Liposomes: quo vadis?

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Liposomes have matured as a delivery system for therapeutic agents. It has taken two decades to develop the liposome carrier concept to a pharmaceutical product level, but commercial preparations are now available in important disease areas. In this review, the authors provide their perspective on where the field is going and where opportunities can be found for rational improvement of drug therapy with liposomes.

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▼ The liposome field has made great progress in recent years. The introduction of drug- or antigen-bearing liposomes as parenteral formulations in therapy has led to a steep learning curve for the scientists involved over the past two decades. There have been many hurdles in the liposome development process. These concerned a lack of knowledge both of the way liposomes and their contents are handled by the body and of the means to influence their disposition. Also, the safety profile of liposomes had not been established and there were pharmaceutical and technological problems. In this short review we aim to discuss the 'state of the art' of the pharmaceutical liposome field. This is not a comprehensive discussion about the future of the whole field; it is a personal view on where the field is going and where opportunities can be found for rational improvement of drug therapy with liposomes. New therapeutic targets, options for site, time and rate control over delivery of the drug, and technological questions and solutions will be discussed.

Liposomal drug delivery: aims and systems

Why use liposomes?

Liposomes (microparticulate lipoidal vesicles) have been under extensive investigation for more than 20 years as carriers for the improved delivery of a broad spectrum of agents, including chemotherapeutic agents, imaging agents, antigens, immunomodulators, chelating compounds, hemoglobin and cofactors, lipids, and genetic material. The use of the term 'drug' in this article is in a generic context; it does not solely refer to chemotherapeutic agents but rather designates the wide variety of agents that have been encapsulated in liposome formulations for *in vivo* application.

The aim of any drug delivery system is to modulate the pharmacokinetics and/or tissue distribution of the drug in a beneficial way. Among the variety of delivery systems that have been devised over the years are many particulate-carrier systems; for example microspheres, nanoparticles, lipoproteins, micellular systems and liposomes[1]. Of these, most excitement has been engendered by the liposome system. Because of the ability of liposomes to carry a wide variety of substances, their structural versatility and the innocuous nature of their components, liposomes have been studied for many different therapeutic situations. To understand how liposomes can best be used to improve the performance of the enclosed drug, it may be useful to consider the following basic reasons for using liposomes as a drug carrier (Box 1).

Direction. Liposomes can target a drug to the intended site of action in the body, thus enhancing its therapeutic efficacy (drug targeting, site-specific delivery). Liposomes may also direct a drug away from those body sites that are particularly sensitive to the toxic action of it (site-avoidance delivery).

Duration. Liposomes can act as a depot from which the entrapped compound is slowly released over time. Such a sustained release process can be exploited to maintain therapeutic (but nontoxic) drug levels in the bloodstream or at the local administration site for prolonged periods of time. Thus, an increased duration of action and a decreased frequency of administration are beneficial consequences.

Protection. Drugs incorporated in liposomes, in particular those entrapped in the aqueous interior,

 1461-5347/98/$19.00. PII: S1461-5347(98)00007-8

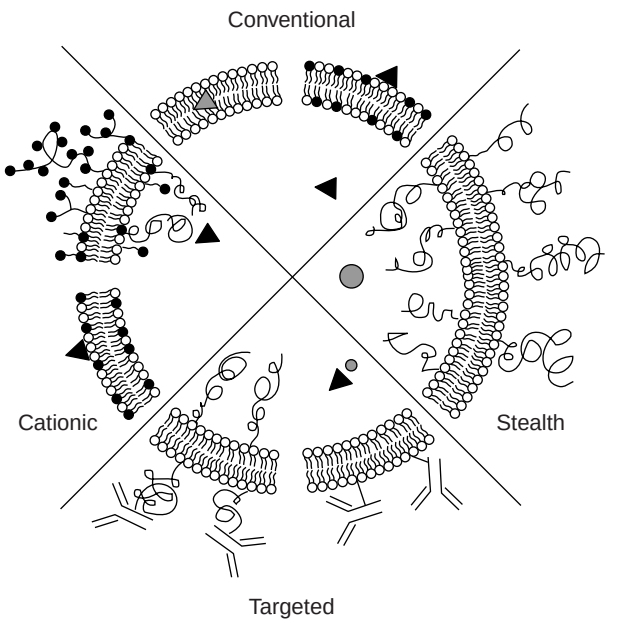


Figure 1. Schematic representation of four major liposome types. Conventional liposomes are either neutral or negatively charged. Sterically stabilized ('stealth') liposomes carry polymer coatings to obtain prolonged circulation times. Immunoliposomes ('antibody targeted') may be either conventional or sterically stabilized. For cationic liposomes, several ways to impose a positive charge are shown (mono-, di- or multivalent interactions). Figure reproduced, with permission, from Ref. 56.

are protected against the action of detrimental factors (e.g. degradative enzymes) present in the host. Conversely, the patient can be protected against detrimental toxic effects of drugs (cf. Duration).

Internalization. Liposomes can interact with target cells in various ways and are therefore able to promote the intracellular delivery of drug molecules that in their 'free' form (i.e. non-encapsulated) would not be able to enter the cellular interior due to unfavorable physicochemical characteristics (e.g. DNA molecules).

Amplification. If the drug is an antigen, liposomes can act as immunological adjuvant in vaccine formulations.

These objectives for using liposomes are not mutually exclusive; often successful applications of liposomes are based on a combination of two or even more, as will become clear from the applications described below.

Classification of liposomes

The liposome system has a major advantage over competing colloidal carrier systems: it allows almost infinite possibilities to alter structural and physicochemical characteristics. This feature of flexibility enables the formulation scientist to modify liposome behavior *in vivo* and to tailor liposome formulations to specific therapeutic needs. In an attempt to classify the plethora of possible liposome versions, four major liposome types can be broadly distinguished on the basis of composition and *in vivo* application (Figure 1).

Box 1. Basic reasons for using liposomes as drug carriers

- Direction
- Duration
- Protection
- Internalization
- Amplification

Conventional liposomes. These can be defined as liposomes that are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. Most early work on liposomes as a drug-carrier system employed this type of liposomes. Conventional liposomes are a family of vesicular structures based on lipid bilayers surrounding aqueous compartments. They can vary widely in their physicochemical properties such as size, lipid composition, surface charge and number and fluidity of the phospholipid bilayers. Although manipulation of these properties is a valuable tool to modify, to a certain extent, the *in vivo* behavior of conventional liposomes (i.e. stability, clearance and distribution), some *in vivo* behavioral features are very consistent among different conventional-liposome formulations. Conventional liposomes are characterized by a relatively short blood circulation time. When administered *in vivo* by a variety of parenteral routes (often by intravenous administration), they show a strong tendency to accumulate rapidly in the phagocytic cells of the mononuclear phagocyte system (MPS), also often referred to as the reticuloendothelial system (RES). The major organs of accumulation are the liver and the spleen, both in terms of total uptake and uptake per gram of tissue. An abundance of MPS macrophages and a rich blood supply are the primary reasons for the preponderance of particles in the liver and the spleen. A logical therapeutic translation of this MPS-directed distribution behavior is that conventional liposomes may be attractive candidates for drug delivery to MPS macrophages (Table 1). Indeed, the literature contains many examples of successful applications of conventional liposomes for the delivery of antimicrobial agents to infected macrophages[2]. Another interesting application of macrophage targeting involves the delivery of immunomodulators to increase the capacity of macrophages to kill neoplastic cells[3,4] and to increase resistance against

Table 1. Classification of liposomes based on composition and application.

