparticles[26] |
| | Carteolol (Nanocapsules/ Nanoparticles) | Improved IOP lowering effect, being this effect superior for nanocapsules than for nanoparticles. Reduction of cardiovascular side effects for nanocapsules[15] |
| | Cyclosporin A (Nanocapsules) | Increased and more prolonged cyclosporin corneal levels as compared with those corresponding to an oily cyclosporin solution[32] |
| | Indomethacin (Nanocapsules/ Nanoparticles) | Improved bioavailability (e.g. cornea, aqueous humour and iris-ciliary body drug levels) as compared with that of indomethacin-loaded Microparticles (6 $\mu$m), and that of a control solution[30] |

**Table 1** (*Continued*)

| Polymer type[a] | Drug (System type) | *In vivo* results[b] (references) |
|---|---|---|
| Chitosan | Cyclosporin A (Nanoparticles) | Higher and more prolonged cyclosporin levels at external ocular tissues (e.g. cornea and conjunctiva) and negligible intraocular and systemic levels, as compared with those corresponding to a cyclosporin suspension in a chitosan solution[47] |

[a]CAP: Cellulose acetate phthalate; PIPAA: Poly(isopropylacrylamide); Eudragits®: Copolymers of ethylacrylate, methyl-methacrylate and chlorotrimethyl-ammonioethyl-methacrylate; PECL: Poly-epsilon-caprolactone; PLA: Poly(lactic acid); PACA: Poly(alquilcyanoacrylate).
[b]IOP: intraocular pressure.

### 2.1.1. *Acrylic polymers-based nanoparticles*

The first study reporting the potential of polyacrylic nanoparticles for ocular drug delivery was published by Wood *et al.*[5] More specifically, these authors found that PACA nanoparticles were significantly retained in the precorneal area, and therefore could act as nanoreservoires for extended drug delivery. Indeed, this improved retention of the carrier at the ocular surface was the explanation for the increased drug concentration in the cornea, and for the enhanced and/or prolonged pharmacological effect reported for antiglaucoma drugs, such as pilocarpine,[11–13] betaxolol[14] and carteolol,[15] the aminoglucoside amikacine[16] and the immunosuppressive peptide cyclosporin A.[17] Moreover, some authors found that the retention and residence time of nanoparticles was significantly increased in inflamed ocular tissues.[18] This was attributed to an enhanced epithelial permeability of the swollen conjunctival tissue. There have also been examples for which the "ocular bioavailability" of the drug associated to PACA nanoparticles was reduced, compared with that of the free drug. This negative result has been attributed to the great affinity of the drug (progesterone) for the polymer, and consequently, to its deficient delivery from the carrier.[19]

Unfortunately, despite the reported efficacy of PACA nanoparticles for enhancing the "ocular bioavailability" of drugs, the study reported by Zimmer *et al.* in 1991 evidenced that the nanoparticles entered the corneal epithelial cells, causing a disruption of cell membranes.[20] Whether this negative result was due to specific experimental conditions of the reported study, or to the intrinsic nature of the PACA and its degradation products, remains to be clarified. Nevertheless, this could possibly provide the explanation for the little attention that PACA nanoparticles have attracted over the last decade, for this specific application.

Interestingly, the toxicity reported for PACAs has not dissuaded the research on the other types of acrylic polymers such as copolymers of ethylacrylate, methylmethacrylate and chlorotrimethyl-ammonioethyl-methacrylate (Eudragits®)[21–23] and polyacrylamide.[24] In the case of Eudragit® nanoparticles, the *in vivo* results showed an enhanced bioavailability (aqueous humour drug levels) of the anti inflamatory drugs (e.g. ibuprofen, flurbiprofen), as well as an improved pharmacological response.[21–23] On the other hand, using epinephrine-loaded polyacrylamide nanoparticles, it was possible to prolong the intraocular pressure (IOP) lowering effect caused by epinephrine.[24] While these studies underline the positive interaction of acrylic nanoparticles with the ocular mucosa, further studies in terms of tolerance and toxicity are required to assess the practical application of these nanoparticles.

### 2.1.2. *Polyester-based nanoparticles and nanocapsules*

The results obtained for PACA nanoparticles stimulated the search for new nanoparticulate carriers made of different polymers. Within this context, poly-ε-caprolactone (PECL) and the copolymers of lactic and glycolic acid (PLGA) have received a great deal of attention. The choice of these polymers was based on their broad history of safe use in humans as suture materials and implants. Moreover, at present, there is significant evidence of the adequate ocular tolerability of nanoparticles composed of these materials.[25]

According to our knowledge, the first reports on the efficacy of polyester for topical ocular drug delivery were published in 1992.[26,27] Marchal-Heussler *et al.*[26] compared the performance of nanoparticles made of PACA, PECL and PLGA containing betaxolol as a model drug, with that of the commercial eye drops. The results showed that under the experimental conditions assayed, all nanoparticulate systems yielded an improved pharmacological response (i.e. intraocular pressure-lowering effect), compared with an aqueous eye drop control formulation of the drug, with the optimum responses being ascribed to PECL nanocapsules. In this case, the improved pharmacological effect was believed to be due to the agglomeration of these hydrophobic nanoparticles in the conjunctival sac, thus forming a depot from which the drug is slowly delivered to the precorneal area.

Simultaneously to this work, we reported the efficacy of PECL nanocapsules consisting of an oily core and a PECL wall for the ocular delivery of metipranolol. The results of this study performed in rabbits led us to conclude that PECL nanocapsules were not only able to increase the pharmacological effect of this drug, but also able to reduce the cardiovascular side effects associated with its systemic absorption.[27,28] This positive behavior suggested that the nanocapsules enhanced the drug transport across the cornea and reduced the systemic absorption

through the conjunctiva. In our attempt to elucidate the mechanism of the action of these nanocapsules, we labeled them with rhodamine B and followed their interaction with the rabbit cornea and conjunctiva by the use of confocal laser scanning microscopy. The results of these *ex vivo* studies clearly evidenced that PECL nanocapsules were able to penetrate the corneal epithelial cells, and that they also exhibited a preference for the cornea *vs* the conjunctiva.[29] Consequently, this differentiated interaction with both the epithelial surfaces could be taken as an adequate explanation for their reported ability to enhance corneal penetration and reduce systemic absorption. More importantly, no evidence of membrane alteration or signs of toxicity were detected in this study.

With the intention of investigating the determinants of the interaction of these particles with the ocular mucosa, and thus of their performance as ocular drug carriers, we compared the efficacy of PECL nanoparticles and nanocapsules (200 nm) with that of PECL microparticles (6 $\mu$m). The results clearly evidenced that the nanoscale size was critical with regard to the ability of the particles to enhance the ocular bioavailability of indomethacin[30] (Fig. 2). Consequently, these results led us to hypothesize that nanoparticles have a greater ability than microparticles to interact with the corneal epithelium cells. A similar conclusion was extracted recently for the interaction of the polyester particles with the conjunctival cells.[31] In specific, these authors found that the *in vitro* uptake of nanoparticles by primary cultured rabbit conjunctival epithelial cells was more important than that of the microparticles.

The positive results obtained for metipranolol and indomethacin encouraged us to test the performance of these nanosystems for the topical delivery of the

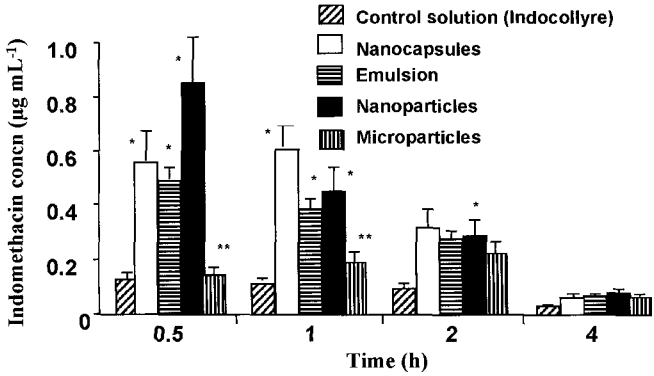


**Fig. 2.** Indomethacin concentrations attained in the aqueous humor following the topical application, in rabbits, of indomethacin-loaded carriers and a control drug solution (Mean values ± SD, $n = 3,4$) (*$P < 0.05$ compared with Indocollyre; **$P < 0.05$ compared with colloidal suspensions) (Reprinted from Ref. 30, with permission from Pharmaceutical Press.)

immunosuppressive peptide cyclosporin A. Interestingly, following topical administration of PECL nanocapsules containing cyclosporin A, we observed corneal levels of the drug which were five times higher than those provided by an oily solution (topical formulation of cyclosporin typically used).[32] These high levels were not, however, translated into high drug concentrations in the aqueous humor; a result that was attributed to the important hydrophobicity of this peptide and its tendency to associate with lypophylic components.

Therefore, at present, there is a proof-of-concept of the efficacy of polyester nanocapsules for enhancing the concentration of topically applied drugs in the corneal epithelium. Whether this enhanced concentration may or may not lead to a favored accumulation of the drug in the inner eye is expected to be largely dependent on the physicochemical characteristics of the drug.

### 2.1.3. *Polysaccharide-based nanoparticles*

The polyester polymers described above are hydrophobic polymers that need to be biodegraded into hydrophilic oligomers in order to be eliminated from the body. A very different class of polymers, which has only received attention in the last few years, is the one represented by the hydrophilic polysaccharides. Hyaluronic acid and chitosan are two types of polysaccharides which have opened new prospects in the ocular drug delivery area. The choice of hyaluronic acid has been justified by its bioadhesive character,[33,34] but also by its well known safety profile. In fact, hyaluronic acid is already being used as a substitute for vitreous humor in intraocular surgery, since it constitutes a basic component of the vitreous body.[35] On the other hand, chitosan is a polycationic biopolymer which exhibits several favorable biological properties for ocular drug administration.[3] These properties include mucoadhesiveness,[36,37] biodegradability in the rich lysozyme-containing mucus (i.e. ocular mucosa),[38–40] and also wound healing and antimicrobial activity.[41–43]

Despite the number of articles showing the efficacy of hyaluronic acid solutions for improving the retention of drugs applied topically onto the eye,[33,34,44] the only particulate formulation that has been tested *in vivo* was composed of microparticles (1–10 $\mu$m) rather than nanoparticles. These hyaluronate microparticles were shown to increase the residence time of the model drug methylprednisolone at the ocular surface of the rabbit eye.[45] Taking into account the reported influence of the size on the interaction of particles with the ocular mucosa, we have recently designed nanoparticles consisting of hyaluronic acid and chitosan.[46] At present, we know that these nanoparticles are stable upon incubation in simulated lachrymal fluids and *in vivo* studies are in progress in order to evaluate their mechanism of interaction with the ocular mucosa.

Chitosan has also received significant attention in the ophthalmic field.[3] One of the chitosan-based systems that has exhibited an interesting behavior following topical ocular administration, is the one consisting of chitosan nanoparticles. These nanoparticles have been tested on the rabbit model for their ability to enhance the concentration of cyclosporin A at the level of the ocular mucosa. As expected, the results showed that the chitosan nanoparticles were able to increase the concentrations of cyclosporin A in the external ocular tissues (cornea and conjunctiva) significantly for up to 48 hr post-instillation (Fig. 3). Despite this enhanced retention of the drug in the external tissues, the levels attained in the internal ocular structures (i.e. aqueous humor, iris and ciliary body) and in the blood were negligible. Consequently, these results suggested the utility of this new formulation for the treatment of surface eye diseases, i.e. dry eye or inflammatory diseases.[47] These high drug concentrations restricted to the periocular tissues were later explained by a high corneal and conjunctival surface retention of chitosan nanoparticles. Indeed, in a study consisting of evaluating the concentration of fluorescent chitosan, either in the form of nanoparticles or as a solution, in cornea and conjunctiva, we could conclude that the affinity of chitosan for the ocular surface is greater when it is in a particulate form.[48] This conclusion invites interesting prospects with regard to the potential of chitosan nanoparticles as drug carriers for topical ocular administration. Keeping this in mind, we tested the acute tolerance of chitosan nanoparticles following topical instillation to rabbits very recently. The results gave evidence of an excellent tolerance, without any sign of irritation or damage of the ocular surface structures.[49]

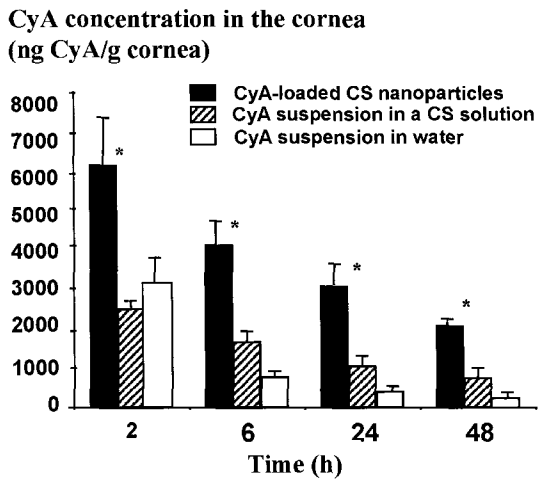


**Fig. 3.** Cyclosporin (CyA) concentration in the cornea after topical administration in rabbits of CyA-loaded chitosan (CS) nanoparticles and control formulations consisting of a CyA suspension in a CS aqueous solution and a CyA suspension in water (*statistically significant differences, $P < 0.05$). (Reprinted from Ref. 47, with permission from Elsevier.)

