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Emerging Biotherapeutic Technologies

Developments in liposomal drug delivery systems

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Liposomes are the leading drug delivery systems for the systemic (iv.) administration of drugs. There are now liposomal formulations of conventional drugs that have received clinical approval and many others in clinical trials that bring benefits of reduced toxicity and enhanced efficacy for the treatment of cancer and other life-threatening diseases. The mechanisms giving rise to the therapeutic advantages of liposomes, such as the ability of long-circulating liposomes to preferentially accumulate at disease sites including tumours, sites of infection and sites of inflammation are increasingly well understood. Further, liposome-based formulations of genetic drugs such as antisense oligonucleotides and plasmids for gene therapy that have clear potential for systemic utility are increasingly available. This paper reviews the liposomal drug delivery field, summarises the success of liposomes for the delivery of small molecules and indicates how this success is being built on to design effective carriers for genetic drugs.

Keywords: adjuvant, doxorubicin, drug carrier, gene therapy, lipoplexes, liposomes, oligonucleotides, targeting

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1. Introduction

Thirty-six years have passed since liposomes were first discovered [1]. In this time liposomes have evolved from a model for biomembranes to drug carriers with clinical utility. The range of medical applications of liposomes extends from chemotherapy of cancer and fungal infections to vaccines and most recently to gene therapy. Liposomal drugs and vaccines on the market include the anticancer drugs doxorubicin and daunorubicin as well as the antifungal drug amphotericin B (AmpB) and vaccines against hepatitis A and influenza. Gene therapy is still in its infancy and liposomal gene carriers have only progressed to the clinical trial stage. With the widening range of applications of liposomal carrier systems the number of biopharmaceutical companies which focus on liposomal drug delivery or incorporate liposome carrier technology into their technology platform is rapidly increasing (see Tables 4-7).

Due to their versatile nature, liposomes can be used for diverse applications, which impose quite different requirements on the carrier. Liposomes form spontaneously in aqueous solutions and components can be added or removed in a modular way. As a result of this, their properties can be tailored to the respective application. This cannot always be readily accomplished, which will be made clear by this review.

The first part of this review provides essential background information according to the motto, 'Only by remembering the past are we able to learn from the present' [2]. Initially, an explanation of what it is that makes liposomes so versatile, what happens when liposomes are injected into the bloodstream and what the physicochemical parameters are that affect the behaviour of liposomes *in vivo* is given. Understanding these factors is important for the design of liposomes with specific tissue accumulation and elimination characteristics. For example, liposomes are naturally removed from the blood by fixed

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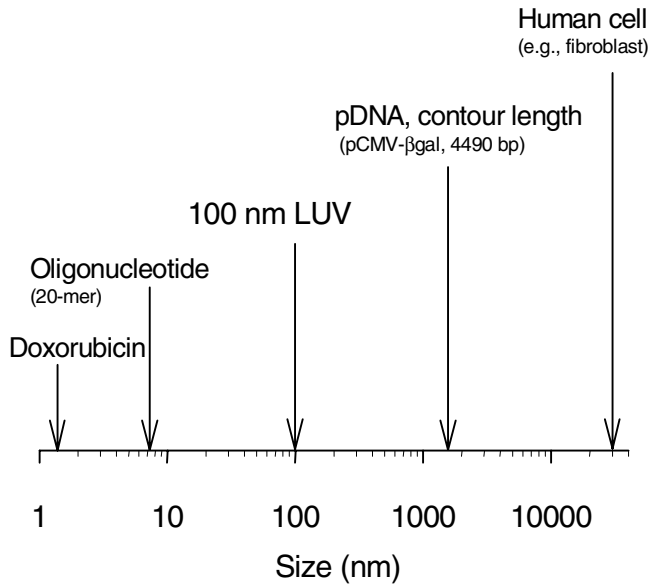

Figure 1. Comparison of the size of a 100 nm large unilamellar liposome with the sizes of different drugs such as doxorubicin a 20-mer oligonucleotide and a 4490 bp plasmid. The longest dimension of the plasmid can range between 300 - 500 nm.
LUV: Large unilamellar vesicle.

macrophages in the liver and spleen. This is an advantage when this cell population is the intended target, as is the case for many intracellular parasites, which localise in phagocytic cells. These cells are also a target when liposomes are used as immunological adjuvants. However, when alternate target sites of disease are being considered this presents a major obstacle.

The other parts of the review address specific applications of liposomal drug delivery systems. These applications include sections on liposomes as carriers for conventional drugs such as the anticancer drug doxorubicin or the antifungal drug AmpB, a section on liposomal vaccines and the adjuvant-activity of liposomes and finally a section on liposomes as carriers for genetic drugs. Each section gives an introduction into the respective area followed by a discussion of clinical applications and a summary of future directions.

2. Background

2.1 What is a liposome?

Liposomes consist of one or more concentric lipid bilayers, which enclose an internal aqueous volume(s). For drug delivery applications liposomes are usually unilamellar and range in diameter from about 50 - 150 nm. Larger liposomes are rapidly removed from the blood circulation. Liposomes are unique in their ability to accommodate drugs, which differ widely in physicochemical properties such as polarity, charge and size. Sites in liposomes where these drugs can localise include the liposome bilayer with its hydrophobic hydrocarbon chain core, its large polar surface which can be neutral or charged, and the internal aqueous space. The word drug is used as a generic term and refers to conventional drugs such as the antifungal agent AmpB and the anticancer drug doxorubicin as well as to genetic drugs such as therapeutic oligonucleotides and plasmid DNA (pDNA). A comparison of the size of a 100 nm large unilamellar vesicle (LUV) with the sizes of some of these drugs and with that of the target, a human cell, is presented in Figure 1. Values for geometric parameters such as the internal aqueous volume, the membrane volume and the surface area are listed in Table 1.