Liposome type	Major application
Conventional liposomes	Macrophage targeting
	Local depot
	Vaccination
Long-circulating liposomes	Selective targeting to pathological areas
	Circulating microreservoir
Immunoliposomes	Specific targeting
Cationic liposomes	Gene delivery

infectious microorganisms[5]. Conventional liposomes have also been used for antigen delivery (Table 1). Liposome-based vaccines have proved effective in experimental models against viral, bacterial and parasitic infections[6–8], as well as against tumors[9]. Several liposomal vaccines have been tested in humans, and one of these, a liposomal hepatitis-A vaccine, has received marketing approval in Switzerland[10,11].

Long-circulating liposomes. The development of these represented a milestone in liposomal drug delivery research[12]. In spite of the above-mentioned valuable applications for conventional liposomes, the fast and efficient elimination of conventional liposomes from the circulation by liver and spleen macrophages has seriously compromised their application for the treatment of the wide range of diseases involving other tissues. The advent of new formulations of liposomes that can persist for prolonged periods of time in the bloodstream led to a revival of interest in liposomal delivery systems at the end of the 1980s. In fact, the long-circulating liposomes opened a realm of new therapeutic opportunities that were up to then unrealistic because of efficient MPS uptake of conventional liposomes. Perhaps the most important key feature of long-circulating liposomes is that they are able to extravasate at body sites where the permeability of the vascular wall is increased. Fortunately, regions of increased capillary permeability include pathological areas such as solid tumors and sites of infection and inflammation. It is illustrative for the importance of the long-circulation concept that the only two liposomal anticancer products that are approved for human use (Table 2) are based on the use of long-circulating liposomes for tumor-selective delivery of antitumor drugs (Doxil, DaunoXome). At present the most popular way to produce long-circulating liposomes is to attach hydrophilic polymer polyethylene glycol (PEG) covalently to the outer surface (Figure 1). Such PEG-coated liposomes are also called 'stealth' or 'sterically stabilized' liposomes, the former term referring to their MPS-escaping capability, the latter term to the steric stabilization mechanism held responsible for the induction of long (half-life in humans ~48 h) circulation times (Figure 2). Steric stabilization results from the local surface concentration of highly hydrated PEG groups that create a steric barrier against interactions with molecular and cellular components in the biological environment[13].

Immunoliposomes have specific antibodies or antibody fragments (like Fab′ or single chain-antibodies) on their surface to enhance target site binding (Figure 1). Although immunoliposome systems have been investigated for various therapeutic applications, the primary focus has been the targeted delivery of anticancer agents[14]. As for any particle in the bloodstream, it is difficult for immunoliposomes to leave the blood compartment at sites other than the liver and the spleen. Therefore, to guarantee accessibility of the target receptors, local administration in body cavities has received some interest (Figure 3). Successful attempts have been made to prolong the half-life of immunoliposomes after intravenous administration by coating with PEG, thus giving them a greater chance to reach target sites other than MPS macrophages (Figure 4).

Cationic liposomes represent the youngest member of the liposome family (Figure 1). They are front-line runners among

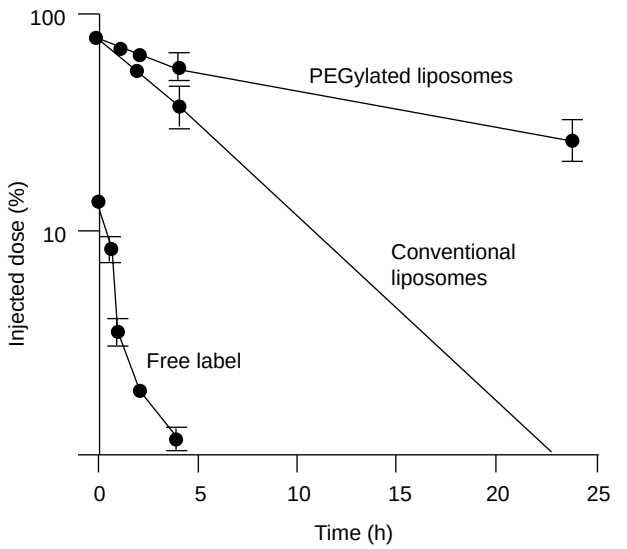


Figure 2. Blood circulation kinetics of conventional liposomes and long-circulating (PEGylated) liposomes after intravenous administration to rats. ^{67}Ga-DF was used as label to follow the fate of the liposomes in the rat. The metal-complexing agent desferal (DF) was required to achieve retention of ^{67}Ga within the liposomes. The rapid clearance of the free label (^{67}Ga-DF) is shown for comparison. Figure adapted, with permission, from Ref. 57.

Table 2. Commercial liposome/lipid-based products.

Product	Drug	Particle type	Indication	Manufacturer
AmBisome	Amphotericin B	Liposome (long-circulating)	Serious fungal infections	NeXstar Pharmaceuticals
Abelcet	Amphotericin B	Lipid complex (ribbon)	Serious fungal infections	The Liposome Company
Amphocil	Amphotericin B	Lipid complex (disc)	Serious fungal infections	Sequus Pharmaceuticals
Doxila[a]	Doxorubicin	PEG-liposome (long-circulating)	Kaposi's sarcoma	Sequus Pharmaceuticals
DaunoXome	Daunorubicin	Liposome (long-circulating)	Kaposi's sarcoma	NeXstar Pharmaceuticals

[a]Marketed as Caelyx in Europe.

the delivery systems under development for improving the delivery of genetic material[15,16]. Their cationic lipid components interact with, and neutralize, the negatively-charged DNA, thereby condensing the DNA into a more compact structure. The resulting lipid–DNA complexes, rather than DNA encapsulated within liposomes, provide protection and promote cellular internalization and expression of the condensed plasmid.

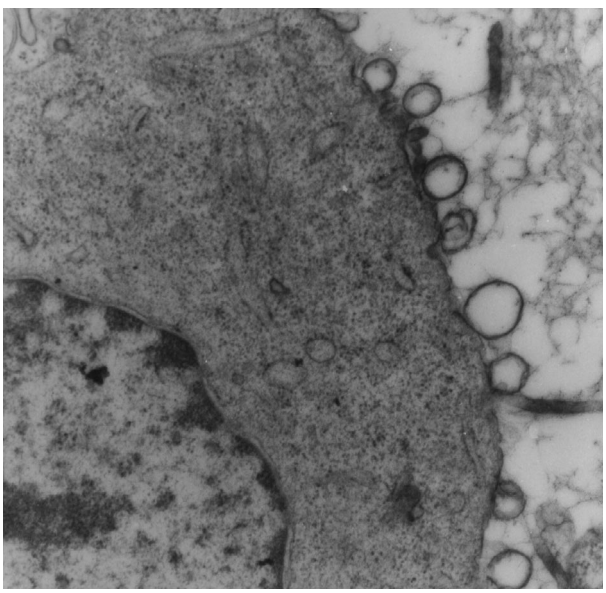


Figure 3. Electron micrograph showing specific immunoliposomes bound to their target, a human ovarian cancer cell present in the peritoneal cavity of a nude mouse. The immunoliposomes expose Fab' fragments of the murine monoclonal antibody OV-TL3 against an antigenic site on the surface of the cancer cell. The immunoliposomes were administered intraperitoneally.