## 2.2. *Second nanoparticles generation: The coating approach*

The previously described nanoparticular polymer-based carriers, are shown to increase the intensity and contact time of drugs with the eye. Moreover, in some cases, this improved contact led to an enhanced intraocular penetration of drugs. Despite the difficulties for comparing the performance of the first-generation nanosystems, it is obvious that their interaction with the ocular surface is determined not only by the nanoscale size, but also by the surface composition of the nanomatrice. Taking this into account, a different approach has arisen based on the principle of providing to the nanocarrier, a polymer coating that favors its interaction with the ocular mucosa. Using this approach, it is additionally possible to select the adequate core composition in order to facilitate the entrapment and protection of the desired drug. Moreover, one can envisage the design of a nanocarrier with a differentiated interaction with the cornea and conjunctiva. An element that could be taken in consideration to achieve this aim is the presence of the mucus layer covering the conjuctival epithelium (i.e. where the goblet cells are) and its reduced amount onto the corneal surface. In this sense, it is important to keep in mind that the interaction with the cornea would be the choice for the drugs whose target is located in the inner eye. In contrast, the improved interaction and controlled release at the conjunctival level could offer a potential for the treatment of surface ocular diseases. Table 2 summarizes the characteristics of the different coated nanostructures developed under these bases.

### 2.2.1. *Polyacrylic coating*

The first "coating approach" was intended to confer the nanosystems with a mucoadhesive character. Theoretically, the coating with mucoadhesive polymers could markedly prolong the residence time of the nanocarriers, since their clearance from the eye surface would be controlled by the much slower rate of mucus turnover than the tear turnover rate.

The simplest approach towards this aim has been the suspension of the nanocarrier in an aqueous solution containing a mucoadhesive polymer. Indeed, Zimmer *et al.*[50] observed that the co-administration of pilocarpine-loaded albumin nanoparticles with bioadhesive polymers such as polyacrylic acid (Carbopol®), hyaluronic acid, mucin or sodium carboximethylcellulose, led to an enhancement of the intraocular pressure lowering effect in rabbits. The efficacy of this approach was also tested for PACA nanoparticles in an *ex vivo* study using bovine corneas. The results showed that the corneal penetration of cyclosporin A, entrapped in PACA nanoparticles, was improved when the nanoparticles were suspended in a polyacrylic acid gel.[51]

**Table 2** Polymer-coated nanoparticulate compositions used in ocular drug delivery (topical administration).

| Polymer coating[a] | Core[b] composition | Associated drug/marker | In vivo results (references) |
|---|---|---|---|
| Polyacrylic acid | Albumin Nanoparticles | Pilocarpine | Enhanced intraocular pressure lowering effect and duration of miosis[50] |
| Chitosan | PECL/oil Nanocapsules | Indomethacin | Improved drug "ocular bioavailability" (corneal and aqueous humor drug levels)[20] |
| Chitosan | PECL/oil Nanocapsules | Rhodamine | Enhanced retention of the nanocapsules on the ocular surface[54] |
| Hyaluronic acid | PECL nanoparticles | — | Not reported[52] |
| PEG | PACA Nanoparticles | Acyclovir | Improved drug "ocular bioavailability" (aqueous humour drug levels)[59] |
| PEG | PLA Nanoparticles | Acyclovir | Improved drug "ocular bioavailability" (aqueous humour drug levels)[25] |
| PEG | PECL nanocapsules | Rhodamine | Evidence of the ability of PEG-coated nanocapsules to cross the corneal epithelium layers[54] |

[a]PEG: Poly(ethyleneglycol).
[b]PECL: Poly-epsilon-caprolactone; PLA: Poly(lactic acid); PACA: Poly(alquilcyanoacrylate).

### 2.2.2. *Polysaccharide coating*

As indicated in the previous section covering the nanocarriers of first generation, two polysaccharides have attracted special attention as mucoadhesive materials for ocular application: hyaluronic acid and chitosan. Apart from the simple dispersion of the core material into an aqueous polymer solution described above, the first attempt to efficiently coat nanoparticles with hyaluronic acid was described by Barbault-Foucher *et al.*[52] These authors described different strategies for the formation of hyaluronate-coated poly-ε-caprolactone (PECL) nanoparticles intended for ocular drug delivery. These strategies were simple adsorption, ionic promoted interaction and chain entanglement during the nanoparticles fabrication process. While the *in vivo* efficacy of these strategies remains to be investigated, this publication shows the versatility of the coating approach procedure.

The mucoadhesive polysaccharide chitosan has also been identified as a successful candidate for the "coating approach". The mucoadhesive properties of

chitosan have generally been ascribed to its polycationic nature, which promotes the interaction with the negatively charged ocular mucosa. However, the cationic nature should not be taken as the only factor determinant of the mucoadhesive properties of polymer-coated nanocarriers. In fact, in a previous study, we have shown that the performance of PECL nanoparticles coated with two different polycationic polymers (poly-L-lysine and chitosan) was drastically different. Concretely, we observed that PECL nanoparticles coated with chitosan were significantly more efficient at increasing the corneal uptake of the encapsulated molecule ($^{14}$C-indomethacin) in rabbits, than those coated with poly-L-lysine.[53] Therefore, these results led us to conclude that it was the intrinsic mucoadhesive character of chitosan, not exclusively ascribed to its positive charge, that is the reason for its successful behavior.

More recently, we attempted to investigate *ex vivo* (isolated rabbit cornea) and *in vivo* the mechanism of interaction of chitosan-coated PECL nanocapsules with the cornea.[54] The results of this study showed that rhodamine encapsulated in these systems had an improved transport across the cornea, compared with that of the marker alone, or in combination with blank nanocapsules (Fig. 4). Moreover, the examination of the corneas treated with fluorescent nanocapsules by confocal microscopy suggested that CS-coated nanocapsules have a lower penetration

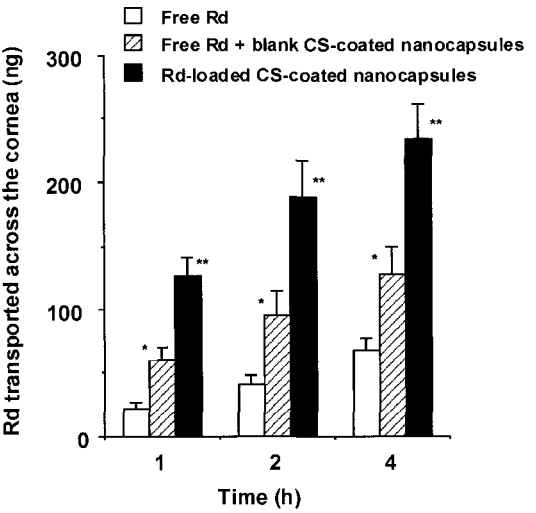


**Fig. 4.** Rhodamine (Rd) amount transported across the rabbit cornea in an *ex vivo* study for Rd-loaded chitosan (CS) coated nanocapsules and control formulations consisting of a Rd solution and a physical mixture of free Rd and blank CS-coated nanocapsules (*Statistically significant different from free Rd). (**Statistically significant different from free Rd and free Rd plus blank CS-coated nanocapsules) (Reprinted from Ref. 54, with permission from Elsevier).

extent than the non-coated PECL nanocapsules, a result that could be attributed to the increased surface retention of the nanocapsules in the mucus layer. Therefore, overall, the results obtained until now with nanoparticulate carriers coated with mucoadhesive polymers permit us to conclude the efficacy of this approach, in terms of increasing the retention of the nanoparticles at the eye surface. This improved retention could be translated depending on the solubility properties of the drug encapsulated to a more important corneal penetration, or in a greater retention on the ocular surface.

### 2.2.3. *Polyethyleneglycol (PEG) coating*

A very different alternative in the "coating approach" has been the one intended to provide the nanoparticulate carrier with an improved stability upon contact with the mucosal fluids. In fact, both the mucus layer and the lachrymal fluids are very rich in enzymes and proteins, which may be attached to the nanoparticles and promote their aggregation.[55] Poly(ethylene glycol) (PEG) appears to be an ideal candidate for such purpose, since it has been widely used to prevent the interaction of colloidal carriers with proteins. For example, in a study performed by us,[56] we observed that a PEG coating around PLA nanoparticles prevented their aggregation in the presence of lysozyme (highly concentrated in the mucus layer). On the other hand, this stabilizing effect has been the main explanation for the successful behavior of PEG-coated PLA nanoparticles as transmucosal drug carriers (i.e. after nasal and oral administration).[57,58] Therefore, from these studies we suggested that the presence of a hydrophilic PEG layer onto the surface of polyester nanoparticles could result in an enhanced stability and, hence, to an improved interaction of these nanosystems with the ocular mucosa.

The first report on the positive effect of the PEG coating approach on ocular drug administration was published by Fresta *et al.*[59] These authors evaluated the ocular bioavailability of acyclovir-loaded PEG-coated PACA nanoparticles and observed a significant increase of the drug levels in the aqueous humor, when comparing these systems with an aqueous acyclovir suspension and with a physical mixture of the free drug and the blank PEG-coated PACA nanoparticles. Interestingly, in this work, the authors did not consider the possibility of an improved stability. Rather, they suggested that the PEG-coated particles might have an improved mucoadhesion. However, no studies were reported to verify this mechanistic hypothesis. In a more recent work, the same group reported the efficacy of the PEG coating but with a different core (PLA nanoparticles), in terms of increasing the "ocular bioavailability" (aqueous humor drug levels) of acyclovir.[25] Moreover, these authors observed that the positive effect of the PEG coating disappeared, when the mucus layer was removed using N-acetylcysteine, prior to the administration of these systems to

rabbits. This observation led the authors to suggest that the mucoadhesion of the PEG-coated nanocarriers may play a role in its mechanism of action. However, this result could also be understood as a consequence of the stabilizing effect provided by PEG in the mucosal surfaces; this effect being not visible in the absence of mucus.

In order to obtain further insights into the interaction of PEG-coated nanoparticles with the cornea, we have recently compared their behavior with that of the uncoated PECL nanocapsules and the chitosan-coated nanocapsules. The confocal images showed that the three types of nanocapsules were able to enter the corneal epithelium. However, their penetration depth followed the ranking of PEG-coated nanocapsules > uncoated nanocapsules > chitosan-coated nanocapsules.[54] The more important corneal penetration of PEG-coated nanocapsules, as compared with that of the non-coated ones, was suggested to be a consequence of their improved stability in the mucosal fluids. The chitosan coating is also known to affect the stability of colloidal particles positively in the presence of the proteins such as lysozyme.[56] However, in this case, the superficial retention of chitosan-coated nanocapsules (described above) could also be understood as a result of their mucoadhesive character.

Overall, the results obtained so far with nanoparticulate drug carriers, coated with hydrophilic polymers, indicate that depending on their nature, these polymers are able to increase the stability and/or the mucoadhesion of the nanocarrier. It could also be presumed that an increase in the mucoadhesion should lead to an accumulation of the drug carrier at the ocular surface. Also, in the case of drugs with adequate permeability properties, it should lead to an increase and prolongation in the corneal penetration. Similarly, an increase in the stability is expected to lead to a more important interaction and transport of the nanoparticles across the corneal epithelium. Moreover, these results suggest that both the extent of the interaction and the penetration depth of the nanocarriers with the cornea, can be modulated by providing them with an appropriate coating. Despite the need of additional mechanistic studies as evidence, the results reported so far provided some basis for the development of strategies intended as an efficient drug targeting to specific ocular structures, as discussed below.