2.2 The versatile nature of liposomes

2.2.1 Liposome stability and the gel-to-liquid-crystalline phase transition

The stability of the liposomal membrane, i.e., its mechanical strength as well as its function as a permeability barrier, depends on the packing of the hydrocarbon chains of the lipid molecules (see Section 2.2.3) [3]. Charge-neutral liposomes with tightly packed membranes exhibit increased drug retention and circulation half-life *in vivo* (see Section 2.3.2). The tight packing reduces the binding/insertion of proteins, which destabilise the membrane and mark the liposomes for removal by phagocytic cells (see Section 2.3.1).

The hydrocarbon chains of bilayer-forming lipids such as phosphatidylcholines (PC) undergo an abrupt transition from a highly ordered, tightly packed arrangement, the so-called gel state, to one which is less ordered and less tightly packed, the

Table 1. Characteristics of 100 nm large unilamellar liposomes. The values presented in this table were calculated using 0.6 nm²/lipid molecule for the lipid headgroup area and molecular weights between 630 - 760/lipid molecule.

Radius (nm)	50
Bilayer thickness (nm)	5
Number of molecules/liposome	95000
Number of liposomes/μmol lipid	6.3×10^{12}
Molecular weight	$6\text{-}7 \times 10^{7}$
Internal aqueous volume/liposome (l)	3.8×10^{-19}
Internal aqueous volume (μl/μmol lipid)	2.4
Internal volume/membrane volume	2.7
Total surface area (m^2/μmol lipid)	0.36
Outside/inside lipid ratio (%)	54/46

liquid-crystalline state, when the temperature is raised [4]. Examples of lipids that are in the gel state at room temperature are saturated lipids with hydrocarbon chains comprising more than 14 carbon atoms such as 1,2-distearoylphosphatidylcholine (DSPC) and egg sphingomyelin (ESM). Unsaturated lipids including egg phosphatidylcholine (EPC) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) are in the liquid-crystalline form at room temperature. The stability (as defined above) of liquid-crystalline bilayers can be dramatically increased through incorporation of cholesterol (chol). Although liposomes formed from saturated lipids are stronger and less permeable than their unsaturated counterparts they can nevertheless also be rapidly cleared from the blood circulation *in vivo*. This was attributed to the high rigidity of these membranes, which can result in packing defects [5]. Cholesterol has the ability to inhibit the crystallisation of the hydrocarbon chains of saturated lipids to form a gel state system. Such lipid mixtures form highly ordered membranes with fluid-like characteristics [6]. Liposomes composed of gel state lipids and cholesterol are one of the preferred drug carrier systems for *in vivo* applications (see Section 2.3.2).

2.2.2 Lipids can adopt a variety of different structures

In addition to an ability to adopt gel or liquid-crystalline bilayer organisation, lipids can also adopt entirely different liquid-crystalline structures upon hydration including the micellar structures formed by lysolipids and fatty acids and the inverted hexagonal phase formed by dioleoylphosphatidylethanolamine (DOPE) [4,7,8]. Lipid phase behaviour can be modulated by changes in hydration, state of ionisation (pH and ionic strength), presence of divalent cations and temperature [7]. The modulation of the structural preferences of lipids through these factors can be exploited for liposomal drug delivery. It allows the preparation of liposomes whose stability is conditional on external or environmental parameters (see below).

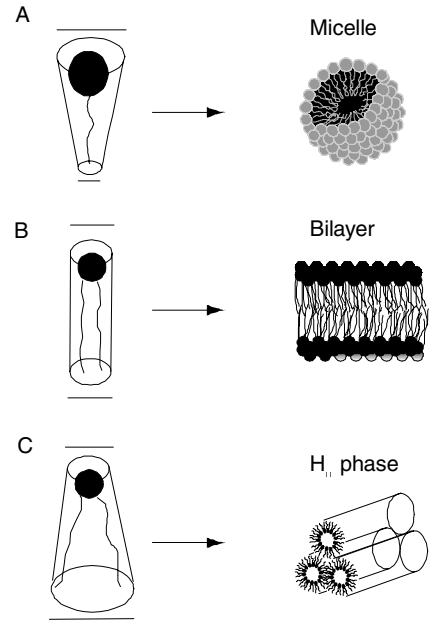

Figure 2. Graphical representation of the shape concept, which is used to explain the structural preferences of lipids. **A.** Lipids with a large headgroup area and a small hydrocarbon area have a cone-like geometry and self-assemble into micelles. **B.** Lipids that are cylindrical in shape, having nearly equal headgroup and hydrocarbon cross-sectional area, self-assemble into lipid bilayers. **C.** Alternatively, lipids with small headgroup areas adopt 'inverted' lipid phases such as the inverted hexagonal (H_{II}) phase or cubic phases. Mixtures of nonbilayer micellar lipids and nonbilayer H_{II} phase preferring lipids can adopt bilayer phases due to shape complementarity effects [10]. In addition, mixtures of oppositely charged lipids, which form bilayer structures in isolation, can spontaneously form the H_{II} phase [13-15]. The behaviour of mixed anionic and cationic lipid systems can be rationalised as arising from the reduction in effective lipid headgroup size following formation of cationic-anionic tetra-acyl zwitterions, which have a molecular shape compatible with the formation of the H_{II} phase.

'Molecular shape' arguments have been used to rationalise the phase behaviour of lipids [4]. A graphical representation of this concept is presented in Figure 2.