Pharmaceutical aspects

The liposome does not exist. Liposomes are a family of vesicular structures differing widely in their characteristics. The mechanical and surface properties of liposomes can be modulated by selecting the proper bilayer components. Over the years, phosphatidylcholine (PC) has emerged as the major lipid component of pharmaceutical liposomes. The rigidity and permeability of the bilayer strongly depends on the type and quality of PC and additional bilayer lipids used. The alkyl-chain length and degree of unsaturation play a major role, for example, a

Box 2. Liposome classification based on pharmaceutical aspects[a]

Based on structural parameters

- MLV, multilamellar large vesicles – >0.5 μm
- OLV, oligolamellar vesicles – 0.1–1 μm
- UV, unilamellar vesicles (all size range)
- SUV, small unilamellar vesicles – 20–100 nm
- MUV, medium sized unilamellar vesicles
- LUV, large unilamellar vesicles – >100 nm
- GUV, giant unilamellar vesicles (vesicles with diameters >1 μm)
- MVV, multivesicular vesicles (usually large >1 μm)

Based on method of liposome preparation

- REV, single or oligolamellar vesicles made by reverse-phase evaporation method
- MLV-REV, multilamellar vesicles made by the reverse-phase evaporation method
- SPLV, stable plurilamellar vesicles
- FATMLV, frozen and thawed MLV
- VET, vesicles prepared by extrusion methods
- DRV, dehydration-rehydration vesicles

[a]Based on Ref. 24.

Table 3. Quality control assays of liposomal formulations.

Assay	Methodology/analytical target
Characterization	
pH	pH meter
Osmolarity	Osmometer
Phospholipid concentration	Lipid phosphorus content/HPLC
Phospholipid composition	TLC and HPLC
Cholesterol concentration	Cholesterol oxidase assay and HPLC
Drug concentration	Appropriate method
Chemical stability	
pH	pH meter
Phospholipid peroxidation	Conjugated dienes, lipid peroxides and FA composition (GLC)
Phospholipid hydrolysis	HPLC, TLC and FA concentration
Cholesterol autooxidation	HPLC, TLC
Antioxidant degradation	HPLC, TLC
Physical stability	
Vesicle size distribution: submicron range	DLS
micron range	Coulter Counter, light microscopy, laser diffraction and GEC
Electrical surface potential and surface pH	Zeta-potential measurements and pH sensitive probes
Numbers of bilayers	SAXS, NMR
Percentage of free drug	GEC, IEC and protamine precipitation
Dilution-dependent drug release	Retention loss on dilution
Relevant body fluid induced leakage	GEC, IEC and protamine precipitation
Biological characterization	
Sterility	Aerobic and anaerobic cultures
Pyrogenicity	Rabbit or LAL test
Animal toxicity	Monitor survival, histology and pathology

Based on Ref. 24. DLS, dynamic light scattering; FA, fatty acid; GEC, gel exclusion chromatography; GLC, gas–liquid chromatography; IEC, ion exchange chromatography; LAL, Limulus Amoebocyte Lysate; NMR, nuclear magnetic resonance; SAXS, small angle X-ray scattering; TLC, thin layer chromatography.

C_{18}, saturated alkyl chain produces rigid bilayers with low permeability at body temperature. The presence of cholesterol also tends to rigidify bilayers. Figure 5 shows the different approaches to produce liposomes. Liposomes produced by these techniques have different physicochemical characteristics (see Box 2). These differences will have an impact on their behavior *in vivo* (disposition) and *in vitro* (e.g. sterilization and shelf-life). More information on the relationship between preparation method and liposome morphology can be seen in Box 3.

Pharmaceutical technology: hurdles

Problems encountered in the development of pharmaceutical liposomes are listed in Box 4.

Raw materials. Phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) from natural sources are often used as phospholipids for parenteral liposome preparations. These phospholipids have a source-dependent composition of acyl chains. The composition is even batch-dependent; for example, there are considerable differences in acyl-chain composition between egg PC and soy bean PC. Phospholipid supplies can contain lyso-phospholipids; where one acyl-chain is left at the C1-position of glycerophosphocholine and the other is removed by, for example, ester hydrolysis during storage. Moreover, peroxidation can occur if unsaturated bonds are present in the acyl chains, as is the case in egg PC and soy bean PC.

In the early 1980s the quality of lipids of several suppliers could vary considerably; both in quantitative and qualitative terms. Nowadays, a few suppliers provide the global market with high-quality products. Quality is ensured by improved purification schemes, the introduction of validated analytical techniques and a better insight into lipid degradation mechanisms

Box 3. Methods for the preparation of pharmaceutical liposomes[a]

Hydration stage

Mechanical methods

- Vortexing or hand shaking of phospholipid dispersions (MLV)
- 'Microfluidizer' technique (mainly SUV)
- High-shear homogenization (mainly SUV)

Methods based on replacement of organic solvent(s) by aqueous media

- Removal of organic solvent(s) before hydration (MLV, OLV, SUV)
- Reverse-phase evaporation (LUV, OLV, MLV)
- Use of water immiscible solvents: ether and petroleumether infusion (solvent vaporization) (MLV, OLV, LUV)
- Use of water miscible solvents such as ethanol injection (MLV, OLV, SUV)

Methods based on detergent removal

- Gel exclusion chromatography (SUV)
- 'Slow' dialysis (LUV, OLV, MLV)
- Fast dilution (LUV, OLV)

Methods based on size transformation and fusion

- Spontaneous fusion of SUV in the gel phase (LUV)
- Freeze-thawing (MLV)
- Freeze-drying (MLV)

Sizing stage

High pressure extrusion
Low pressure extrusion
Ultrasonic treatment

Removal of non-encapsulated material

Dialysis
Ultracentrifugation
Gel-permeation chromatography
Ion-exchange resins

[a]Based on Ref. 24; for abbreviations see Box 2.

Box 4. Problems encountered in the development process of liposomes as drug carrier system

- Poor quality of the raw material, the phospholipids
- Poor characterization of the physicochemical properties of the liposomes
- 'Pay load' is too low
- Shelf-life is too short
- Scale-up problems
- Absence of any data on safety of these carrier systems on chronic use

leading to better shelf-life conditions (Table 3)[17,18]. Interestingly, over the years, the price per unit has dropped considerably while the quality has improved.

Physicochemical properties. Liposome behavior *in vitro* and *in vivo* strongly depends on their size, bilayer rigidity, charge and morphology (i.e. unilamellar, multilamellar, multivesicular). Therefore, a full physicochemical characterization of pharmaceutical liposomes is required in early stages of research (Table 3). In a later development stage, the outcomes of the listed quality control assays can be used to obtain regulatory approval for the liposome product. A selection can then be used to ensure batch-to-batch consistency.