## 2.3. *Third nanoparticles generation: Towards functionalized nanocarriers*

The new tendency in the design of the new drug delivery systems is directed towards integrating several drug delivery technologies, in order to provide the system with unique properties. In the particular case of the ocular drug delivery, the design of highly sophisticated drug delivery nanosystems could benefit from

the knowledge gained from the application of such systems to other trasmucosal routes of administration. As described in the previous chapters of this book, some of the present efforts on the design of more specialized nanocarriers go through functionalizing their surface. This means that the design of nanoparticles with surface characteristics allowing their functionalization with specific targeting moieties, is able to selectively direct the nanocarrier to the predetermined ocular structures.

Among the targeting moieties described till now, lectins may represent an interesting option for targeting the ocular mucosa. Lectins are glycoproteins capable of recognizing and binding reversibly to specific carbohydrate moieties which are present on cell surfaces and mucin. In fact, lectin-like molecules are known to be important in the adhesion of micro-organisms to mucosal surfaces.[60] Therefore, lectins clearly differ from conventional mucoadhesive materials which interact non-specifically with the mucus or simply adhere to biological surfaces.[61] Some examples of lectins are wheat germ agglutinin and phasoleus vulgaris agglutinin, which bind specifically to N-acetylgalactosamine and mannose receptors, respectively.

With regard to the potential of lectins as targeting moieties for ocular drug delivery, it is a known fact that lectins can bind to the corneal and conjunctival surfaces and also to some constituents of the tear film.[62,63] From our knowledge, despite this information, there is no evidence of the potential of the targeted systems in the ocular drug delivery field.

A different category of targeting ligands is represented by the monoclonal antibodies. The initial attempts towards the monoclonal antibody-based targeting approach have been directed to the treatment of ocular herpes simplex virus (HSV) infection. More specifically, Norley *et al.*[64] proposed the attachment of monoclonal antibodies (anti-glycoprotein D of HSV) to liposomes, in order to achieve the targeted delivery of antiviral drugs to the infected corneal cells. The results of this work showed the ability of these "immunoliposomes" to preferentially bind to virus-infected corneal cells *in vitro*. However, there are no *in vivo* data available to support the efficacy of this targeting strategy, thus far.

The lack of reported success of the monoclonal antibody-based targeting approach could be related to the late clinical development of antibodies. Nevertheless, the enormous effort devoted to the development of antibodies for therapeutic or diagnosis purposes in the last few years[65] opens optimistic prospects with regard to their use as targeting moieties for nanoparticulate carriers.

Within this context, PEG-coated nanoparticles offer interesting opportunities for the functionalization with ligands such as lectins[66] and monoclonal antibodies.[67] Additionally, as polysaccharides present many available reactive groups, active targeting could also be attained by grafting ligands onto the polysaccharide-coated nanoparticles.

## 3. Nanoparticulate Polymer Compositions as Subconjuctival Drug Delivery Systems

The subconjunctival route has been proposed as an alternative to the topical drug delivery route, in order to force the retention of a significant amount of drug in the eye. The drug molecules locate underneath the conjunctival epithelium are supposed to diffuse through the sclera and reach the inner eye. There is no doubt that the most important limitation of this modality of administration is the poor acceptability by the patients. Therefore, the use of controlled release micro and nanoparticles was thought to be a good approach in order to reduce the number of injections. Despite the logic of this approach, no improved pharmacological and/or therapeutical effects have been reported so far for either micro or nanoparticles. For example, the subconjunctival injection of cyclosporin-loaded PLGA microparticles failed to improve the response of this drug.[68] On the other hand, to the best of our knowledge, there has been no report on the efficacy of nanoparticles for the delivery of drugs at the subconjunctival level. The study reported by Amrite *et al.*[69] showed that the model fluorescent nanoparticles (20 nm) and microparticles (2 $\mu$m) administered subconjunctivally were not able to cross the sclera, remaining at the injection site.

## 4. Nanoparticulate Polymer Compositions as Intravitreal Drug Delivery Systems

Most diseases affecting the posterior segment of the eye are chronic in nature and require prolonged drug administration. These diseases are one of the major causes of blindness in the developed world. Unfortunately, the described difficulty of reaching effective drug levels at intraocular structures represents a major limitation associated to these therapies (i.e. treatment of proliferative vitreoretinopathy, endophthalmitis, recurrent uveitis, acute retinal necrosis, choroidal neovascularization and cytomegalovirus retinitis). In these severe situations, the intravitreal injection becomes the route of choice for drug delivery. However, in clinical practice, this modality of administration has important draw-backs: (i) poor patient acceptability, which may lead to failure of therapy; (ii) rapid drug elimination from vitreous humor (i.e. removal to the systemic circulation along with the aqueous humor drainage, active secretion from the retina); (iii) possible retinal toxicity of certain potent drugs; (iv) potential hazards associated with repeated intravitreal injection, such as the clouding of the vitreous humor, retinal detachment, lens damage and endophthalmitis.

The above indicated problems illustrate the need for the design of adequate controlled release systems which could minimize the frequency of injection. Vitrasert® is an example of a commercially available sustained release intraocular device for ganciclovir, which has been approved for use in patients suffering from cytomegalovirus (CMV). This implant is a reservoir system consisting of a magnesium stearate core containing the drug, and a coating of ethylenevinyl acetate polymers. Apart from the necessity of surgical removal,[70] additional problems observed for this device include endophthalmitis, retinal detachment, dislocation of implant and poor intravitreal drug levels due to its placement in the suprachoroidal space.[71]

Within this context, biodegradable micro and nanoparticles appear to offer advantages when compared with large devices, since they can be injected through a needle, thus avoiding the necessity of a surgical procedure. Among the particulate carriers investigated to date for intraocular drug delivery, those made of biodegradable polyesters such as poly(lactic/glycolic acid) (PLGA) are expected to offer a significant potential. In fact, there are already a number of reports on the biodegradability, tolerability and efficacy of PLGA intraocular implants[72,73] and microparticles (for a review, see Ref. 74).

With regard to the specific potential of nanoparticles, in two studies published in 1994[75,76] aimed at evaluating the interaction of PLA microparticles (0.2–1 $\mu$m) with retinal pigment epithelium cells, it was found that these particles were internalized by the above mentioned cells. This finding was justified by the known phagocytic activity of these cells, which, on the other hand, are essential for the maintenance of retinal metabolism and visual acuity. This initial observation was corroborated in a more recent study which evidenced that PLGA nanoparticles were localized within these cells even at 4 months post-administration.[77] Moreover, these authors observed that PLGA nanoparticles were well tolerated following intravitreal injection to rats. Therefore, these publications showed the potential of nanoparticles no only as simple depot controlled release systems but as intracellular controlled delivery systems for bioactive molecules.

Surprisingly, despite the attractive features of PLGA nanoparticles as intraocular delivery systems, the information reporting the success of this approach is scarce. For example, in a very recent publication it was shown that PLGA nanoparticles could work as gene delivery systems to the posterior segment of the eye.[78] Concretely, the plasmid encoding the red nuclear fluorescent protein (RNFP) was associated to PLGA nanoparticles and then injected into the vitreous cavity of rabbits. The results showed an important level of RNFP expression within the retinal pigment epithelial cells, thus indicating the adequate internalization and delivery of the plasmid into the cells. On the basis of these findings, these authors suggested the potential of nanoparticles for designing future gene-based ocular therapy strategies.

Another type of nanoparticles that has been investigated for intravitreal drug delivery is the one consisting of PACA. More specifically, these nanoparticles were tested for their ability to deliver $^{3}$H-acyclovir and ganciclovir for extended periods of time following intravitreal injection to rabbits. The drug concentrations attained in the vitreous and retina were high and steady for up to 10 days.[79] Unfortunately, these positive results were counteracted by the negative reaction observed in the lens (opacification) and in the aqueous humour (turbidity).

With respect to the alteration of the normal physiological conditions of the eye, one of the problems that could be expected from the use of micro and nanospheres is their instability in the vitreous humor. Indeed, although no stability study has been reported, it could be accepted that, as in the case of other biological fluids, i.e. lachrymal fluid, nanoparticles may suffer an aggregation process mediated by their interaction with proteins. One of the alternatives to resolve this problem could be the PEG coating approach described in the previous sections. Interestingly, while this approach has not been applied to PLGA nanoparticles yet, some evidence of its efficacy has been reported for PEG coated-poly(hexadecyl cyanoacrylate) nanoparticles.[80] These PEG-coated nanoparticles, containing tamoxifen, have shown promising results for the treatment of experimental autoimmune uveitis, although no comparison was made between PEG coated and non-coated nanoparticles. Therefore, and in spite of these promising results, the potential application of these nanoparticles will greatly depend on their tolerability and biodegradability in the ocular environment.

Finally, non-polymeric nanoparticles have also been reported for intraocular drug delivery. Concretely, Merodio *et al.*[81] evaluated the ocular toxicity induced by the prolonged presence of the ganciclovir-loaded albumin nanoparticles after their intravitreal injection to rats. These authors detected the presence of these systems in the vitreous cavity for up to two weeks after their intraocular injection. In addition, according to the authors, the histological evaluation of these adjacent tissues revealed a good tolerance.

In summary, the reports of the potential of nanoparticles as intraocular drug delivery systems indicate that while their characteristics appear to be appealing for such application, further studies are necessary to assess important issues that include their stability and biodistribution in the intraocular cavity, as well as their biocompatibility and absence of toxic reactions or alterations of the normal function of the eye.

## 5. Conclusions and Outlook

Despite extensive research in the field, the major problem in ocular drug delivery is the attainment of an optimal drug concentration at the intended site of action for

a sufficient period of time. The site of action maybe located on the eye surface or in the inner ocular structures. The important barriers that need to be overcome in order to reach the target site limits not only the number of medications available for the treatment of ocular diseases, but also the extent to which those available can be used without incurring undesirable systemic side effects. From the results described in this chapter, it is possible to conclude that nanoparticles offer great chances of solving these limitations, while still benefiting from their topical administration as eye drops. Indeed, nanoparticles, depending on their composition, are significantly retained on the ocular mucosa, and from this location, they deliver the associated drugs for extended periods of time. This situation normally results in an enhanced and prolonged therapeutic response, and also in a decrease in the side effects. The results reported so far have also evidenced that both the extent of interaction and the penetration depth of the colloidal systems with the cornea, can be modulated by the selection of an appropriate coating. In addition to these beneficial effects associated with the topical ocular administration, nanoparticles offer an interesting potential in terms of improving intraocular drug administration. This potential includes not only the prolongation of the residence time of drugs in the eye, but also their targeting to the retinal cells.

Finally, significant efforts are currently underway to develop highly sophisticated nanoparticles functionalized with specific targeting ligands (i.e. lectins and antibodies). Advances in this area are expected to open new avenues for the diagnostic and therapy (including gene therapy) of ocular disorders.

## Acknowledgments

The authors would like to thank the Spanish Ministery of Education and Science for the financial support of some of the studies described in this chapter (Refs. MAT2004-04792-C02-02 and SAF2004-08319-C02-01).

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# 28

# Nanoparticles and Microparticles as Vaccine Adjuvants

*Janet R. Wendorf, Manmohan Singh and Derek T. O'Hagan*

## 1. Introduction

One of the most important current issues in vaccinology is the need for new adjuvants and delivery systems. Many of the vaccines currently in development are based on purified subunit proteins, recombinant molecules, synthetic peptides or plasmid DNA. Unfortunately, it is clear that this new generation of vaccines will be less immunogenic than traditional vaccines, and will require better adjuvants and delivery systems to induce optimal immune responses.[1,2] In addition, non-living vaccines have generally proven ineffective at inducing potent cell mediated immunity (CMI), particularly of the Th1 type. T helper cells can be classified into Th2 and Th1 subtypes, mainly based on their cytokine production profile, with Th1 responses being characterized by the production of $\gamma$ interferon. Th1 responses are likely to allow the development of vaccines against important infectious diseases, including HCV and HIV.