DOPE is the most widely used non-bilayer-forming lipid in liposomal drug delivery. It is an essential component of cationic liposome/pDNA complexes and of pH-sensitive liposomes. DOPE forms a bilayer phase below 10°C, while at elevated temperatures it adopts the H_{II} phase [9]. DOPE can form liposomes in the presence of stabilising lipids such as PCs, cholesterol, cationic lipids, detergents and polyethylene glycol (PEG)-lipid constructs [10-12]. If ionisable lipids such as 1,2-dioleoylphosphatidylserine, cholesteryl hemisuccinate (CHEMS) or fatty acids are incorporated into bilayer phases with DOPE, the stability of the bilayer can be conditional on the pH, which can control the structural preferences of the ionisable lipid [11,16-18]. Loss of the stabilising function results

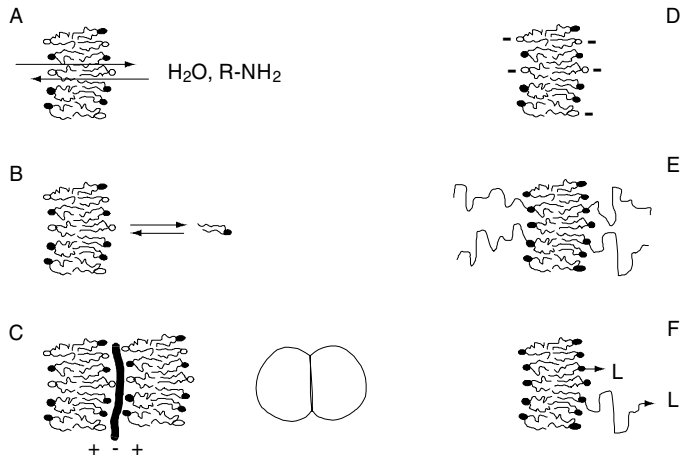

Figure 3. Membrane processes (A - C) and surface characteristics of lipid membranes (D - F) important for liposomal drug delivery. A. The lipid membrane is a selective permeability barrier. Water and amines can rapidly permeate across the lipid membrane while ions such as sodium and chloride cannot. **B.** Lipids can exchange between membranes at different rates. A large lipid headgroup, short chain length and increasing degree of unsaturation facilitate lipid exchange. **C.** The interaction of multivalent ions and polyelectrolytes with oppositely charged liposomes leads to aggregation. Membrane adhesion can lead to membrane fusion and/or rupture and can trigger further structural rearrangements (D - F). The surface characteristics of the liposomal membrane can be easily modified. **D.** Incorporation of charged lipids allows changing of the surface charge. **E.** Incorporation of poly(ethylene glycol) lipid conjugates provides a steric barrier which prevents liposome aggregation and interactions with proteins and cells *in vivo*. **F.** The liposomal membrane can be modified to display targeting ligands either directly coupled to the surface or to the distal ends of poly(ethylene glycol)-lipids.

in H_{II} phase formation and triggers contents release from liposomes (pH-sensitive liposomes). The stabilising function can also be lost through chemical modification such as low pH induced hydrolysis or enzymatic cleavage [19].

2.2.3 *Permeability properties of lipid membranes*

The permeability properties of the liposomal membrane determine how well a drug is retained in the liposome interior (see Section 3.2).

The lipid bilayer acts as a selective permeability barrier (**Figure 3A**) [4,20]. Membrane permeability varies greatly for various types of solutes, with permeability coefficients (p) ranging from about 10^{-2} - 10^{-4} cm/sec for water to about 10^{-11} - 10^{-14} cm/sec for ions such as Cl^- and Na^+ [3]. Significant but smaller variations are observed for bilayers of different lipid composition [21,22].

Differences in permeability are best illustrated in terms of the time required for release of one-half of liposome-entrapped material ($t_{1/2}$). The halftime for release can be calculated according to $t_{1/2} = 0.693R/3p$, where R is the radius of the liposomes [4]. For a 100 nm LUV, $t_{1/2}$ is 11 ms for $p = 10^{-4}$ cm/sec as in the example of water, whereas for $p = 10^{-14}$ cm/sec, $t_{1/2}$ is approximately 3.6 years as for cations such as Na^+. These variations in permeability allow the establishment of osmotic gradients and electrochemical potentials across a lipid bilayer in response to an asymmetric distribution of impermeable and permeable agents. For example, liposomes encapsulating Na^+ can be induced to swell or shrink by placing them in solutions of lower or higher salt concentrations, respectively.

A number of different factors affect the permeability of water, non-electrolytes and electrolytes. For example, the electrical potential at the membrane surface will affect the ability of charged ions to cross. Negatively charged lipids will repel anions from and attract cations to, the lipid-water interface. One factor which affects all three classes of permeants mentioned above, is the order of the membrane. Generally, the more ordered and hence tightly packed the membrane, the less permeable it is [21]. As described above, the increase in order can be established either by increasing the saturation of the fatty acid acyl chains or by introducing cholesterol. Gel-state membranes are particularly impermeable. A steep rise in membrane permeability can be found near the gel-to-liquid-crystalline phase transition [4]. The increased membrane permeability is a consequence of defects, which arise through the co-existence of gel and liquid-crystalline domains. This feature of increased permeability near the phase transition temperature is utilised in temperature-sensitive liposomes and is described in greater detail in Section 3.4.1.

2.2.4 *Lipid exchange*

Lipid exchange offers another way of controlling the stability of the liposomal membrane (**Figure 3B**). Exchange of bilayer-stabilising lipids out of the membrane results in progressive membrane destabilisation. Furthermore, certain lipids can also be post-inserted into the liposome membrane. This offers the possibility to modify the surface properties of liposomes

after they have been formed (see Section 3.4.2).

Spontaneous lipid exchange rates between membranes vary widely. Half-times ranging from seconds to several days or weeks have been observed for lyso-PCs, POPC and long chain saturated lipids such as DSPC, respectively [23,24]. In general, the exchange rate of lipids increases with decreasing chain length and increasing degree of unsaturation [24,25]. Conjugation of large hydrophilic molecules such as PEG, polylysine or proteins to lipids markedly accelerates their rates of intermembrane transfer (5- to 25-fold) [26,27]. Typical exchange half-times of PEG-ceramides with acyl chain lengths ranging from 8 - 20 carbons are from several minutes to about 3 days [28]. These differences in exchange times allow, for example, adjustment of the rate at which membrane-stabilising PEG-lipids dissociate from liposomes [29].