Pay load. After finishing the hydration stage of the liposome preparation process, non-liposome-associated drug is removed (see Box 3). Polar drugs and drugs that don't have an electric charge opposite to the (usually negatively charged) bilayer show poor encapsulation after hydrating the lipids: there is 'a pay load problem'. Active or remote loading strategies have been developed increasing encapsulation efficiencies up to 100% (Ref. 19). The driving force is a pH gradient over the bilayer. Doxorubicin can be loaded up to 100% in liposomes by using an ammonium sulfate gradient (inducing a pH gradient)[20]. These active loading strategies, in principle, allow empty liposomes to be loaded with a drug 'at the patient's bedside'. This is an interesting option if a labile drug or labile liposomes need to be used.

Shelf-life. The meaning of the term 'stability' strongly depends on the interpretation of the professional target group. For a biochemist, a shelf-life of a week at −70°C may be acceptable. For a pharmaceutical product, a minimum shelf-life of two years, preferably without refrigerator cooling, is a primary requirement. Liposome shelf-life may be limited because of two factors. First, physical instability – drug leakage from or through the bilayer and liposome aggregation or fusion. Second, chemical instability – hydrolysis of the ester bonds or oxidation of unsaturated acyl groups.

Zuidam and Grit[17,18,21] systematically studied chemical shelf-life problems. Oxidation can be prevented by excluding oxygen from the injection vial, by addition of an anti-oxidant (e.g. vitamin E) or by selection of saturated acyl-chains in the phospholipid. Minimizing hydrolysis is possible by selecting an environmental pH of 6.5 and low temperatures. If those conditions cannot be met, (freeze) drying may be considered. Liposomes can be successfully freeze dried if the proper lyoprotectant is used and proper freeze-drying conditions are chosen. Disaccharides are excellent lyoprotectants. They prevent aggregation and fusion upon reconstituting the cake. It is not always possible to avoid drug leakage from liposomes after a freeze-drying–rehydration cycle, but recent insights into the mechanism of lyoprotection improve the chances for success[22].

Scale-up. Laboratory-scale liposome preparation methods are listed in Box 3. Several of these preparation approaches have

been scaled up to industrial scale. If possible, the use of a high-shear homogenizer for the production of small vesicles is a first choice. No organic solvents are required to dissolve the lipids first, nor are detergents necessary to hydrate the lipids, and there is easy access to the appropriate (commercially available) equipment. Problems with this high-shear homogenizing approach may arise if more than one lipid bilayer component is present. Alternative upscalable technologies are:

- the detergent removal method, where the detergent is removed from the mixed micelles containing lipid bilayer components;
- the ethanol injection method, where the lipids are dissolved in ethanol and then mixed with the aqueous phase; and finally,
- the bilayer forming lipids can be lyophilized in the presence of the (lipophilic) drug. Upon hydration the highly porous lipid cake forms liposomes (MLV >1 μm) and the lipophilic drug is liposome associated[23].

Issues that are related to the parenteral administration of liposomes are the product sterility and the absence of pyrogens. The preferred way of sterilizing liposomes is by autoclaving. This is a realistic option; if the pH conditions are optimal, the drug is heat stable and lipophilic. Otherwise, reliance on filtration through membranes with 0.2 μm pores or aseptic production procedures are necessary. Standard procedures for pyrogen-free production of parenterals can be utilized. Limulus amoebocyte lysate (LAL) tests on pharmaceutical liposomes should be thoroughly validated[18,24].

Safety data. Safety of liposomes has been debated by several groups[25–27]. For the present generation of pharmaceutical liposomes containing highly potent drugs with a narrow therapeutic window (e.g. cytostatics and fungicides) no safety problems directly related to the liposomes have been observed. However, changes in their side-effect profile may occur. For example, the 'hand-and-foot' syndrome (see below) observed after administration of long-circulating doxorubicin liposomes is not found after administration of free doxorubicin in standard protocols.

Attachment of functional groups to the liposome surface

For active targeting approaches and for covalently attaching PEG to the surface of liposomes, different 'bridging' approaches have been developed. The 'homing devices' used are often peptides or proteins. The relevant coupling chemistry is fairly well established; heterobifunctional cross-linking agents limit protein–protein and liposome–liposome interactions. Homing devices can be coupled either to anchor molecules in existing bilayers or to lipid molecules before formation of the liposome. The disadvantage of coupling homing device and lipid anchor molecule before liposome formation is that the homing device is exposed to the rather harsh conditions, such as high shear forces, heat or organic solvents, that are used to create a pharmaceutically acceptable liposome. Recently homing proteins have been covalently attached to the end group of PEG attached to bilayers in attempts to combine long-circulation properties and target-homing capacity (Figure 4)[28–31].

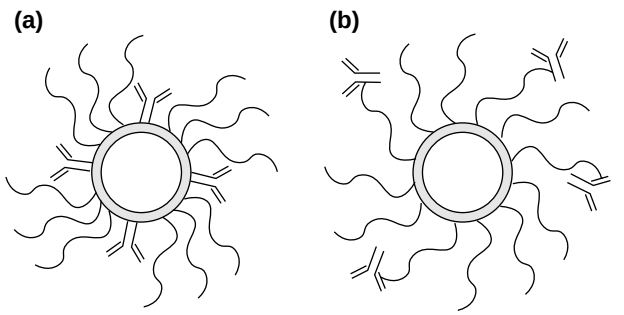


Figure 4. Immobilization of antibody on PEG-liposomes by (a) direct coupling to the liposome surface (which may provide steric hindrance to antigen binding) and (b) coupling to the terminal ends of the PEG chains (which does not give a steric hindrance problem). Figure reproduced, with permission, from Ref. 58.

Commercial products

Industrial liposome formulations approved for parenteral use in humans are listed in Table 2. The therapeutic indications are in two diseases categories: serious fungal infections and cancer. The corresponding dispersions contain the polyene antibiotic, amphotericin B and the anthracycline drugs, doxorubicin or daunorubicin.