Immunological adjuvants were originally described by Ramon[3] as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone." This broad definition encompasses a very wide range of materials.[4] However, despite extensive evaluation of a large number of candidates over many years, the only adjuvants currently approved by the US Food and Drug Administration are aluminum based mineral salts, generically known

as alum. Alum has a good safety record but comparative studies show that it is a weak adjuvant for antibody induction to recombinant protein vaccines and induces a Th2, rather than a Th1 response.[5] In addition, Alum is not effective in inducing mucosal IgA antibody responses. Moreover, alum adjuvants can induce IgE antibody responses and have been associated with allergic reactions in some subjects.[5,6] Although Alum has been used as an adjuvant for many years, its mechanism of action remains poorly defined. It was originally thought to provide a "depot" effect, resulting in the persistence of antigen at the injection site. However, more recent studies involving radio-labeled antigens suggest that Alum does not establish a depot at the injection site.[7] Recent work has indicated that Alum upregulates co-stimulatory signals on human monocytes and promotes the release of IL-4.[8] Alum adsorption may also contribute to a reduction in toxicity for some vaccines, due to the adsorption of contaminating LPS.[9] A key issue in adjuvant development is toxicity, since safety concerns have restricted the development of adjuvants ever since Alum was first introduced more than 50 years ago.[10] Many experimental adjuvants have advanced to clinical trials and some have demonstrated high potency, but most have proven too toxic for routine clinical use. For standard prophylactic immunization in healthy individuals, only adjuvants that induce minimal adverse effects will prove acceptable. Additional practical issues that are important for adjuvant development include biodegradability, stability, ease of manufacture, cost and applicability to a wide range of vaccines.

Adjuvants can be used to improve the immune response to vaccine antigens in several different ways, including (1) increasing the immunogenicity of weak antigens, (2) enhancing the speed and duration of the immune response, (3) modulating antibody avidity, specificity, isotype or subclass distribution, (4) stimulating CTL, (5) promoting the induction of mucosal immunity, (6) enhancing immune responses in immunologically immature, or senescent individuals, (7) decreasing the dose of antigen in the vaccine to reduce costs, or (8) helping to overcome antigen competition in combination vaccines.

The mechanisms of most adjuvants still remains poorly understood, since immunization often activates a complex cascade of responses, and the principle mechanism of the adjuvant is often difficult to discern clearly. However, if one accepts the geographical concept of immune reactivity, in which antigens that do not reach the local lymph nodes do not induce responses,[11] it becomes easier to propose mechanistic interpretations for some adjuvants, particularly those based on a "delivery" mechanism such as nanoparticles and microparticles. If antigens which do not reach lymph nodes do not induce responses, then any adjuvant which enhances delivery of antigen into the cells that traffic to the lymph node may enhance the response. A subset of dendritic cells (DC) are thought to be the key

cells which circulate in peripheral tissues and act as "sentinels", being responsible for the uptake of antigens and their transfer to lymph nodes, where they are then presented to T cells. Circulating immature DC are efficient for antigen uptake, while mature DC are efficient at antigen presentation to T cells. Hence, promoting antigen uptake into DC, trafficking to lymph nodes and DC maturation are thought to be the key components to the generation of potent immune responses. DC are thought to be the most effective antigen presenting cells (APC), although macrophages can also function in this role.

It can be argued that the role of adjuvants for recombinant vaccines is to ensure that the vaccine resembles infection closely enough to initiate a potent immune response.[12] In addition, the innate immune system directs the balance of humoral and CMI,[13] and adjuvants can control the type of acquired immune response induced.[14] Adjuvants can be divided into different broad groups based on their principal modes of action, depending on whether or not they have direct immunostimulatory effects on APC, or whether they function as antigen delivery systems. Particulate adjuvants (e.g. emulsions, microparticles, iscoms, liposomes, virosomes and virus-like particles) have comparable dimensions to the pathogens which the immune system evolved to combat. Immunostimulatory adjuvants may also be included in particulate delivery systems to enhance the level of response, or to focus the response through a desired pathway, e.g. Th1. In addition, formulating potent immunostimulatory adjuvants into delivery systems through restricting the systemic circulation of the adjuvant may limit adverse events. Nanoparticle and microparticle adjuvants generally act as delivery systems, although the materials they are made of may also have some adjuvant effect.

In the studies from 1976 onwards, Kreuter *et al.*[15] described the use of polymeric nanoparticles (50 nm to 300 nm in size) as adjuvants for adsorbed and entrapped vaccines. However, the polymethyl methacrylates used in these studies are degraded *in vivo* only very slowly. Faster degrading particles prepared with the more biocompatible poly-lactic acid (PLA) and poly (lactide-co-glycolide) (PLG) polymers have subsequently been extensively investigated as adjuvants. Size was shown to be an important parameter affecting the immunogenicity of microparticles, since smaller particles ($< 10\,\mu$m) were significantly more immunogenic than larger ones.[16,17] With PLG particles of size 1–10 $\mu$m (mean of 3.5 $\mu$m) compared with 10–110 $\mu$m (mean of 54.5 $\mu$m) with entrapped staphylococcal enterotoxin B, the serum IgG response was much higher with the smaller particles.[16] With a model antigen entrapped in PLA particles, there was an increased antibody response with particles $< 5\,\mu$m compared with particles with mean sizes larger than 5 $\mu$m.[18] The effect of particle size on immunogenicity is likely to be a consequence of enhanced uptake into lymphatics and greater uptake into antigen presenting cells for the

smaller sized particles, since only microspheres $< 5\,\mu$m were transported to the spleen.[19] The advantages of particles with a mean size of less than 5 microns for optimal immune responses has been demonstrated on a number of occasions, but the data supporting the use of nanoparticles is less convincing. In addition to the inherent immunogenicity of nano- versus microparticles, carrier capacity and the efficiency of antigen entrapment in different formulations also needs careful consideration.

The dividing line between nanoparticles and microparticles is ill defined, with some sources considering 1000 nm particles to be nanoparticles,[20] while the United States patent office has the class definition for nanotechnology using the scale 1–100 nm or slightly larger. In this chapter, the evidence supporting the advantages of nanoparticles versus microparticles will be critically assessed. Previous reviews have focused on alternative delivery routes, including nasal[21] and oral immunization.[22] In addition, the use of nanoparticles for DNA vaccines,[23] the stability of vaccines following microencapsulation,[24,25] the cellular uptake of nanoparticles and microparticles,[26] and the overall role of adjuvants in the immune response[27,28] has also been reviewed. Although a wide variety of microparticles and nanoparticles have been developed as possible vaccine adjuvants, we will focus primarily on systems where *in vivo* immune responses have been reported following systemic immunization, although mucosal immunization will be briefly covered.

## 2. Nanoparticle and Microparticle Preparation Methods

There are many biodegradable or biologically compatible polymers that have been used for the preparation of nanoparticles and microparticles. Different methods have been used for particle formation and these methods have optimal size ranges as well as suitability for use with different polymeric agents. The preparation methods used, the typical size ranges, polymeric materials and antigens that have been evaluated are summarized in Table 1.

### 2.1. *Nanoparticles and microparticles made from polyesters*

Nanoparticles and microparticles made from poly (lactide-co-glycolide) (PLG) and poly (lactide) (PLA) and their derivatives have been widely investigated for vaccine delivery. Using preformed polymers, particles with entrapped antigens can be prepared from a variety of emulsification evaporation methods. These methods are based on the formation of a multiple emulsion (water in oil in water emulsion) from which the oil phase, an organic solvent used to dissolve the polymer, is evaporated, resulting in the preparation of an aqueous suspension of particles. This technique

**Table 1** Summary of various particle preparation methods using different polymers and mixing conditions.

| Material | Method | Size Range | Antigen Type | Comments | References |
|---|---|---|---|---|---|
| PLG or PLA | emulsification evaporation | 200 nm–10 $\mu$m | DNA, protein, small molecules | smaller particles with increased surfactant concentration | [29, 30, 35, 36] |
| PLG or PLA | solvent displacement | 80 nm–500 nm | protein, small molecules | smaller particles with lower polymer concentrations | [38–41] |
| poly(cyanoacrylate) | chain polymerization | 90 nm–800 nm | protein | concerns about residual monomers | [46, 47] |
| subramolecular biovectors | crosslinking | 60 nm | protein | | [48, 49] |
| chitosan | sodium sulfate precipitation | 300 nm–1 $\mu$m | protein | | [52] |
| chitosan-DNA | complexation | 20 nm–500 nm | DNA | complex forms spontanously with correct ratio | [54] |
| carbon nanotubes | modification for covalent attachment | nano | protein, small molecules | undetermined biological compatibility | [55] |
| calcium phosphate | various | 100 nm–1.2 $\mu$m | DNA, protein | | [57, 58] |

generally has a lower limit for the particle preparation of about 0.5 $\mu$m (500 nm), although 200 nm particles can be created with a low PLG concentration and an increased surfactant concentration.[29] PLG particles, with encapsulated model proteins having diameters as small as 320 nm, can also be formed by sonicating the emulsions.[30] Various alternative approaches have also been described based on emulsions, including spray drying[31] and phase separation.[32,33] However, one of the drawbacks of microencapsulation of antigens is the instability of the antigen due to the exposure to solvents and the high shear force during microparticle preparation.[25,34] An alternate approach to encapsulation is to adsorb the antigen onto the microparticles after the particle has been formed, avoiding the exposure of the antigen to solvents and high shear.[35–37]

The solvent displacement method (also sometimes referred to as interfacial deposition) was first described by Fessi *et al.*[38,39] and allows the preparation of nanoparticles from preformed polymers. A water-miscible solvent (i.e. acetone) is used to dissolve the polymer with magnetic stirring, which is then added to an aqueous solution. The nanoparticles are formed by diffusion and the solvent is eliminated by evaporation. Depending on the solvent, polymer type, polymer concentration and addition of emulsifiers, these particles can range in size from 80 nm to 500 nm or larger.[40,41] The antigens for encapsulation need to be water soluble and compatible with the water-miscible solvent. However, the encapsulation efficiency is often low for water-soluble molecules.[42] Nanocapsules, with an internal oil core, can be formed when a small volume of oil is introduced into the organic phase, and these can be used to dissolve less water-soluble antigens, and to increase encapsulation efficiency.[38] However, one limitation of this approach is the preparation of low particle concentrations, due to the dilute initial polymer concentrations necessary in producing 100 nm particles. In addition to PLG, other polymers used to prepare nanoparticles include poly-$\epsilon$-caprolactone (PCL)[43,44] and sulfobutylated poly(vinyl alcohol)-g-PLG.[36,45]

Chain polymerization of modified cyanoacrylate monomers has also been used to make particles with a diameter of 100 nm,[46] and with an additional polysaccharide coating, particles ranging in size from 93 to 800 nm can be produced.[47] Polymerization preparation of nanoparticles has also been used for methyl methacrylate polymers.[15] However, with the polymerization approach to nanoparticles preparation, there are concerns about the levels of residual monomers in the final formulation. Supramolecular Biovectors ($^{TM}$SMBV) are positively charged particles with a polysaccharide core surrounded by a phospholipidic layer, with a mean size of 60 nm.[48,49] They are made from maltodextrins that were crosslinked with 2,3-epoxychloropropane and branched with glycidyl trimethylammonium to form a gel, which was then homogenized to give the nanoparticles, with the lipids added for the layer outside. Antigens were then adsorbed to the nanoparticles.

### 2.2. *Nanoparticles and microparticles made with chitosan*

Chitosan is a natural biodegradable polymer of glucosamine and N-acetylglucosamine. It is made from the partial de-acetylation of chitin which is found in the shells of crustaceans.[50] It has been shown to be an effective adjuvant for the intranasal delivery of vaccines, enhancing T-cell response and antibody levels when used as a soluble polymer.[51] Chitosan is cationic and readily binds negatively charged materials, including DNA and the sialic acid found on cell surfaces.