2.2.5 *Liposome adhesion*

Adhesion and adhesion-mediated processes such as membrane fusion and rupture dominate the interaction of multivalent ions and polyelectrolytes with oppositely charged liposomes (**Figure 3C**) [30,31]. Addition of Ca^{2+} or pDNA to oppositely charged liposomes leads to aggregation and can trigger further structural rearrangements [32-34]. These processes are responsible for the structural diversity of lipoplexes [34].

Generally, adhesion is followed by rapid spreading of the contact area and deformation of the liposomes as they flatten against each other. This places the bilayer under increased tension. If the tension (adhesion energy) is high enough the stress imposed on the lipid membrane can be relieved either by fusion (increase in area/volume ratio) and/or rupture (volume loss). Most bilayers rupture when the area is increased by about 3% [35]. Upon bilayer rupture vesicles collapse flattening against each other to form multilamellar stacks. Co-surfactants such as alcohols and detergents can modulate the structural rearrangements occurring upon interaction of cationic liposomes with DNA or oligonucleotides [36,37]. Entrapment procedures for and oligonucleotides, which rely on the use of co-surfactants, will be described in Section 5.2.1.

2.2.6 *Modification of the liposome surface*

By varying the lipids used to produce liposomes, liposomes with specific properties can be designed (**Figure 3D - F**). Zwitterionic lipids such as PC can generate neutral liposomes. A negative surface charge can be generated if negatively charged lipids such as phosphatidylserines are used (**Figure 3D**) [20]. Antibodies can be covalently coupled to the liposome surface to allow targeting of liposomes to specific cells (**Figure 3F**, see Section 3.4.2). Coating the liposome surface with a hydrophilic polymer layer represents an effective way to increase the time liposomes remain in the circulation [38-41]. The extension in circulation lifetime results in passive accumulation of these liposomes at disease sites such as tumours and sites of infection (see Section 2.3.2). The coating with polymer is usually accomplished by incorporation of poly(ethylene glycol) conjugated lipids into the liposome membrane (**Figure 3E**). This type of liposome is also referred to as sterically stabilised liposomes (see Section 2.3.2).

2.3 Liposomes *in vivo*

Over the last 30 years much information has been gained concerning the behaviour of liposomes *in vivo* (for reviews see [42-48]). It was found that clearance of liposomes from the circulation and their biodistribution depend on the physicochemical properties of the liposomes such as liposome size, surface charge and bilayer packing, as well as on other factors such as dose and route of administration [42,44-47]. The inter-relationships between these factors are best illustrated when considering the physiological and anatomical barriers liposomes encounter *en route* to a disease site, for example a solid tumour. These barriers are shown in **Figure 4**. The iv. route of administration was chosen as an example since it is the most common and universal route, which allows at the same time to target the primary tumour as well as sites of metastatic tumour growth.

In order to transport drugs to or into tumour cells liposomes must avoid interactions with circulating cells and proteins in the blood, and uptake by phagocytic cells, which are responsible for their removal from the circulation (**Figure 4A**). They must then leave the vasculature (extravasate) at the site of tumour growth (**Figure 4B**). Liposomes have then to cross the space between the vasculature and the tumour (interstitial space) and enter the tumour mass (**Figure 4C**). There, dependent on the drug being delivered, the liposomes have to be taken up into the tumour cells and facilitate the delivery of the drug to its intracellular site of action (**Figure 4D**). For conventional drugs there is no absolute need for the liposomes to associate with the tumour cells and to be taken up into the cells. Drug released within the tumour, or even in tissue nearby, can diffuse and kill target cells in adjacent areas (bystander effect). Genetic drugs such as pDNA on the other hand have to be delivered into the target cells. These large highly charged molecules are not readily taken up by cells and lack stability in the extracellular and intracellular environments.

2.3.1 *Liposome clearance*

Immediately after iv. injection, liposomes become coated by proteins circulating in the blood. Some of these proteins compromise the integrity of the lipid bilayer causing rapid leakage of liposome contents. Others promote recognition and subsequent elimination of liposomes from the blood. For example, liposomes composed of unsaturated lipids such as EPC rapidly lose their membrane integrity through lipid transfer to lipoproteins and disintegrate [49]. This process involves insertion of $ApoA_1$, an apolipoprotein found predominantly in the high-density lipoprotein fraction, into the lipid bilayer [50]. Other proteins called opsonins, mark liposomes for removal through phagocytic cells [43,44]. Examples of opsonins include components of the complement system (C3b, iC3b), IgG, β2 glycoprotein-1 and fibronectin [43,44]. The removal of foreign