Lipid formulations of amphotericin B

Invasive fungal infections are among the most important causes of morbidity and mortality in immunocompromised patients, particularly in cancer patients treated with intensive chemotherapy and in AIDS patients. Parenteral administration of amphotericin B is still the therapy of choice. However, the clinical use of amphotericin B is fraught with problems of poor tolerability. Among the many possible side effects, nephrotoxicity is the most severe and often limits the dose. The frequency of treatment failure is high, especially in persistently granulocytopenic patients. In the past decade, numerous reports pointed to the value of lipid-based formulations in increasing the therapeutic index of amphotericin B (Refs 32,33). Promising preclinical results, along with technological advances in liposome production technology, have led to the clinical application of several lipid-based formulations of amphotericin B. The industrial formulations differ widely in

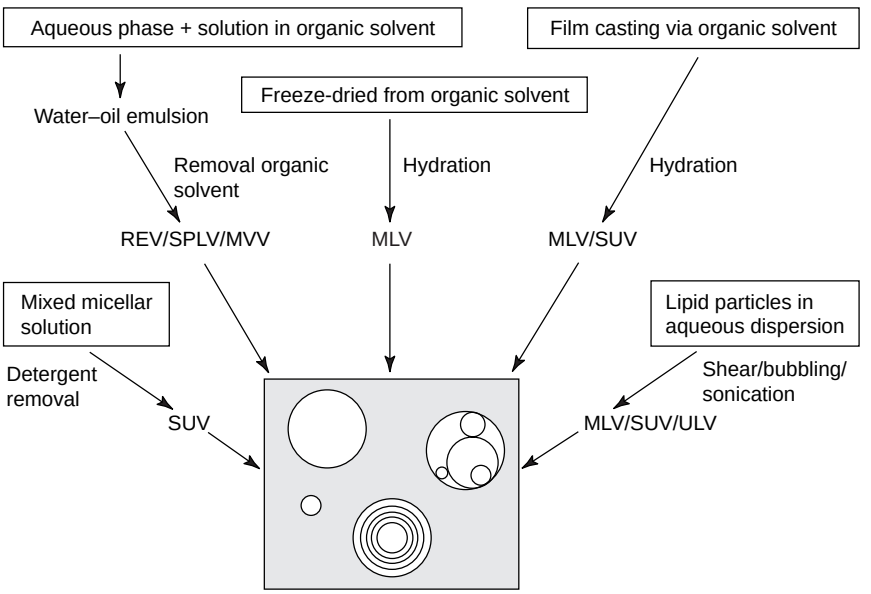


Figure 5. Schematic diagram of regularly used methods for liposome preparation. The commonly obtained type of vesicle is indicated. MLV, (classical) multilamellar vesicles; MVV, multivesicular vesicles; REV, reverse-phase evaporation vesicles; SPLV, stable plurilamellar vesicles; SUV, small unilamellar vesicles; ULV, unilamellar vesicles. Figure adapted, with permission, from Ref. 20.

composition and physicochemical properties and are not all liposomes: AmBisome has a liposomal bilayer structure, others do not contain bilayers but are colloidal drug–lipid complexes. Amphocil has a disc-like structure (Figure 6), while Abelcet has a ribbon-like structure (Figure 7). In spite of the large differences in structural features, all formulations have been shown to reduce toxicity greatly, allowing higher doses to be given and thereby improving clinical efficacy.

Liposomal antineoplastic agents

The ability of long-circulating liposomes to localize preferentially in tumors radically changed the approach to liposome-based anticancer therapy. Both Doxil and DaunoXome belong to the class of long-circulating liposomes. Doxil liposomes are PEGylated (Figure 1). DaunoXome liposomes derive their long-circulation time from the selection of a particular rigid bilayer composition in combination with a relatively small liposome size. Both products can boost substantially the anthracycline levels in tumors and their antitumor activity, as demonstrated in a wide variety of animal and human xenograft tumor models[12,34–36]. This passive type of tumor targeting appears to rely on the phenomenon that prolonged circulation enables the liposomes to extravasate through tumor microvasculature exhibiting increased permeability; for example, during the process of angiogenesis (Figure 8)[37]. Currently, Doxil and DaunoXome have been approved for 'just' one indication, the treatment of refractory AIDS-related Kaposi's sarcoma, but clinical studies in other solid tumor types are well under way[38].

The clinical studies performed with Doxil have illustrated an important, basic point that should be considered when developing a targeted liposome product. The restricted biodistribution of liposome-associated drugs can result in the distribution of a drug away from some sensitive nontarget tissues, such as the heart in the case of anthracyclines. However, the altered biodistribution can also lead to the appearance of new toxic effects. Quite unexpectedly, skin eruptions on the palms of the hands and soles of the feet (hand-and-foot syndrome) have been observed in patients treated with Doxil[38]. Fortunately, this form of skin toxicity is usually mild and rarely necessitates discontinuation of treatment.

New therapeutic options: selected examples

Conventional liposomes: immunotherapeutic applications

In the field of therapeutic cancer vaccination, cytokines are extensively studied as adjuvants in tumor vaccines[39]. An elegant approach for enhancing the antitumor immune response is to vaccinate with cytokine-gene-transfected tumor cells capable of secreting cytokines. As shown in Figure 9, simple addition of cytokine-liposomes to the cancer vaccine can provide similar adjuvant activity as compared to *ex vivo* transfected and reinjected tumor cells[40]. From a technological point of view (as both the slow-release characteristics of liposomes can be tailored and industrial-scale production of liposomes is possible), liposome incorporation may be preferred over cytokine-gene transfection of tumor cells for sustained cytokine delivery in a paracrine way. Liposomal delivery can also be used to improve antigen processing and presentation. Recently, we observed in rhesus monkeys that liposomal encapsulation of synthetic antigenic peptides can improve *in vivo* loading of major-histocompatibility-complex (MHC) molecules on antigen-presenting cells and thereby their presentation to T cells (Figure 10)[41]. This immunomodulatory

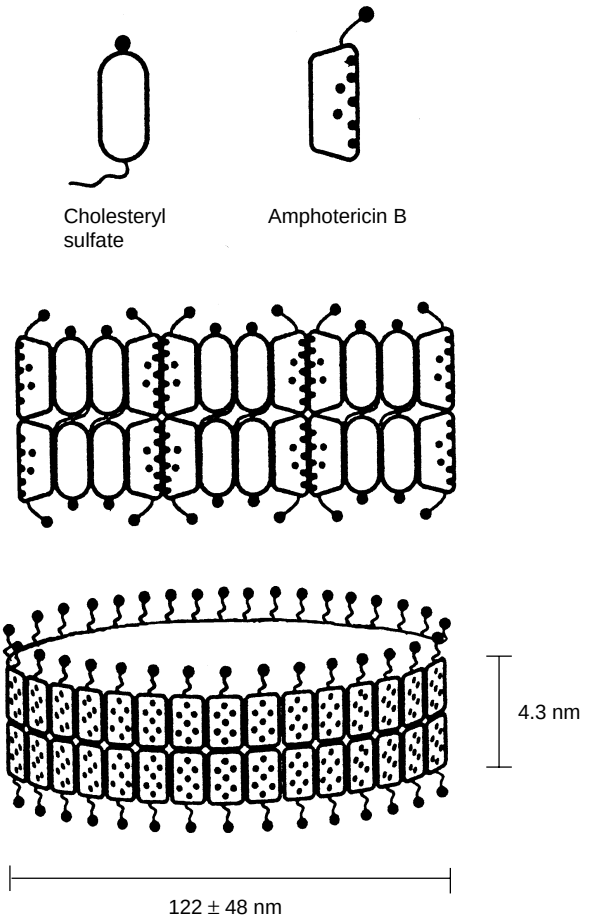


Figure 6. Structural features of Amphocil. Interaction of amphotericin B with cholesteryl sulfate forms rigid and tightly packed lipid layers that are unable to bend sufficiently to form closed vesicular structures. Because of the presence of a polar hydroxyl region, amphotericin B probably forms a shield at the disc edges and is oriented so that the seven hydroxyl groups along its side are in contact with the polar aqueous environment.

function of liposomes may be exploited for therapeutic intervention in a variety of diseases, for example, to modulate T-cell responses in autoimmune diseases.