Chitosan and chitosan coated particles can be made using several methods. Using a chitosan with a low molecular weight, 700 nm chitosan particles can be formed by sodium sulfate precipitation. With sonication, the particles can be reduced in size to 300 nm.[52] Chitosan coated poly-$\epsilon$-caprolactone particles were made using the solvent displacement method described above, with sizes ranging from 230–500 nm.[44] With an emulsification method, chitosan was dissolved in the external phase to form 500 nm PLG particles with a chitosan coating.[53]

Nanoparticles in the range of 20 nm to 500 nm can be formed spontaneously upon mixing of chitosan with DNA. The zeta potential (surface charge) can vary from negative to positive, depending on the ratio of DNA to chitosan, although some particles were unstable and showed aggregation.[54]

### 2.3. *Other nanoparticles and microparticles*

Functionalized carbon nanotubes have been investigated as particles for vaccine delivery. Through organic modification, multiple sites for covalent attachment can be made available for small molecules, sugars, peptides or proteins.[55] The biological compatibility of carbon nanotubes is not certain yet, since they are not biodegradable. Another nanoparticle preparation method uses emulsifying wax of cetyl alcohol and polysorbate in combination with SDS to create microemulsions, which are then cooled to form particles ranging in size for 90 to 425 nm, depending on the SDS concentration.[56] Calcium phosphate particles have been studied for vaccine delivery, but are usually $> 10\,\mu$m in size. However, calcium phosphate nanoparticles have also been studied, although the reported mean size was $< 1.2\,\mu$m, and these formulations were called "nanoparticles".[57] Alternative calcium phosphate particles of mean size 100–120 nm have been used to encapsulate plasmid DNA for gene therapy application.[58]

## 3. Adjuvant Effect of Nanoparticles and Microparticles

The adjuvant effect achieved as a consequence of the association of antigens with particles has been known for many years.[15] The enhanced immunogenicity of particulate antigens is unsurprising, since pathogens are particulates of similar

dimensions and the immune system has evolved to deal with these.[59] Particulate delivery systems present multiple copies of antigens to the immune system and promote trapping and retention of antigens in the local lymph nodes. Moreover, particles are taken up by macrophages and dendritic cells, leading to enhanced antigen presentation and the release of cytokines, so as to promote the induction of an immune response. Antigen uptake by APC is enhanced by the association of antigen with polymeric particles, or by the use of polymers or proteins which self-assemble into particles. A particularly attractive feature of particles is their ability to control the rate of release of entrapped antigens. Many alternative antigen delivery systems that are available are particulates, including liposomes, ISCOM's, micelles and emulsions.[59,60]

Aluminum adjuvants have several limitations which has encouraged the search for alternative approaches. Aluminium adjuvants are not effective for all antigens, induce some local reactions, induce IgE antibody responses and generally fail to induce cell-mediated immunity, particularly cytotoxic T-cell responses. In the early studies, microparticles with entrapped protein[61,62] and peptide[63] antigens were shown to induce cytotoxic T lymphocyte (CTL) responses in mice following systemic[61] and mucosal immunization.[62] Microparticles also induced a delayed-type hypersensitivity (DTH) response,[62] which is thought to be mediated by Th1 cells, and potent T cell proliferative responses. The limited data available on the induction of cytokine responses in cells from animals immunized with microencapsulated antigens indicates that microparticles preferentially induce a Th1 type response.[61,64] Hence, microparticles may possess some inherent advantages over the more established Alum based adjuvants. Macrophages have been reported to be responsible for phagocytosis and the presentation of particulate antigens through the cytosolic MHC class I restricted pathway.[65] However, dendritic cells are also likely to play an important role in the presentation of particulate antigens and the release of cytokines to promote a Th1 type response.[66]

The effect of particle size on antibody induction and cell mediated immunity has been investigated, and it has been concluded that 1 $\mu$m particles are generally better than larger ones. However, the data supporting the benefit of nanoparticles over microparticles is considerably less convincing.

## 3.1. *Nanoparticles and microparticles as mucosal adjuvants*

Mucosal administration of vaccines offers a number of advantages over the traditional approach to vaccine delivery, which normally involves systemic injection using a needle and syringe. Mucosal delivery would eliminate the possibility of infections caused by inadequately sterilized needles or the re-use of needles. Also, mucosal vaccines might result in the induction of mucosal immunity at the sites

where many pathogens initially infect hosts. Mucosal delivery most commonly involves oral and intranasal (i.n.) immunization, although alternate routes are also available. The potency of particles for mucosal delivery is generally dependent on their ability to be taken up across the mucosal epithelium. In many studies, the uptake of particles by the mucosal associated lymphoid tissues (MALTs) of the Peyer's patches in the gut and the MALT of the respiratory tract have been demonstrated, albeit a very inefficient process. Moreover, there is good evidence that the composition of the particles impacts efficiency of uptake, including evidence that the binding of PLG microparticles to M cells of the Peyer's patches is less efficient than the binding of latex particles.[67,68]

A number of alternative approaches have been evaluated for the mucosal delivery of vaccines using particulate carriers of various mean sizes. Chitosan particles of 300–350 nm with associated tetanus toxoid (TT) were administered i.n. in mice, and induced significantly higher serum IgG responses compared with free antigen.[69] In addition, sulfobutylated poly (vinyl alcohol)-g-PLG particles of mean size 100 nm with adsorbed TT were administered orally and i.n., as well as enhanced serum IgA (and IgG for the i.n.) antibodies, compared with soluble controls.[36] SMBV nanoparticles with Hepatitis B surface antigen administered i.n. induced cytotoxic T lymphocyte (CTL) responses and higher serum IgG antibody responses than soluble protein.[70] Calcium phosphate particles (size $< 1.2\,\mu$m) were used for the mucosal delivery of a herpes simplex virus type 2 antigen and they induced greater mucosal IgA and IgG, and systemic IgG responses, compared with soluble antigen.[57] However, the calcium phosphate particles were sized before the final protein coating, so it is unclear what size the particles were when they were actually administered. Overall, although these various observations support the contention that particulate antigens are better than soluble antigens for mucosal delivery, all approaches appear to fall short of any likelihood of commercial development. Moreover, the rationale for preparing nanoparticles rather than the more established microparticles is not necessarily clear.

Data from studies evaluating the effect of particle size on the induction of mucosal immunity, following mucosal administration of vaccines, has offered conflicting outcomes, depending upon the specific polymeric system evaluated (Table 2). In one study, with the model antigen ovalbumin (OVA) adsorbed to chitosan particles of varying sizes (400 nm, 1 $\mu$m, 3 $\mu$m) for i.n. administration, higher IgA responses were seen with 400 nm and 1 $\mu$m particles, compared with the 3 $\mu$m particles. However, there was no difference in the response with the 400 nm and the 1 $\mu$m sized particles.[52] The antigen adsorption efficiency was similar for all particles, although no information is reported on the antigen release profile. The conclusion that smaller nanoparticles were more immunogenic than larger microparticles was not confirmed by the paper.

**Table 2** Summary of various particles with different sizes showing the mucosal adjuvant effect.

| Particle Material | Antigen | Sizes | Route | Result | Reference |
|---|---|---|---|---|---|
| chitosan | model protein (ovalbumin) | 400 nm, 1 $\mu$m, 3 $\mu$m | i.n. | comparable IgA for smaller sizes, both greater than control/3 $\mu$m | [52] |
| PLG | model protein (BSA) | 200 nm, 500 nm, 1000 nm | i.n. | IgG responses of 1000 nm > 500 nm > 200 nm | [71] |
| PLG | model protein (BSA) | 200 nm, 500 nm, 1000 nm | oral | IgG responses of 1000 nm > 500 nm ~ 200 nm | [71] |
| PEG-PLA | protein (TT) | 200 nm, 1.5 $\mu$m | i.n. | comparable IgG and IgA responses for both sizes | [72] |
| sulfobutylated pva-g-plg | protein (TT) | 100 nm, 500 nm, 1500 nm | oral | IgG and IgA responses of 100 nm > 500 nm, none for 1500 nm | [36] |
| sulfobutylated pva-g-plg | protein (TT) | 100 nm, 500 nm, 1500 nm | i.n. | comparable IgG/IgA response of 100 nm, 500 nm, none for 1500 nm | [36] |
| chitosan | protein (TT) | 350 nm | i.n. | higher IgG and IgA compared to free antigen | [69] |
| SMBV | protein (HBsAg) | 60 nm | i.n. | high CTL and IgG compared to free protein | [70] |
| calcium phosphate | protein (HSV-2) | <1.2 $\mu$m | mucosal | higher IgG and IgA compared to free antigen | [57] |

It is also known that particle charge is important for particles to be transferred to the APCs and the uptake by the MALT.[68] This would suggest that positively charged chitosan particles may behave differently than negatively charged particles. Therefore, similar responses with micron and sub-micron particles may not apply to all particle types. Chitosan particles are biodegradable and may have an inherent immunostimulatory effect which pther polyesters (PLGA) lack. Thus, Chitosan may be a promising candidate for a particulate system of size 1 micron, although there is no justification for chitosan nanoparticles at this moment.

Another study evaluated the effect of particle size, using a specialized method of size 100 nm with adsorbed TT administered orally and i.n. This nanoparticle

formulation induced increased serum IgA and IgG antibody responses in comparison with soluble antigen control.[36] Kamm *et al.*[36] also examined 100, 500 and 1500 nm particles. Following oral administration, the highest serum IgG and IgA responses were found with 100 nm particles and the responses were progressively lower for the 500 nm and 1500 nm nanoparticles. Following nasal administration, the 100 nm and 500 nm particles induced comparable IgG and IgA responses, which were higher than the responses with 1500 nm particles. The authors speculated that the different size dependence observed for the different routes is due to the different translocation mechanisms in the NALT, as compared with the GALT. The overall observations were that sub-micron nanoparticles were more immunogenic than larger particles. The SBPVA-PLG may be preferred to PLA for its faster degradation rate, however, the grafting chemistry required, renders it much less suitable for commercial development.

In contrast to the studies described above, it has also been claimed in some studies that 1 $\mu$m particles are more effective than nanoparticles. With BSA encapsulated into biodegradable "nanospheres" administered i.n. and orally, 1000 nm PLG particles elicited higher serum IgG responses than 200 nm and 500 nm particles (71). For i.n. administration, the 500 nm particles also induced higher serum IgG responses than the 200 nm particles. The IgG2a/IgG1 ratios with the different particle sizes were similar and higher than antigen alone and that with alum.[71] From *in vitro* release studies, it was found that the 1000 nm particles did have a different release profile from the 200 nm and 500 nm particles, which may account for some of the differences seen *in vivo*. For the oral studies, the authors hypothesized that 200 nm and 500 nm particles are more readily absorbed through the intestinal wall than 1000 nm particles, absorbed almost exclusively by Peyer's patch cells, leading to higher immune responses for the larger particles. The two studies comparing particle size,[36,71] reached differing conclusions, but it is unclear whether this was due to the differences in polymers used for the studies, the different antigens used, or that of different formulations.

Particle type as well as particle size can also have a strong influence on immune responses. Using 200 nm PEG-PLA particles, 200 nm PLA particles and 1.5 $\mu$m PEG-PLA particles with encapsulated TT administered i.n., it was found that the 200 nm and 1.5 $\mu$m PEG-PLA induced higher IgG and IgA antibody responses, compared with the PLA particles.[72] Although the two particle sizes were comparable, but the PEG-PLA particles performed better than PLA particles of the same size. The PEG-PLA particles are less hydrophobic than the PLA particles. This may explain some of the differences as particle uptake is strongly influenced by the hydrophobicity of the polymer. The authors also hypothesized that the difference between the particle types is related to the propensity of the PLA nanoparticles to aggregate *in vitro*, indicative of the relative stabilities of the particles in the mucosal fluids. Although

these particles are interesting, the PEG-PLA polymer is not commercialy available, thereby hindering development. The material of the particle system is an important factor to consider when comparing particles.

## 3.2. *Nanoparticles and microparticles as systemic adjuvants*

The use of nanoparticles as systemic adjuvants is summarized in Table 3. The advantage of particulates for vaccine delivery, compared with soluble antigens or alum, has been investigated in a number of studies and has been extensively evaluated by several groups (2,24). In one study, TT and CpG (a known immunostimulatory oligonucleotide) were co-encapsulated within PLG nanospheres of mean size 290–310 nm and were evaluated in mice. This formulation resulted in the induction of an enhanced antigen specific T-cell proliferative response, in comparison with the soluble antigen plus CpG.[73] The co-delivery of TT and CpG within PLG nanoparticles induced very strong serum IgG response. This is another instance of nanoparticles out performing soluble antigen. However, the rationale for preparing nanoparticles rather than the more established microparticles is not necessarily clear.