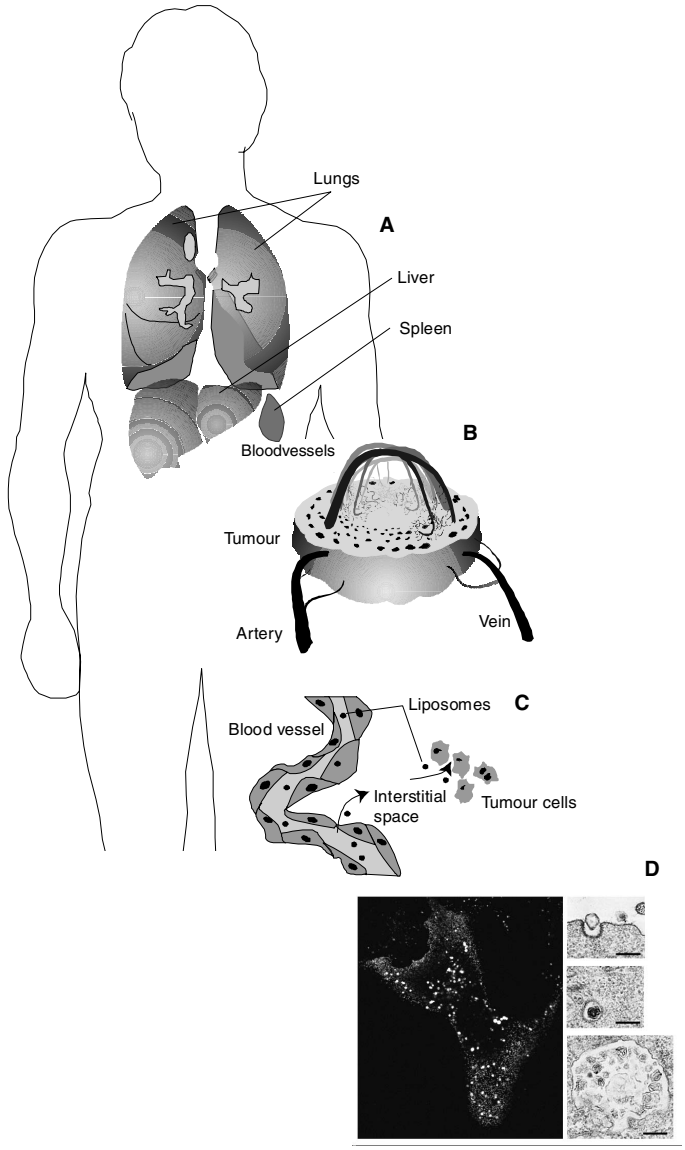

Figure 4. Behaviour of liposomes *in vivo*. A. Following injection of liposomes into the bloodstream, proteins in the blood mark liposomes as foreign matter. The majority of liposomes are subsequently cleared in the liver and spleen. Lipoplexes also tend to accumulate at very high levels in the lungs mainly through trapping in the lung capillaries. **B.** Only liposomes, which evade recognition and remain in the blood circulation for extended periods of time, can accumulate at a distal tumour site. **C.** This accumulation is a consequence of the local structure of the vasculature in these regions. It exhibits large openings through which liposomes can r each the interstitial space of the tumour. The uneven distribution of leaky capillaries in a tumour and the high interstitial pressure (lacking lymphatic system) can prevent an even distribution of liposomes within a solid tumour. **D.** Liposomes as well as lipoplexes that bind to (tumour) cells are taken up into the cell through endocytosis [58-60]. Along the endocytic route, liposomes encounter compartments of progressively more acidic pH and are degraded together with their contents once they reach the lysosomes [61]. The right hand side of **D** shows electron micrographs of the endocytic uptake of gold-labelled liposomes *via* clathrin-coated pits and their progression from endocytic vesicles to endosomes and lysosomes. The bars represent 200 nm. The degradation of hydrolytically sensitive material in lysosomes is a main barrier in lipid-based gene delivery. Depicted on the left-hand side of **D** is a COS-7 cell that has endocytosed rhodamine-PE-labeled liposomes. The discrete fluorescent spots inside the cell indicate the endosomal and lysosomal localisation of liposomes. The dark area in the centre of the cell is the cell nucleus. The right hand-side portion of Figure 4D was reproduced with permission from Straubinger *et al.* [58].

matter including liposomes is carried out by the mononuclear phagocyte system (MPS), in particular the resident macrophages of the liver (Kupffer cells), spleen, lung and bone marrow. The bulk of the injected liposomes accumulate in the liver and spleen (**Figure 3A**) [47].

2.3.2 Circulation lifetime and passive disease-site targeting

One of the most important discoveries in liposomal drug delivery has been that liposomes that exhibit long circulation lifetimes tend to accumulate at sites of disease such as tumours, infection or inflammation [40,51-55]. This is a consequence of the structure of the microvasculature in these regions (**Figure 3B**). It has large openings (up to 500 nm) through which liposomes can permeate [56,57].

The main properties governing circulation lifetimes of liposomes are size, lipid composition and dose [46]. For example, small liposomes (< 200 nm) are cleared less rapidly than large liposomes. Liposome stability (drug retention) in the blood and clearance from the blood circulation are indirectly related to the amount of protein binding [44]. Positively or negatively charged surfaces as well as membranes composed of unsaturated lipids show increased levels of protein binding and are rapidly removed from circulation [44,53]. However, there are exceptions such as lipids where the charge is screened by a bulky headgroup such as in GM_1 [52,62].

Charge-neutral lipids in conjunction with cholesterol (tightly packed membranes) as well as sterical stabilisation (steric barrier) reduce the levels of protein binding. Thus, liposomes formed from mixtures of saturated PCs or ESM with cholesterol, which are 100 nm or smaller in size, exhibit circulation half-lives of several hours and longer and have been shown to accumulate at tumour sites [52,63,64]. In some cases circulation lifetimes can be further increased by inclusion of a PEG surface coating [38-41]. The polymer acts as a steric barrier and reduces the level of plasma protein binding and uptake by phagocytic cells [46,65,66]. This forms the basis of sterically stabilised or stealth liposomes. The properties of conventional and sterically stabilised liposomes are summarised in **Table 2**.

3. Liposomal carriers for conventional drugs

Encapsulation of drugs in liposomes has several advantages. Stable encapsulation of drugs in liposomes changes the drug elimination characteristics (pharmacokinetics) and biodistribution. For example, free drugs injected into the bloodstream usually have a large volume of distribution and as a consequence exhibit significant toxicity for healthy tissues. Encapsulation of drugs in liposomes can reduce the volume of distribution and decrease toxic side effects in healthy tissues. Furthermore, increased circulation lifetimes result in higher levels of accumulation at disease sites as compared to free drug. This can result in increased efficacy if the drug is bioavailable (released from the liposomes).