Long-circulating liposomes: applications outside oncology

The ability of long-circulating liposomes to persist in the blood and to localize in sites of pathology also provides applications for drug delivery outside the oncology field. Other examples can be found in recent evaluations of their usefulness for delivery of scintigraphic agents (Figure 11)[42–44], antibiotics[45,46] and cytokines[47,48]. Their persistence in blood suggests that they may also prove effective to provide slow release of therapeutics such as biomacromolecules into the systemic circulation. One study of this approach has been reported using the peptide hormone vasopressin[49].

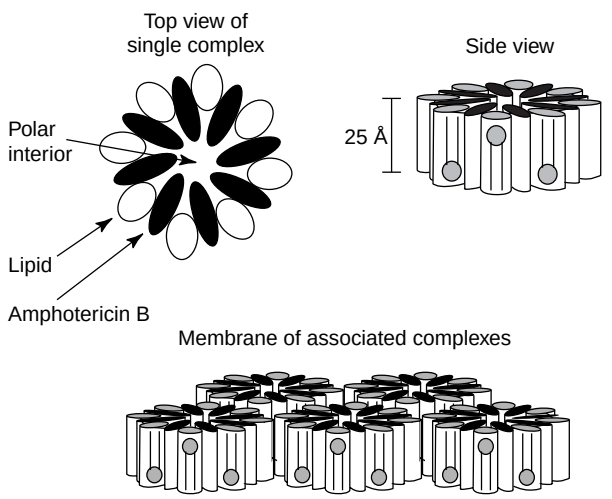


Figure 7. Putative structure of Abelcet. Amphotericin B and lipid are arranged in a 1:1 interdigitated complex. One possible arrangement would require drug–lipid pairs to be arranged in cylinders. The hydrophobic polyene region of the drug would be aligned with the lipid hydrocarbon chains, and the polar hydroxyl groups would face towards the center of the cylinder pore. The cylinders would align side by side, thus creating a ribbon-like appearance. Figure reproduced, with permission, from Ref. 60.

Immunoliposomes: target-cell dragging

Immunoliposomes are combinations of liposomes with immunoglobulin (fragments) covalently attached as a homing device. Upon intravenous injection, readily accessible targets are located in the blood compartment – that is, circulating blood cells and endothelial cells lining the blood pool. An interesting approach coined 'target-cell dragging' aims to attach immunoliposomes to specific circulating target cells in the blood. The immunoliposome 'coat' on a target cell will identify the target cell as a 'foreign' particle and the complex will rapidly be taken up by macrophages where the 'dragged' cell will be destroyed[50].

Immunoliposomes: improvement of ADEPT

Antibody-directed enzyme prodrug therapy (ADEPT) is a two-step therapeutic approach designed to generate a high concentration of anticancer molecules only in close proximity to tumor cell membranes[51,52]. The first step usually entails an antibody in an antibody–enzyme conjugate that binds to antigens preferentially expressed on tumor cells, or present in the tumor interstitium. The second step involves the injection of a nontoxic prodrug, which is matched with the enzyme, after completion of the enzyme targeting step and clearance of the conjugates from blood and normal tissues. Near the tumor cell membrane, the prodrug is converted into a cytotoxic drug by the targeted enzyme. At present, many ADEPT systems have

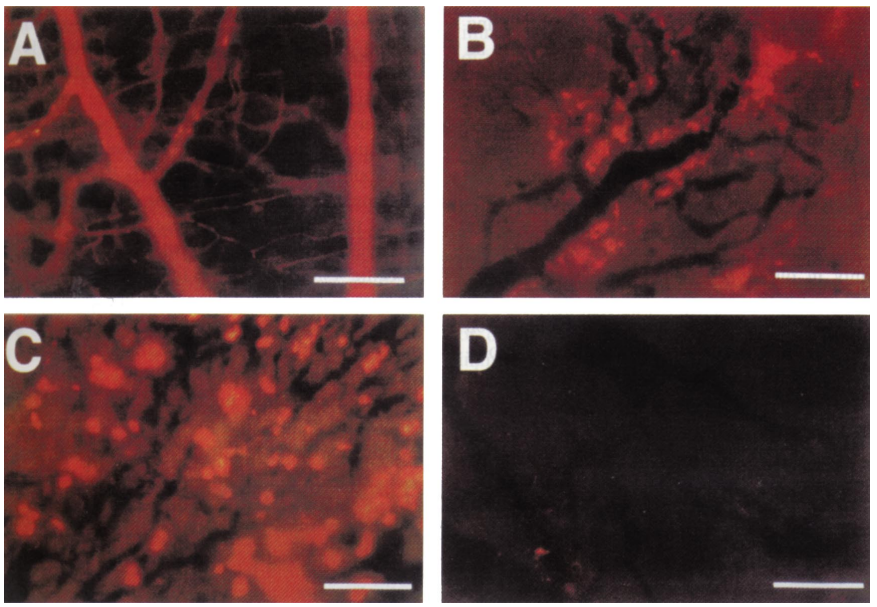
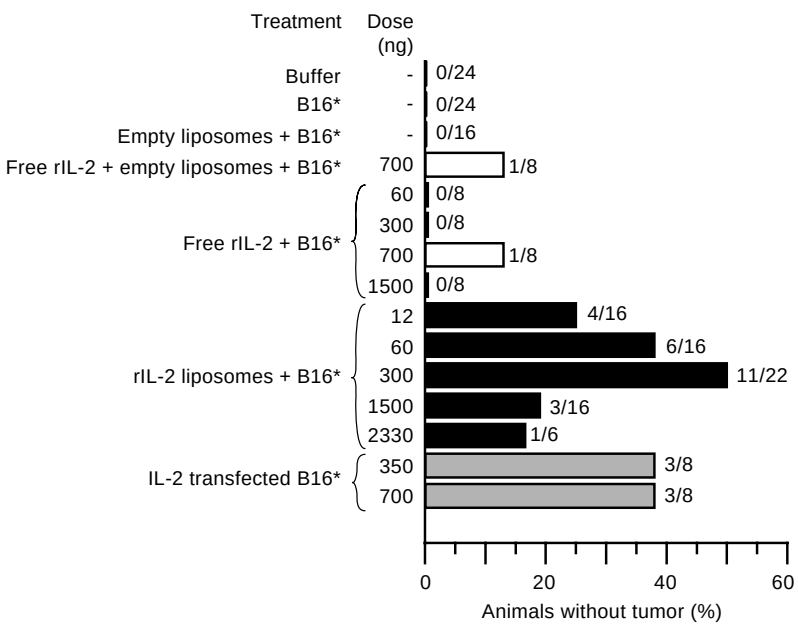


Figure 8. Transvascular transport in dorsal skin and tumors of rodents. Liposomes were labeled with the fluorescent label rhodamine. (a) There is hardly any extravasation of 90 nm diameter liposomes from normal vessels. (b) Heterogeneous extravasation of 90 nm diameter liposomes from human colon carcinoma xenogaft (LS174T) tumor vessels, 48 h after injection. Note that some vessels are leaky as indicated by the yellow fluorescence, while others are not. Extravasated liposomes do not diffuse far from blood vessels. (c) Liposomes of about 400 nm diameter (yellow fluorescent spots) extravasate adequately from LS174T through LS174T tumor vessels. (d) Liposomes of about 600 nm diameter do not extravasate, suggesting that LS174T vessels have pore-size cut-off of ~500 nm. Figure reproduced, with permission, from Ref. 37.