**Table 3** Summary of various particles with different sizes showing the systemic adjuvant effect of these formulations.

| Particle Material | Antigen | Sizes | Route | Result | Reference |
|---|---|---|---|---|---|
| chitosan | model protein (ovalbumin) | 400 nm, 1 $\mu$m | i.p. | comparable IgG for both sizes and greater than control | [52] |
| PLG | model protein (BSA) | 200 nm, 500 nm, 1000 nm | s.c. | IgG responses of 1000 nm > 500 nm ~ 200 nm | [71] |
| PLA | protein (TT) | 630 nm, 4 $\mu$m | i.m. | comparable titers, both sizes less effective than alum | [76] |
| carbon nanotubes | peptide (fmdv) | diameter ~ 15–60 nm length ~ 500 nm | i.p. | more neutralizing antibodies compared to free antigen | [55] |
| PLG | protein (TT) + CpG | 290–310 nm | s.c. | higher IgG, Th1type compared to free antigen; enhanced T-cell proliferation | [73] |

Similarly, in another study, using an alternate carbon polymer, a non-biodegradable, inorganic nano-scale particle was investigated. Carbon nanotubes with covalently linked peptides from a foot-and-mouth disease virus were administered to mice i.p. and the carbon nanotubes elicited high levels of virus-neutralizing antibodies, compared with the free peptide control.[55] Carbon nanotubes introduce many problems. They are not biodegradable and may be toxic; hence they are not a good choice for vaccine formulation.[74,75]

Data from studies evaluating the effect of particle size on the induction of systemic immunity have offered conflicting outcomes. A single dose of PLA particles with TT encapsulated (4 $\mu$m and 630 nm) induced lower anti-TT titers than two doses of alum. There were no significant difference between the larger and smaller particles.[76] The lower response with the particle formulation may be due to the limitation of having only one dose in particles, as compared with two doses on the alum. The lower response with the PLA formulation may also be due to the encapsulation process compromising of antigen stability.[34,77]

The effect of particle size using BSA as an antigen was also evaluated by Gutierro *et al.*[71] The model antigen BSA was encapsulated into PLG particles of varying sizes (i.e. 1000 nm, 200 nm and 500 nm) and administered s.c. This elicited higher serum IgG with the 1000 nm, compared with the smaller particles.[71] The *in vitro* release profile was different for 1000 nm PLG particles which may account for the differences in responses. In another similar study, no size dependent effect was found with a model antigen OVA adsorbed to chitosan particles of varying sizes 400 nm, 1 $\mu$m. Higher serum IgG responses were seen with the particles compared with the soluble antigen, but there was no difference between the two sizes.[52]

Both studies described above used model antigens and the size conclusions may not be applicable beyond model antigens. Also, for some vaccines, antibody response may not directly correlate with protective efficacy of the vaccine. One major difference between these two studies is the net surface charge of the particles. It has been shown *in vitro* that positively charged particles (poly-L-lysine coated polystyrene) induced higher phagocytosis in dendritic cells. Surface charge as well as particle size might influence uptake by macrophages and dendritic cells.[78] Therefore, the difference in size dependent behavior observed may also be more prominent involving particles of a specific charge.

Recent studies have also shown that nanoparticles may exert an adjuvant effect for the induction of cell mediated immunity compared with soluble antigen.[70,79] Particle size may be an important parameter influencing the efficacy of microparticles as adjuvants for CTL induction. Nixon *et al.*[63] showed that microparticles $< 500$ nm induce better CTL responses than microparticles $> 2\,\mu$m.[63] In another study, a non-degradable polystyrene nanoparticle with OVA antigen covalently bound was administered i.d. in mice. It was seen that 40 nm particles induced

highest T-cell responses in comparison with the 2 $\mu$m size.[80] However, in this paper, the initial particle size is reported, but the size after covalent attachment of the antigen is not reported. Therefore, the conclusion cannot be confirmed by the findings reported.

## 4. Delivery of DNA Using Nanoparticles and Microparticles

The previous sections were mainly focused on the delivery of proteins or peptides, but DNA nanoparticles and microparticles have also been used. Nanoparticle studies with DNA as adjuvants are summarized in Table 4. DNA plasmids are weakly immunogenic and particles may help boost the immune response. Cationic PLG particles with adsorbed CTAB of sizes 300 nm, 1 $\mu$m, and 30 $\mu$m were used to adsorb an HIV-1 DNA plasmid and delivered i.m. The 300 nm and 1 $\mu$m particles induced, significantly enhanced IgG titers, compared with naked DNA and the 30 $\mu$m particles. The 1 $\mu$m particles were capable of inducing potent CTL responses, whereas naked DNA failed to induce CTL activity.[81] It appeared in this study that the 300 nm particles were better than the 1 $\mu$m particles. An additional study with more animals confirmed that the 1 $\mu$m and 300 nm PLG particles with adsorbed plasmid DNA, induced comparable IgG serum titers (Fig. 1). There was no advantage for the smaller sized particles. It is believed that the efficient delivery of DNA to APCs is

**Table 4** Various particle based delivery systems for DNA showing the mucosal systemic adjuvant effect of these formulations.

| Particle Material | Antigen | Sizes | Route | Result | Reference |
|---|---|---|---|---|---|
| PLG (CTAB) | DNA (HIV-1) | 300 nm, 1 $\mu$m, 3 $\mu$m | i.m. | higher IgG for 300 nm, 1 $\mu$m compared to free DNA | [81] |
| chitosan | DNA (peanut allergen) | 150–300 nm | oral | higher IgA and IgG2a response compared to free DNA | [82] |
| chitosan | DNA (tuberculosis) | 376 nm | pulmonary | increased IFN-$\gamma$ compared to DNA alone of i.m. Administration | [83] |
| polylysine-g-imidazoleatic acid | DNA (HIV-2 env) | 140 nm | i.d. | higher IgM, IgG, IgA compared to free antigen | [84] |

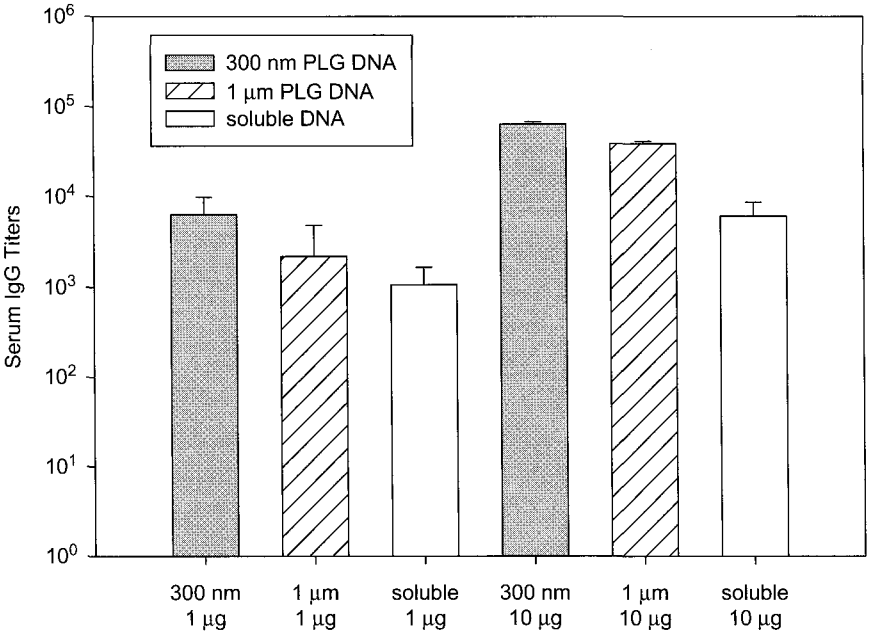


**Fig. 1.** Serum IgG titers in groups ($n = 20$ for particles, $n = 10$ for soluble) of BALB/c mice immunized with either PLG-CTAB-p55 gag DNA of size 300 nm or 1 $\mu$m or DNA alone at two dose levels of 10 $\mu$g and 1 $\mu$g. Antibody titers are geometric mean titers $\pm$ SE at 2 weeks post-second immunization (week 6) time point after immunizations at day 0 and day 28. The response from the 300 nm and 1 $\mu$m particles at both dose levels are not significantly different.

an important component of the adjuvant effect, since larger microparticles $> 30$ $\mu$m did not elicit a strong immune response.

The association of DNA with nanoparticles and microparticles has been shown to be more effective than naked DNA. Chitosan-DNA particles (150–300 nm) of peanut allergen gene delivered orally, produced secretory IgA and serum IgG2a, compared with no detectable response with naked DNA.[82] The pulmonary delivery of chitosan-DNA particles (average size of 376 nm) with plasmid DNA encoding T-cell epitopes from mycobacterium tuberculosis, were able to induce the maturation of dendritic cells and the increased level of IFN-$\gamma$ secretion, compared with the plasmid in solution or i.m. delivery.[83] In another DNA study, polylysine-graft imidazoleacetic acid complexed with DNA with a diameter of 140 nm was used for the HIV env plasmid.[84] It was found that IgG, IgM and IgA responses were increased several folds, compared with naked DNA. It was also speculated that this formulation may also help protect the DNA from nuclease degradation.[84] Based

on the evidence in the literature, the rationale for preparing nanoparticles rather than the more established microparticles is not necessarily clear with the DNA formulations.

## 5. Conclusions

A number of systems with different types of antigen (proteins, peptides, DNA) have been investigated with the particles ranging in size from 50 nm to 1000 nm. For most systems, the critical particle size is $< 5\,\mu$m, with particles in the range of 100 nm to 1 $\mu$m, often inducing comparable immune responses. In some cases, depending on the route of delivery, there may be increased immune responses with the smaller nanoparticles. For i.n. administration, there was no evidence that 100 nm particles were better than micron particles. For oral administration, some studies found enhanced responses with nanoparticles, compared with microparticles, while other studies found equivalence, or that microparticles elicited higher responses. Overall, the evidence for nanoparticles ($\sim 100$ nm) outperforming microparticles (1–2 $\mu$m) for enhanced immunogenicity is weak. Further examination is needed to support nanoparticles as a better formulation in place of microparticles. Also, some of the studies carried out were done model antigens and the same results may/may not apply to relevant antigens where vaccine efficacy is determined. However, there are some advantages to nanoparticles compared with microparticles that have not been directly addressed. For instance, smaller nanoparticles (sub-200 nm) can be sterile filtered, allowing the particle preparation to be a non-sterile process with terminal sterilization.

The distinction between nanoparticles and microparticles is usually made by the authors and there is no consistency in what constitutes a nanoparticle, and this needs careful consideration when comparing results from the literature. The important size measurement point is immediately prior to administration, post-lyophilization, or other processing, and this is not always reported. More relevant endpoints such as protective efficacy may be crucial in distinguishing between nanoparticles and microparticles. Nanoparticles and microparticles both constitute a very effective vaccine delivery system. Some of these formulations are currently in pre-clinical and clinical evaluations.

## Acknowledgments

We would like to acknowledge the contributions of our colleagues in Chiron Corporation to the ideas contained in the chapter, particularly, all the members of the Vaccine Adjuvants and Delivery Group.

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# 29

# Pharmaceutical Nanocarriers in Treatment and Imaging of Infection

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Nanoscaled carrier systems can be used in the treatment and imaging of infectious diseases. To optimize accumulation of the carrier at the site of infection, the characteristics of the nanocarriers should complement the pathophysiological processes that play a role during infection.

As carriers are recognized as foreign materials, they can be employed for targeting the mononuclear phagocyte system (MPS). The MPS consists of cells that are specialized in the clearance of the body of foreign particles. As such, these cells are involved in the clearance of microorganisms and they form an important replication site for infectious organisms to survive intracellularly.

If carriers can avoid recognition by the MPS, it allows them to take advantage of the enhanced capillary permeability that is one of the hallmarks of the inflammatory reaction coinciding with infection. Together with the local efflux of plasma, nanoscaled carriers can enter the inflamed area through convective forces.

Finally, the carrier can be locally administered to interact specifically with tissues, cells or microorganisms that are present at the site of infection.

## 1. Introduction

Pharmaceutical nanocarrier systems include liposomes, nanoparticles, micelles and emulsions, and they can be used in the treatment and imaging of infectious diseases.[1–3] These systems are used to improve the degree of localization or the

persistence of encapsulated antimicrobial drugs or imaging molecules at the site of infection by altering the molecules' pharmacokinetics and tissue distribution profiles. To optimize accumulation of the carrier at the site of infection, factors on the side of the carrier, as well as on the side of the infected host should be taken into account. Ideally, the characteristics of the nanocarriers should be tailored to complement the pathophysiological processes that play a role during infection.[4–6]

Three strategies to target sites of infection have been employed: (1) the use of carriers that are recognized as foreign materials; (2) the use of carriers that avoid recognition as foreign materials, and (3) local delivery of carriers. These targeting strategies are discussed in the light of recent developments in delivery strategies and the introduction of new antimicrobial agents such as the nubiotics for bacterial infections and siRNA for viral infections.