Two problems become immediately obvious when trying to encapsulate drugs into liposomes. First, encapsulation becomes more difficult and inefficient as the size of the drug increases. For example, the longest dimension of a 4490 bp plasmid is between 300 - 500 nm and exceeds the diameter of a 100 nm liposome. Encapsulation techniques for this class of molecules will be described in Section 5.2.1. Second, the encapsulation efficiencies and drug-to-lipid ratios achieved by 'passive' encapsulation techniques such as lipid film hydration are low. Lipid film hydration is the simplest method for the encapsulation of water soluble drugs. A lipid film is hydrated with an aqueous solution containing the drug. In this case the upper limits for encapsulation are determined by the lipid and drug concentrations. Lipid dispersions containing more than 100 mM lipid are difficult to formulate and some drugs are only available in small quantities or are insoluble at higher concentrations. The highest obtainable encapsulation efficiency is therefore < 25% (100 mM lipid).

The development of liposomal drugs with clinical utility relied on the development of techniques, which allowed the rapid generation of homogeneous small liposomes and efficient accumulation of drugs into liposomes. This was made possible by the extrusion technique and the pH gradient loading techniques, which were developed in the late 1980s and early 1990s. The first liposomal drug formulation on the US market was the anticancer drug doxorubicin encapsulated in sterically stabilised liposomes (Doxil®). Doxil® was approved by the FDA in 1995. It should be noted that it can take between 5 - 10 years and US $ 50 - 100 million to bring a liposomal drug from the research and development stage to the market.

3.1 Drug loading techniques

The methods by which drugs can be loaded into liposomes depend on the properties of the drugs and the lipids. Hydrophobic drugs can partition into the lipid hydrocarbon region and hydrophilic water-soluble drugs can be trapped in the interior aqueous compartment. In reality, relatively few drugs segregate exclusively into hydrocarbon or aqueous compartments. AmpB is an example of a drug, which associates with the lipid membrane. The partitioning of AmpB into and its exchange rate out of the liposome membrane is highly dependent on the lipid composition [67-69]. Incorporation of negatively charged lipids increases the stability of association with the membrane [69].

Extremely high drug-to-lipid ratios and trapping efficiencies that are independent of lipid composition can be achieved with pH gradient loading techniques [70-73]. Many anticancer and antibiotic drugs are weak bases and can be accumulated in liposomes in response to a transmembrane pH gradient (for a review see [74]). Examples of anticancer drugs include the anthracyclines doxorubicin and daunorubicin, vinca alkaloids such as vincristine and camptothecins such as topotecan. Drug-to-lipid ratios as high as 0.2 mol/mol, corresponding to about 20,000 molecules per 100 nm LUV can be readily

Table 2. Properties of conventional and sterically stabilised liposomes.

Conventional liposomes	Sterically stabilised liposomes
50 - 100 nm in size	< 150 nm in size
Composed of lipids with high phase transition temperatures such as DSPC and egg sphingomyelin in combination with 35 - 50 mol% cholesterol.	Liposome surface coated with poly(ethylene glycol).
Reduction of plasma protein binding: charge-neutral surface and tightly packed membrane.	Reduction of plasma protein binding: steric exclusion from the liposome surface.
Pharmacokinetics and tissue distribution depends on size, surface charge and membrane packing. Clearance kinetics is dose-dependent and non-linear (saturation due to the limited capacity of the MPS).	Clearance kinetics is dose-independent, log-linear and non-saturable. Long circulation lifetimes and tumour accumulation can be obtained with fluid-phase lipids.
Circulation half-lives between 5 - 12 h can be obtained (highly dependent on dose).	Circulation half-life is > 24 h in rats and even longer in humans.

obtained for doxorubicin (**Figure 5B**). The pH loading techniques are also referred to as active or remote loading techniques since the drug is actively taken up into the liposomes. The procedure involves two steps, the generation of the pH gradient with low intraliposomal pH and the subsequent loading of the drug. Transmembrane proton gradients can be generated by a variety of ways. Liposomes can be prepared in a low pH buffer such as a pH 4 citrate buffer followed by exchange of the external buffer solution against a pH 7.5 buffer [70]. Alternatively, ionophores can be used in conjunction with cation gradients (high internal cation concentrations) [73]. Ionophores such as nigericin and A23187 couple the outward movement of monovalent or divalent cations, respectively, to the inward movement of protons thus acidifying the liposome interior. Finally, liposomes can be prepared in the presence of high concentrations of a weak base such as ammonium sulfate [72]. Removal of the external ammonium salt solution results in the generation of a pH gradient according to the same principle, which is also responsible for the subsequent drug loading process. The principle of pH gradient loading techniques is described **in Figure 5A**. It should also be noted that formation of a drug metal ion complex in the liposome interior can be utilised to load doxorubicin [75]. In this case complex formation is the driving force for accumulation.

3.2 Drug retention

A liposomal drug carrier of clinical utility must be able to efficiently balance stability in circulation (drug retention) with the ability to make the drug bioavailable at the disease site (drug release). The lipid composition as well as the nature of the drug are the main factors which determine stable association of the drug with liposomes.

Membrane permeability is regulated by the lipid composition. Including cholesterol in the membrane and/or increasing the saturation and length of the fatty acid chains reduces the likelihood that entrapped solutes will leak from the liposome. **Figure 6** demonstrates the effect of increasing chain saturation on the leakage rate of doxorubicin from liposomes *in vivo* [76]. The EPC/chol formulation loses half of the entrapped doxorubicin within 1 h while doxorubicin does not significantly leak from DSPC/chol liposomes over a period of 24 h.