Figure 9. Cytokine-liposomes as alternative for cytokine gene-transfected tumor cells in therapeutic cancer vaccination. The murine B16 melanoma model was used to evaluate whether sustained release of interleukin-2 (IL-2) either from liposomes or through transfection of cells with the IL-2 gene results in enhanced systemic antitumor activity. Mice were immunized twice at a seven-day interval with a subcutaneous injection of IL-2 gene-transfected B16 melanoma cells or irradiated B16 melanoma cells (B16*) supplemented with free IL-2 or IL-2 liposomes. Seven days after the second immunization, a challenge with 1×10^5 viable wild-type B16 tumor cells was given subcutaneously at the contralateral flank. The survival and tumor occurrence were surveyed up to 80 days after the tumor challenge. Bars indicate the percentage of animals surviving without a tumor at day 80 after the tumor challenge. Numbers beside bars indicate the number of surviving animals out of the total number of immunized animals. The results show that IL-2 liposomes admixed with tumor cells can be as effective as IL-2 gene-transfected tumor cells in evoking a systemic immune response against a lethal tumor challenge. Note that supplementing the tumor cell vaccine with free IL-2 failed to generate significant systemic protective responses at any dose tested. Figure reproduced, with permission, from Ref. 40.



been tested in preclinical settings and some are entering clinical trials[52]. Recently, we proposed to improve ADEPT by using immunoliposomes as targeted carriers for the prodrug-activating enzymes (immuno-enzymosomes) (Figure 12)[53]. A theoretical advantage of this approach over the use of antibody–enzyme conjugates in ADEPT is that more enzyme molecules can be delivered to the tumor by a single targeted carrier unit. Proof of this concept was obtained *in vitro* using immuno-enzymosomes bearing β-glucuronidase capable of converting anthracycline-glucuronide prodrugs[54].

Cationic lipids - gene therapy

The rate-limiting step to develop a successful gene-based therapeutic medication is the delivery of the desired genetic material

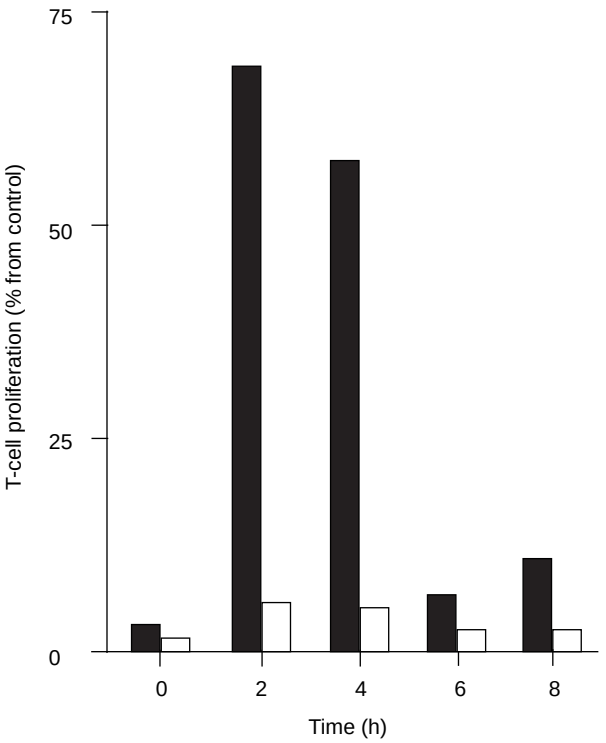


Figure 10. Liposome-mediated delivery of antigenic peptides to major-histocompatibility-complex (MHC) molecules on peripheral blood mononuclear cells (PBMC) in rhesus monkeys. An immune response to an antigenic protein is initiated by the uptake and processing of the antigen by antigen presenting cells (APC), which involves loading of small fragments into MHC molecules equipped with an antigen binding site. T lymphocytes recognize antigens with their antigen receptor (TCR) only in the context of self-MHC class I and II molecules on the APC surface. The data shown deal with an experiment designed to address the question whether liposomal encapsulation of antigenic peptides may improve *in vivo* loading of MHC molecules and thereby their presentation to T cells. T-cell stimulatory peptides (SP) of the mycobacterial 65 kD heat-shock protein were selected as model peptides. The SP peptides bind with high affinity to human as well as rhesus monkey MHC-DR molecules belonging to the HLA-DR3 lineage and can be presented by PBMC from *Mamu-DR3*-positive rhesus monkeys to human T cells. The results show that PBMC (black bars) collected at 1 and 2 h after intravenous injection of liposome-encapsulated SP stimulate proliferation of human T-cell clones, whereas only negligible stimulatory activity was found in plasma (white bars). As i.v. injection of free SP peptides fails to induce proliferation of T-cell clones, it can be concluded that liposome encapsulation of SP peptides facilitates loading of MHC class II molecules on PBMC. These data indicate that liposomes containing antigenic peptides may have therapeutic potential for modulation of T-cell responses in autoimmune and other MHC-associated diseases. Figure reproduced, with permission, from Ref. 41.

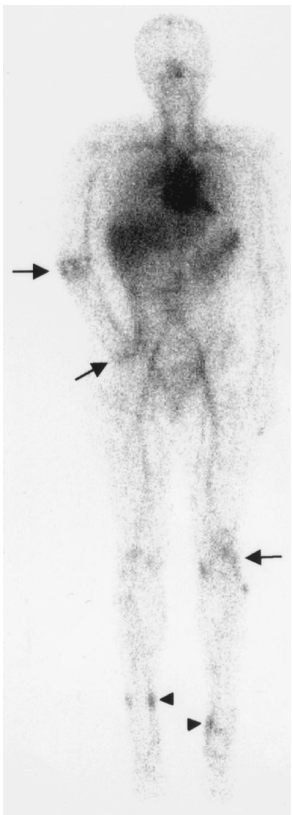


Figure 11. ^{99m}Tc-PEG-liposomes scintigraphy of a 46-year-old female patient with a 3-day history of increasing pain in multiple joints, painful red plaques at the medial site of the ankles and several pustules on the trunk and legs. Anterior whole-body image, 24 h postinjection, shows physiological uptake in the cardiac blood pool, greater veins, liver and spleen. Liposome uptake at pathological sites can be noted along the synovial lining of the left elbow, left wrist and right knee (arrows), and at the medial site of both ankles (arrowheads). Cultures remained negative. She responded favorably to Salazopyrine and was eventually diagnosed as having unexplained polyarthritis and pustulosis. The patient participated in a study on the usefulness of ^{99m}Tc-labeled PEG-liposomes for detection of infection and inflammation, performed by Dr E.T.M. Dams, Dr W.J.G. Oyen, Dr O.C. Boerman and Prof. Dr F.H.M. Corstens of the Dept of Nuclear Medicine, University Hospital, Nijmegen, The Netherlands, in collaboration with Dr G. Storm of the Dept of Pharmaceutics of the University of Utrecht, The Netherlands.