## 2. Carriers that are Easily Recognized as Foreign Materials

Most carriers that are administered *in vivo* are almost always rapidly recognized as foreign materials. Proteins in biological fluids such as activated complement components, opsonize the carriers to facilitate recognition and uptake[7–9] by cells that constitute the MPS. These cells are continuously surveiling the body to detect and phagocytose these opsonized foreign particles. This avid recognition and uptake mechanism can be employed for targeting purposes. These cells are of interest since many infectious agents are also recognized as foreign materials and end up in the same type of cells. Microorganisms that are able to survive intracellularly may be difficult to reach for conventional antimicrobial treatment, due to the barriers posed by the cellular membranes.[10–13]

One of the first demonstrations of the value of nanoscale carriers for the delivery of drugs to the MPS was delivered by New and colleagues as early as 1978.[14] Liposome-encapsulated antimony was used in this study to treat experimental leishmaniasis, showing several orders of magnitude increase in the therapeutic index. Leishmaniasis is still an endemic disease in several parts of the world and also occurs as an opportunistic infection in immunocompromised patients. Nowadays, treatment of leishmaniasis is complicated by widespread resistance to antimony compounds leading to treatment failure and relapse. Lipid formulations of amphotericin B are currently employed in the clinic as possible option for the treatment of visceral and cutaneous leishmaniasis.[15–18]

The lipid formulation is used to facilitate drug accumulation in the macrophages. As such, it is preferred over free amphotericin B deoxycholate (Fungizone®). The nanoparticulate structures in this formulation are unstable in the blood stream, leading to higher toxicity especially in the kidneys.[16,19]

Oil-in water-emulsions of submicron sizes, also known as lipid nanospheres that have been loaded with piperine, have been described as a new formulation for the treatment of visceral leishmaniasis.[20] All nanosphere formulations applied were more effective than the free drug in reducing parasite counts in all organs investigated in a model of *Leishmania donovani* infection in Balb/c mice at a dose of 5 mg/kg. The most effective formulation also contained the cationic lipid stearylamine. Inclusion of charged lipids usually promotes phagocyte uptake of lipidic carrier systems, which probably explains the increased therapeutic effect.[21] Alternatively, stearylamine has been described as leishmanicidal agent.[22] Furthermore, it was shown that enzyme levels in blood (as a measure for liver toxicity) and creatinine, and urea levels in the blood (as a measure for renal toxicity) were not altered after administration of the drug in the nanosphere formulations, indicating that toxicity of these formulations in these organs is limited.

Recently, a liposomal pentavalent antimony formulation was described, targeted at the macrophage via the scavenger receptors A and B for the treatment of *Leishmania chagasi* amastigotes.[23] The natural affinity of these receptors for polyanions was used by the inclusion of the negatively charged phospholipid phosphatidylserine in the liposomal membrane. The negative charge mediates receptor-mediated endocytosis. Furthermore, the authors demonstrated that the scavenger receptor was upregulated upon infection, possibly through secretion of transforming growth factor-$\beta$1, which could preferentially increase uptake by infected macrophages. Nevertheless, infected macrophages ingested less liposomes than normal macrophages, possibly because of the high parasite burden per macrophage that could affect normal cell metabolism. Overall, the targeted drug showed a 16-fold higher efficacy, compared with free compound *in vitro*.

The same strategy with phosphatidylserine was used to target liposomes to macrophages infected with *Cryptococcus neoformans*. Incorporation of chloroquine in liposomes resulted in increased delivery to macrophages *in vitro* and *in vivo*. This promoted infected mouse survival and reduced parasite counts in liver and brain.[24]

Mannose was used as a recognition signal for macrophage uptake by the mannose receptor by Medda *et al.*[25] Phospholipid microspheres consisting of polylactic-co-glycolic acid and phosphatidyl ethanol amine were grafted with mannose and subcutaneously injected. In this system, dihydroindolo [2,3-a] indolizine, an antileishmanial agent, was incorporated. The drug was administered at a dose of 3 mg/kg. In *Leishmania donovani* infected hamsters, mannose-grafted microspheres suppressed parasite numbers in the spleen over 90%, whereas free drug only reduced numbers by 26%. Furthermore, it was shown that infection induced changes in the blood levels of hepatic enzymes, creatinine and urea. These changes could largely be prevented by the formulated drug.

Mannose-coated liposomes were also employed to deliver CpG-containing oligodeoxynucleotides to macrophages infected with *Leishmania donovani*.[26] These CpG-oligonucleotides activate macrophages through the presence of Cytidine phosphorothioate Guanosine islands that are strong activators of macrophages via Toll-like receptors, leading to interleukin-12 and interferon-$\gamma$ production. In mice, suffering from visceral leishmaniasis, mannose-coated liposomes containing CpG-oligonucleotides, were more effective in inhibiting amastigote growth than liposome-encapsulated CpG-oligonucleotides or free CpG-oligonucleotides. In the spleen, the mannose-coated liposomal formulation of the CpG-oligonucleotides completely eliminated the parasites, whereas both controls failed to achieve complete eradication.

A dual targeting approach combining tuftsin and nystatin in liposomes was reported by Khan *et al.*[27,28] In their studies, the effects of the liposomal antifungal agents on *Candida albicans* infection in mice was studied. The tuftsin-bearing liposomes were taken up by macrophages by binding to the tuftsin receptor which activated these cells. As these cells were loaded with liposomal nystatin, this may improve their killing of phagocytosed *C. albicans* organisms through the added action of the drug. Alternatively, the macrophages may function as a depot for subsequent prolonged release of the drug. In this study, combination of tuftsin and nystatin in the liposome formulation increased *C. albicans* clearance in liver, spleen, kidneys and lungs, and reduced the numbers of organisms in the blood stream, thereby promoting mice survival, compared with free agents or liposomal formulation of either tuftsin or nystatin alone.

In another study, delivery of immunomodulatory compounds to macrophages was demonstrated. The important role of macrophages in generating pro-inflammatory reactions had been the basis for targeting antisense molecules to these cells.[29] Antisense oligomers against tumor necrosis factor-alpha were incorporated into albumin microspheres. Endotoxin was administered i.v. or *Escherichia coli* was administered i.p. to rats. Animals received 100 $\mu$g antisense TNF and TNF-inhibition and rat survival was measured. Microencapsulated antisense improved survival in these endotoxin and *E. coli* peritonitis models, compared with free antisense. Importantly, delivery by albumin encapsulation prolonged the action of the antisense to 72 hrs.

In a similar approach, Sioud and Sorensen employed the process of RNA interference to silence TNF-production.[30] The process was based on cellular delivery of small interfering RNA (siRNA), small double-stranded RNA molecules that mediate degradation of complementary mRNA sequences. siRNA was administered at a dose of 50 to 100 $\mu$g and was complexed to liposomes that were based on the cationic lipid dioleoyl trimethylammonium propane. The complexes were injected i.p. and shown to prevent the induction of TNF production upon challenge by endotoxin.

The specificity of the inhibition was demonstrated by the fact that the induction of IL-6 expression remained unchanged.

## 3. Carriers that Avoid Recognition as Foreign Materials

The specific accumulation of carriers that are easily recognized as foreign materials by macrophage can offer great benefits when the infectious organism is exclusively localized in these cells. For micro-organisms that are primarily present in other tissues, this fast recognition can become a major obstacle to reach distant sites. Therefore, carriers have been developed to avoid recognition by the MPS, allowing these carrier systems to take advantage of the increased capillary permeability at the site of infection.[31–34] The increased capillary permeability as well as vascular leakage are accompanying inflammatory reactions together with infection. With the local efflux of plasma, plasma proteins and immune cells, nanoscaled carriers can enter the inflamed area through convective forces. This approach has been used in the treatment and imaging of infections.

In magnetic resonance based imaging, the natural tropism of $> 150$ nm iron oxide particles to be rapidly taken up by liver and spleen macrophages prevented imaging of peripheral infections. In the studies by Kaim *et al.*, the usage of ultra-small nanoparticles was explored.[35,36] The nanoparticles consisted of a core of iron oxide crystals that is coated with dextran. The mean particle size was 35 nm. As a result of this small particle size, the blood half-life in rats increased to approximately 2–3 hrs. The authors demonstrated successful imaging of soft tissue infections in rats using this system. They postulate that the mechanism responsible is the uptake by peripheral macrophages in the blood stream, followed by extravasation at the site of infection based on the absence of non-phagocytosed particles in the inflammatory milieu. However, the presence of non-ingested particles may be underestimated due to the cellular accumulation, making it difficult to quantify individual extracellular particles.

Long-circulating liposomes, which have a substantially prolonged circulation time compared with the iron oxide particles that were described above (a half-life of approximately 24 hrs in rats), have also been successfully employed to image a variety of infectious diseases. The use of long-circulating liposomes for imaging purposes has been reviewed recently.[37–39]

Recent advances include the detection of invasive pulmonary aspergillosis at an early stage of infection.[40] The causative fungus *Aspergillus fumigatus* is difficult to detect using conventional imaging agents, and in most cases, only at an advanced stage of the disease. Furthermore, interpretation of CT-scan images is difficult as the supposedly characteristic halo or air crescent-sign of the infection is not always clear. Imaging of intravenously injected $^{99m}$Tc-PEG- liposomes in a rat model of

left-sided invasive pulmonary aspergillosis, demonstrated that 82% of the scintigraphic images revealed the presence of the fungus already at 48-hr inoculation. Active infection was needed for the accumulation of labeled liposomes to occur, as inoculation with saline or killed *Aspergillus*-spores did not cause increased liposome accumulation in the inoculated lung.

The long circulatory half-life of PEG-liposomes results in a gradual increase in accumulation at the site of infection, which is in general considered an advantage as the scintigraphic image improves. Nevertheless, in specific situations when the infection is localized in the vicinity of the heart or large blood vessels, the background activity of the blood remains high for prolonged periods of time, thus hampering visualization. Laverman *et al.* demonstrated that biotin-coated $^{99m}$Tc-PEG-liposomes kept their long-circulating half-life.[41] However, upon injection of avidin, complexes were formed intravenously, resulting in a rapid clearance from the blood stream by MPS-organs. This clearance coincided with a loss of activity in the blood pool, allowing visualization of an experimental abscess in the neck region of the rabbits, previously undetected due to the activity in the heart region.

These long-circulating PEG-liposomes can also be loaded with antimicrobial agents to achieve site-specific drug delivery, as has been recently reviewed.[31,42,43] In our own laboratory, we have evaluated the factors on the side of the host, as well as on the side of the liposome that determine target localization. It appeared that the area under the blood concentration time-curve determined the degree of localization at the site of infection; whereas the level of vascular leakage was the most important determinant on the side of the host.[44–47] Optimized PEG-liposomes were loaded with gentamicin and therapeutic effects were studied in a rat *Klebsiella pneumoniae* model. Rat survival was strongly dependent on the gentamicin formulation. In immunocompetent rats, liposomal gentamicin was superior over the free drug, even though complete therapeutic efficacy could be achieved with multiple administrations of the free drug. In leukopenic rats, a combination of free and liposomal gentamicin showed best therapeutic effects, compared with either treatment alone. It is postulated that liposomal gentamicin produces low therapeutically active drug concentrations in the blood stream, which are insufficient to control the rapidly occurring bacteremia in the case of impaired host defense. However, liposomal gentamicin does localize in the infected lung and leads to local bacterial killing. Therefore, the combination of free drug (producing active antimicrobial levels in the circulation) and liposomal drug (leading to high drug levels in the lung) showed optimal results.[48]

PEG-liposomes were also loaded with a combination of antimicrobial agents that was predicted to interact synergistically based on *in vitro* data.[49] Whether synergistic drug action *in vivo* also occurs when the agents are administered in the free form is questionable, as their differing pharmacokinetic profile and tissue distribution do not correlate with the static drug concentrations present

in the *in vitro* assays. However, the use of a targeted drug carrier such as long-circulating PEG-liposomes, could enforce a parallel tissue distribution resulting in increased concentrations at the site of infection. Gentamicin and ceftazidime were co-encapsulated in long-circulating liposomes, as these agents were demonstrated *in vitro* to act synergistically on *K. pneumoniae*. These liposomes were tested for their ability to prolong the survival of rats infected in the left lung with a high gentamicin/ceftazidime-susceptible or a gentamicin/ceftazidime-resistant *K. pneumoniae*. The high susceptible *K. pneumoniae* could be effectively treated with single doses of lipsomal-gentamicin or liposomal-ceftazidime. Liposomal co-encapsulation of both agents allowed reductions in doses due to a synergistic therapeutic effect of these antibiotics. In the resistant *K. pneumoniae* model, the co-encapsulated agents again resulted in the synergistic activity of both antibiotics, allowing effective treatment of the otherwise lethal pneumonia. Interestingly, in both models, combinations of the two free drugs were merely additive.