Large drug-specific differences in retention have been observed for drugs, which were loaded by pH gradient techniques (same membrane composition). While some drugs such as the anticancer drug doxorubicin are only slowly released from the liposomal carrier, other drugs such as the antibiotic ciprofloxacin leak out rapidly [77]. These differences in leakage rates have been found to be related to the form and location of the drug in liposomes. Doxorubicin, for example, tends to precipitate in the liposome interior in a concentration and counterion-dependent manner [78-81]. Ciprofloxacin on the other hand stays in solution even though the concentration in the liposome interior can exceed its solubility in the bulk aqueous phase by two orders of magnitude [77]. The rapid leakage of this drug is related to its lack of precipitation. Little is known about the factors which determine the solubility of drugs in the aqueous interior of liposomes. Apart from precipitation, incorporation of charged lipids into the membrane has been found to retard the leakage of oppositely charged drugs through stable association with the membrane [82].

The form and location of the drug in the liposome interior together with factors such as membrane permeability determine how quickly the encapsulated drug can respond to disturbances of thermodynamic equilibria such as depletion of the pH gradient. The only easily adjustable parameter for control of drug release rates is the membrane lipid composition.

3.3 Clinical applications

The most advanced applications of liposome-based therapy are in the treatment of cancer and systemic fungal infections. Currently, there are two different doxorubicin formulations, a daunorubicin formulation and liposomal AmpB on the market (**Table 3**). The anthracyclines doxorubicin and daunorubicin are anticancer drugs. AmpB is a polyene antibiotic and the therapy

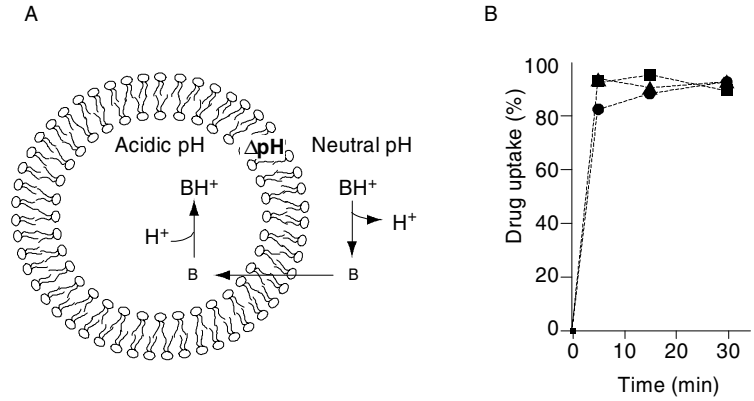

Figure 5. A. Model for the uptake of weakly basic drugs into LUVs in response to a transmembrane pH gradient (inside acidic). For compounds with appropriate pK_a values, a neutral exterior pH results in a mixture of both the protonated DH^+ (membrane impermeable) and protonated D (membrane permeable) forms of the drug. The unprotonated neutral form will tend to diffuse across the membrane until the interior and exterior concentrations are equal. However, an acidic interior results in protonation of the neutral form, thereby driving continued uptake of the compound. Depending on the quantity of the exterior weak base and the buffering capacity of the interior compartment, essentially complete uptake can usually be accomplished. The drug loading process as well as the set-up of a pH gradient using ammonium salts obeys the following relationship: $[H^+]_{in}/[H^+]_{out} = \Delta pH = [DH^+]_{in}/[DH^+]_{out}$, where $[H^+]_{in}$, $[DH^+]_{in}$ and $[H^+]_{out}$, $[DH^+]_{out}$ are the proton concentration and the concentration of the protonated drug in the liposome interior and in the bulk aqueous phase, respectively and ΔpH is the pH gradient. **B.** Accumulation of doxorubicin (●), ciprofloxacin (▲) and vincristine (▼) in liposomes in response to a transmembrane proton gradient. The pH gradient was generated through an ethylammonium sulfate gradient. DSPC/chol (55:45) LUVs were used at a lipid concentration of 5 mM. The initial drug-to-lipid ratios were 0.2 mol/mol for doxorubicin and ciprofloxacin and 0.03 mol/mol for vincristine. This figure was reproduced with permission from Maurer-Spurej *et al.* (1998) [82].

of choice for the treatment of systemic fungal infections. Both groups of drugs distribute extensively into healthy drug-sensitive tissues when given intravenously in free form. The anthracyclines exhibit strong cardiotoxicity in addition to their strong myelosuppressive activity. AmpB, on the other hand, is extremely nephrotoxic. These toxic side effects limit the dose at which these drugs can be administered.

The effect of encapsulation on the pharmacological characteristics of these drugs is shown in **Table 4** using the liposomal doxorubicin formulations Myocet™ and Doxil as typical examples [45-47,54,83,84]. Myocet is doxorubicin encapsulated in EPC/chol liposomes and Doxil is a sterically stabilised liposome formulation. First, the liposomal formulations are cleared less rapidly from circulation than the free drugs. This corresponds to a much larger area under the plasma clearance curve (AUC) than for the free drug. Second, liposome-encapsulated drugs distribute less widely over the body as shown by the smaller volume of distribution (V_d) compared to free drug. There may be large differences in the circulation half-life and retention of liposomal drugs. The smaller plasma AUC for Myocet relative to Doxil reflects both the shorter circulation time of this formulation as well as the increased rate of drug leakage. The higher volume of distribution of Myocet also indicates that doxorubicin is released more rapidly than from Doxil formulations (see also **Figure 6**). The example of Doxil also shows that liposomal drugs can accumulate to a significantly higher level at disease-sites than the drug given in free form at an equal dose.

The main advantage of the liposomal formulations of these drugs lies in the reduction of toxic side effects. Encapsulation redirects the drug away from drug-sensitive tissues such as the heart in the case of the anthracyclines to the organs of the MPS, in particular to the liver and spleen, which are less drug-sensitive.