into the nucleus of the target cell. Figure 13 indicates the functions of the ideal vector system in directing DNA sequences to their target – the nucleus. Two vector-based approaches can be discerned: either the patient receives genetically engineered viruses, or the patient receives genetic information in the form of plasmids complexed with nonviral delivery systems[55]. The relative advantages and disadvantages of viral and nonviral vectors are listed in Box 5. Lipid-based transfection systems developed to date have consisted of a positively charged, hydrophilic headgroup(s) linked to a lipophilic anchor group. Many different cationic lipids have been synthesized and tested. In general, transfection efficiency is still relatively low and improvement is necessary. Greater understanding is required in synthesis of new lipids, building new lipid transfection systems and in establishing the preferred structures. Structure–activity relationships established on the basis of transfection studies *in vitro* have not been consistent with *in vivo* results[15,16]. More sophisticated approaches focus on improving specific stages of the total gene delivery process:

- Homing devices, such as transferrin and galactose, have been attached and introduce cell specificity.
- Fusogenic devices have been incorporated in the DNA–lipid complexes to gain access to the target cell cytoplasm.
- Approaches to induce endosomal escape have been selected (e.g. pH dependent fusion), when the endosomal route was involved in the cellular uptake and trafficking process.

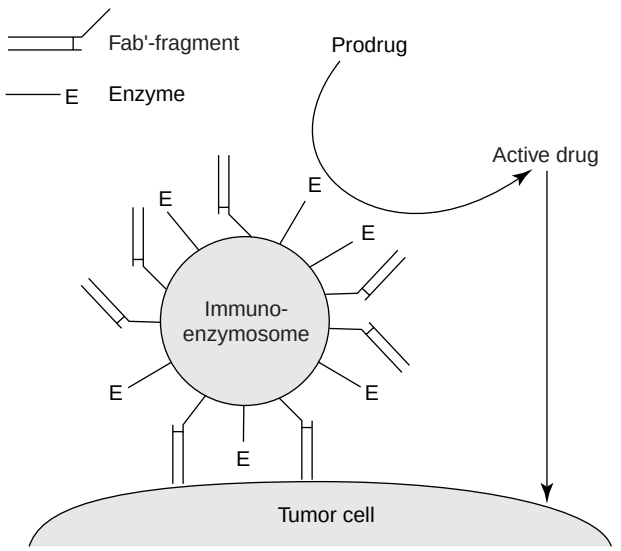


Figure 12. Schematic representation of the concept of antibody-directed enzyme prodrug therapy (ADEPT) with immuno-enzymosomes. The immuno-enzymosomes are first allowed to bind to the target cells. Then a prodrug is given, which is activated by the immuno-enzymosomes in close proximity of the target cell. Subsequently, the active drug can kill the cell.

Box 5. Advantages and disadvantages/concerns of viral and nonviral vectors

Viral vectors

Advantages

- Relatively high transfection efficiency

Disadvantages/concerns

- Immunogenicity, presence of contaminants and safety (e.g. insertional mutagenesis, recombination)
- Vector restricted size limitations for recombinant gene
- Pharmaceutical issues – large-scale production, GMP, reproducibility, stability and cost

Nonviral vectors, such as lipid or polymer based vector systems

Advantages

- Pharmaceutical issues – large-scale production, GMP, reproducibility, stability and cost
- Plasmid independent structure
- Low immunogenicity
- Opportunities for chemical/physical manipulation (e.g. targeting potential)

Disadvantages/concerns

- Relatively low transfection efficiency

- Suggestions have been made to improve intracellular trafficking to the nucleus by utilizing nuclear localization signals (NLS), peptides with a basic amino acid sequence.

Liposomes: quo vadis?

Liposomes have matured as a drug (antigen, gene) delivery system. Commercial preparations to fight life-threatening diseases are now available. The advantage of liposomes over other colloidal carrier systems is their high versatility in terms of their physicochemical behavior *in vivo* and *in vitro*. This is a consequence of a versatile molecular structure and a tendency to form bilayers with different characteristics. Supramolecular structures with many functional options are possible: long-circulating liposomes and 'conventional' liposomes for passive targeting and vaccination, positively charged liposomes for DNA condensation and transfection, and immunoliposomes for active targeting.

Liposomes are vesicles encapsulating water; this an important feature when considering the new generation of biotech drugs. Such protein drugs are sensitive to denaturation, which is encouraged by removal of their water coat. Most other

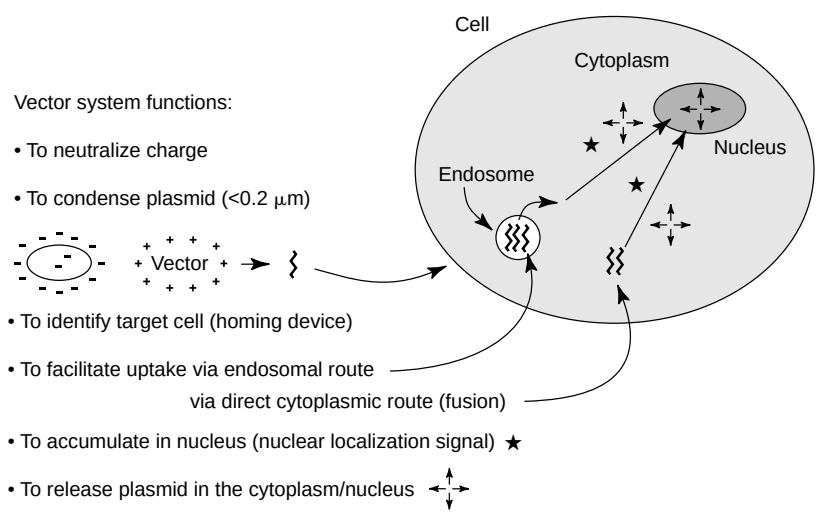


Figure 13. Nonviral vector/carrier system functions. The functions of a vector system are: neutralization of the negative charge of the plasmid; condensation of the plasmid (<0.2 μm); identification of the target cell and homing of the plasmid to the surface of the target cell; to facilitate uptake of the plasmid either through the endocytic route and early escape from the endosome, or via direct uptake in the cytoplasm (fusion with/or destabilization of the cytoplasmic membrane); and to release the plasmid into the cytoplasm or in the nucleus.

pharmaceutical colloidal carrier systems do not offer an aqueous environment.

It has taken two decades to develop the liposome from a concept to a pharmaceutical product, but such a long 'incubation time' is typical for a front-runner product. There was little understanding of the behavior and therapeutic potential of liposomes *in vivo*, and several pharmaceutical problems had to be solved. The development of commercial liposomal products has never failed for pharmaceutical reasons. Where it had failed, it was for therapeutic or economic reasons.

An important take-home message from 'the liposome story' is the critical importance of multidisciplinary research teams. Only through such an approach can liposomes be developed that are both therapeutically beneficial and pharmaceutically acceptable.

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