O-stearylamylopectin was used as a recognition signal for the uptake of PEG-liposomes by lung macrophages.[50] The macrophages in this organ are an important replication site for *Mycobacterium tuberculosis* replication. In a murine model of *M. tuberculosis* infection, administration of liposome-encapsulated isoniazid and rifampicin reduced the number of bacteria in lungs, liver and spleen more efficiently than the free drugs. There was no significant reduction in bacterial numbers in mice treated with free drugs once weekly for 6 weeks, compared with untreated control mice. Liposomal treatment resulted in approximately 90% decrease in bacterial numbers. The enhanced efficacy of the liposome-encapsulated drugs is likely to enhance delivery to macrophages in liver, spleen, and lungs.

In another study focused on osteomyelitis, vancomycin and ciprofloxacin were encapsulated in liposomes.[51] As compared with neutral or anionic liposomes, cationic liposomes entrapped the highest percentage of drugs and showed highest antibacterial activity *in vitro*, probably due to the charge based cell interactions that can occur *in vitro*. The cationic liposomes were tested for therapeutic efficacy in a rabbit model of chronic staphylococcal osteomyelitis. Both free ciprofloxacin or vancomycin over a period of two weeks failed to achieve bone sterilization after i.v. injection. Combination of free ciprofloxacin and vancomycin was more effective only at the expense of renal dysfunction and severe diarrhea. Complete sterilization of the bone was seen in the group treated for two weeks with the combination of drugs in liposomal form, while nephrotoxicity and diarrhea were less frequent. Although the cationic liposomes showed preferable characteristics *in vitro* regarding drug encapsulation and antibacterial effect, it is likely that the use of neutral or PEG-coated liposomes would have improved results as the localization at the site of infection for cationic liposomes is expected to be limited due to the rapid clearance of charged liposome species.

Another application of long-circulating liposomes is that they can be employed as a sustained drug delivery system in the blood stream. When ciprofloxacin is encapsulated in the PEG-liposomes, there is a gradual release of antibiotic from the liposomes.[52,53] As such, the liposomal formulation displayed a prolonged presence in the blood and tissues of ciprofloxacin. As the encapsulated ciprofloxacin is shielded from the tissues by the liposomal membrane, the antibiotic could also be administered at relatively high doses. Interestingly, in the rat pneumonia model with high ciprofloxacin susceptible *K. pneumoniae*, 90% animal survival could not be achieved with the free drug at a once daily dosing schedule where the highest doses could be administered. In contrast, liposome-encapsulated ciprofloxacin injected once daily was still effective at this low frequency dose schedule.

To promote localization of amphotericin B at infectious foci, a number of formulations have been devised trying to achieve prolonged presence of amphotericin B in the blood stream and to shield the toxicity of the drug. Espuelas and colleagues used poly(epsilon-caprolactone) nanospheres coated with poloxamer 188 or mixed micelles with of this surfactant to deliver amphotericin B.[54] Both formulations had an approximately 10-fold lower activity *in vitro* against *C. albicans*; however, the activity towards infected macrophages was similar to that of Fungizone® that is 5-fold reduced. However, the reduced toxicity was paralleled by a 4-fold reduced efficacy. Similar observations have been noted in most studies concerning alternative formulations of amphotericin B, achievement of the reduction of the toxicity of the drug parallels a reduction in therapeutic efficacy.

A study by Fukui *et al.*, however, shows that these two phenomena can be uncoupled.[55] Lipid nanospheres were used to deliver amphotericin B, and this new formulation was compared with the commercially available lipid formulations of amphotericin B. Plasma amphotericin B-levels of Amphocil® or Abelcet® were low, reaching levels below 1 $\mu$g/ml within minutes after intravenous injection in rats at a dose of 1 mg/kg. Amphotericin B levels in the lipid nanosphere formulation were nearly two orders of magnitude higher and similar to those yielded by AmBisome®. Interestingly, in dogs, plasma amphotericin B concentrations after administration in the nanosphere formulation were approximately 3-fold higher than that of AmBisome®. In a rat model of local candidiasis, amphotericin B-containing nanospheres significantly inhibited the growth of *C. albicans*, whereas AmBisome® did not, even though local amphotericin B concentrations were similar. These results were similar as obtained *in vitro* where nanopshere-incorporated amphotericin B was as effective as Fungizone®, while AmBisome® activity was reduced.

Similarly, amphotericin B-containing nanoparticles were designed coated with heparin to achieve localization at the infected site, at the site of lesions.[56] The heparin-coated formulation did achieve 3-fold higher concentrations in the lungs of mice with pulmonary blastomycosis than Fungizone®. However, this could reflect a difference in pharmacokinetics rather than the specific binding at the target site. In

this mouse model, Fungizone® dosed at the maximum tolerated dose (1.2 mg/kg) failed to cure mice, whereas the nanoparticles dosed at 4.8 mg/kg achieved a 50% cure rate.

## 4. Local Application of Carriers

Nanoparticulate drug delivery systems may also be applied locally at the site of infection to increase drug delivery or to act as a local depot from which the drug is gradually released.

A new class of DNA or RNA-based antimicrobial agents (known as "nubiotics") was investigated by Dale *et al.*[57] The compounds are thought to act antimicrobially by virtue of their proton donor capacity. The hydrogen ions would then be responsible for the killing of the bacteria due to membrane depolarization. However, the exact mechanism of action remains yet to be confirmed. In a burn-wound infection caused by *Pseudomonas aeruginosa*-model, the therapeutic effects of neutral liposome-encapsulated nubiotics were investigated. It appeared that intravenously and subcutaneously injected liposome-encapsulated drug at a dose of 20 mg/kg was able to promote the survival of mice, whereas free nubiotics or PBS failed.

Nucleic acid based therapeutics were also employed to treat respiratory syncytial virus.[58] The nucleic acid was an siRNA that was intracellularly produced in the host by an encoding DNA-vector. The siRNA was directed towards the viral NS1 gene. Cell entry of this siRNA will prevent the protein from being synthesized, hence inhibiting viral replication. The DNA-vector was complexed to oligomeric nanometer-size chitosan particles and administered intranasally. siRNA against NS1 were shown to be produced in the lung tissues and protected against respiratory syncytial virus multiplication for at least 4 days. These studies show that this formulation may have prophylactic potential to prevent viral infection.

The results obtained, however, should be interpreted critically, as polyICLC and CpG oligonucleotides are able to mount antiviral responses that are not specific; and the phenomena of the resulting interferon production is also noted in the study using siRNA described above.[59,60] Prophylactic administration of liposome-encapsulated polyICLC completely protected mice against a lethal respiratory challenge of influenza A virus in mice, whereas all animals died in the control group.[61] The antiviral effect was shown to last up to 3 weeks after administration. The strong aspecific activity of liposome-loaded nucleic acids cautions against the straightforward attribution of therapeutic effects to specific mechanisms.

A series of studies used local application of nanoparticles to address *Helicobacter pylori* infection. One study employed mucoadhesive gliadin nanoparticles, containing amoxicillin for increased retention in the stomach, for the eradication of *H. pylori*.[62] Rhodamine labeled particles were administered to rats by gavage to test their gastric mucoadhesive properties. It was found that the mucoadhesive

characteristics of nanoparticles increased with increasing gliadin content. In Mongolian gerbils, the eradication of *H. pylori* was evaluated after oral administration of amoxicillin-loaded nanoparticles. Amoxicillin alone was also able to kill *H. pylori*, but the dose needed was higher than that of the nanoparticles, likely owing to the prolonged presence of the particles in the stomach.

Improvement of drug residence time was also the objective of a study using floating microspheres.[63] Polycarbonate based particles were tested for their floating capacity and they were loaded with acetaminohydroxamic acid. *In vivo* studies on *H. pylori*-infected Mongolian gerbils showed that both free drug and drug-loaded particles displayed antibacterial activity *in vivo*, but particle encapsulation lowered the drug dose required for *H. pylori* eradication, by one order of magnitude, likely as a result of the prolongation of the gastric residence time of the particles.

In another study, lipobeads were used.[64] These beads consist of a polymeric particle core that is coated with a lipid bilayer consisting of phosphatidylethanolamine. The concept aims at binding the lipobeads to *H. pylori* phosphatidylethanolamine receptors. In addition, the particle is loaded with acetohydroxamic acid for gradual drug release in the bacteria's vicinity. In *in vitro* studies, the drug loaded particles were shown to be most efficacious in inhibiting bacterial growth and stomach cell adherence, compared with free drug and empty lipobeads.

Wong *et al.* studied the effects of aerosolized non-PEGylated liposomes encapsulating ciprofloxacin.[65] Levels of drug in the lungs were higher after aerosolization than that of free drug. The therapeutic efficacy of liposome-encapsulated ciprofloxacin was compared with free drug in a mouse model of pulmonary infection by *Francisella tularensis*. At 48 hrs after the mice were infected intranasally with ten times the LD50, they were treated with aerosolized liposome-encapsulated ciprofloxacin or ciprofloxacin in the free form, resulting in 100% survival and 0% survival respectively at the doses chosen.

Tobramycin was encapsulated in fluid liposomes that were administered intratracheally for the treatment of *Pseudomonas aeruginosa* in rats.[66] Similarly, as described above, drug exposure in the lungs was improved upon administration of tobramycin in the liposomal form. Single doses of free drug or liposomal drug were hardly effective, as lung counts of bacteria remained $>10^5$ in 90% of the animals for both formulations. Only after repeated administrations, bacterial numbers $<10^3$ were noted in 10% of the animals treated with free drug and 30% of the animals treated with the liposome-encapsulated drug.

## 5. Concluding Remarks

For MPS-targeted carriers, the new developments in this category indicate an increasing focus on specific targeting of macrophages using receptor-mediated

endocytosis, possibly to increase the cell-type specificity of targeting. It may be expected that future studies will try to address different subpopulations of macrophages (infected *vs* non-infected, activated *vs* quiescent) to increase specificity even more. In addition, increased understanding of the immunologically important role of macrophages has generated interest in modulating their function during inflammation. Especially, the potent and highly specific new technique of RNA interference may offer powerful ways to modulate macrophage function. Nevertheless, other mechanisms such as the activation of macrophages through CpG-oligonucleotides may be less specific, but they can still raise a strong response that may have important clinical benefits.

For long-circulating carrier systems, the gradual accumulation at the sites of infection due to locally increased capillary permeability is an important asset for the imaging studies. The use of biotin labeled liposomes to obliterate this long-circulating characteristic at chosen time-points by the injection of avidin is to remove activity from the blood pool. In certain cases, this may improve the contrast. Long-circulating characteristics may also be used for drug delivery at peripheral sites. This may also be of value for several of the microorganisms surviving inside phagocytes. Although liver and spleen are the prime target organs for phagocyte drug delivery, tissue macrophages may also be of interest where these infections are concerned. As long-circulating carriers are ultimately taken up by deep seated tissue macrophages (in addition to MPS), these carriers can also be used to reach intracellular infections outside the liver and spleen.

Local application of drug delivery systems can have important therapeutic benefits for localized infections. The confinement of *H. pylori* to the stomach offers an ideal opportunity for local delivery. The approaches discussed in this chapter are aimed at increasing stomach residence time, so as to increase drug exposure of the pathogen. The same objective is true for the local delivery in the lung. However, pathogens causing pneumonia may be expected to spread from the primary site of infection to the distant tissues more easily, requiring additional treatment. The local delivery approach may also be of value for nucleic acid-based drugs that are otherwise, due to their inherent instability and charged character, difficult to deliver.

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# INDEX