Not all drugs benefit in the same way from encapsulation. While liposomal formulations of drugs such as doxorubicin and daunorubicin result in reduced toxicities relative to the free drug, other drugs such as vincristine also display enhanced efficacy [45,85-87]. Critical for achieving maximum efficacy is the release rate of the drug from liposomes [85]. Increased accumulation at disease-sites does not necessarily translate into increased efficacy if the drug is not released from the liposomes rapidly enough (drug not bioavailable). Active control of drug release rates can increase the potency of liposomal doxorubicin formulations over conventional liposome formulations (see below).

Many other liposomal drug formulations are in preclinical development and clinical trials. **Table 5** lists examples of such drugs. Future developments will pay particular emphasis to combinations of liposomal drugs with other conventional drugs as well as with macromolecular drugs such as monoclonal antibodies,

Table 3. Approved liposomal and lipid-based drug formulations.

Product	Company	Drug	Composition/size	Therapeutic indication
Cancer				
DaunoXome®	NeXstar/Gilead, www.gilead.com	Daunorubicin	DSPC/chol liposomes, ~ 60 nm	Advanced Kaposi's sarcoma
Doxil®/Caelyx®	Alza Corp. www.alza.com	Doxorubicin	HSPC/chol/PEG-DSPE liposomes, 80 - 120 nm	Metastatic ovarian cancer and advanced Kaposi's sarcoma
Myocet™	The Liposome Company/Elan, www.lipo.com	Doxorubicin	EPC/chol liposomes, ~ 100 nm	Metastatic breast cancer
Infectious diseases				
AmBisome®	NeXstar/Fujisawa www.gilead.com	AmpB	HSPC/chol/DSPG liposomes, 55 - 75 nm	Systemic fungal infections, visceral leish-maniasis
Abelcet®	The Liposome Company/Elan, www.lipo.com	AmpB	Complex with lipids (DMPC, DMPG)	Systemic fungal infections
Amphocil® / Amphotec®	Alza Corp. www.alza.com	AmpB	Complex with cholesteryl sulfate	Systemic fungal infections

AmpB: Amphotericin B; Chol: Cholesterol; DMPC: Dimyristoylphosphatidylcholine; DMPG: Dipalmitoylphosphatidylglycerol; DSPC:1,2-Distearoylphosphatidylcholine; DSPE: Distearoylphosphatidylethanolamine; EPC: Egg phosphatidylcholine; HSPC: Hydrogenated soy phosphatidyl choline; PEG: Polyethylene glycol.

Table 4. Pharmacokinetic parameters and tumour accumulation of different liposomal doxorubicin formulations in humans in comparison to the drug in free form.

Formulation/trade name	Dose (mg/m²)	Plasma AUC [(mg/l).h]	V_d (l)	Clearance [l/h]	TA[c] (µg/g)
Free doxorubicin[a]	25	1	254	45.3	0.8
EPC/chol (55:45)/Myocet™ [b]	25	19.7	18.8	23.3	n.d.
HSPC/chol/PEG-DSPE (56:39:5)/ Doxil®[a]	25	609	4.1	0.08	7.7

chol: Cholesterol; DSPE: Distearoylphosphatidylethanolamine; EPC: Egg phosphatidylcholine; HSPC: Hydrogenated soy phosphatidyl choline; PEG: olyethylene glycol; Plasma AUC: Area under the plasma clearance curve; TA: Tumour accumulation; V_d Volume of distribution.

Data were taken from [a]Gabizon *et al.*, [54]; [b]Cowens *et al.*, [83].

[c]TA was determined in biopsy specimens of Kaposi's sarcoma lesions. The administered dose wa s 40mg/m².

which do not exhibit some of the problems associated with conventional anticancer drugs such as multiple drug resistance [88].

3.4 Future directions

Liposomal drugs can be improved in many ways. In particular, two characteristics of liposomal carriers would significantly increase the potency of liposomal drugs. These features are active control of drug release rates (triggered release) and targeting to specific cells at the disease site. The former would ensure that the drug is bioavailable, whereas the latter would raise the drug concentration at the disease site. An ideal carrier would retain the drug in circulation, avoid rapid clearance by the MPS, preferentially accumulate at the disease-site (targeting) and efficiently release entrapped drug (triggered release).

3.4.1 Active control of drug release rates

Optimising the release rate is crucial to achieve maximum efficacy. The basic reasons for this are obvious. If the drug leaks out of the liposome at a rapid rate, it will all leak out before getting to the disease site and no therapeutic benefit over free drug will be seen. On the other hand, if the drug leaks out of the liposomes very slowly, the drug will get to the tumour but will leak out so slowly that the levels of free drug never reach therapeutic concentrations. All liposomal drug formulations described above rely on passive control of drug release rates. For example, changing the lipid composition from EPC to EPC/chol to DSPC/chol results in a stepwise reduction in drug release rates (fast, medium and slow; **Figure 6**). Active release relies on a triggering mechanism to destabilise the liposomal bilayer once the drug reaches the disease site. The trigger can be a change in environmental factors encountered at disease sites such as the low pH in the interstitial space of many solid tumours or an external trigger such as local heating. The most widely studied approaches utilise either acidification, enzymatic, thermal or photochemical triggering [17,89-92].

Evidence that the therapeutic efficacy of liposomal drugs can be improved by enhancing the drug release from lipo-

somes extravasated into tumour tissue comes from thermosensitive liposomes [93-98]. The heat induced drug release concept is based on the large increase in permeability of liposomal bilayers around their phase transition temperature. Local heating of tumour tissue up to this phase transition temperature will enhance drug release from the liposomes. Other approaches are less developed. For example, pH-sensitive liposomes are usually not very stable in circulation, tend to loose their pH-sensitivity in serum and are rapidly removed from the blood [17].

3.4.2 *Active disease-site targeting*

Active targeting of liposomes to specific cells, for example tumour cells, can be achieved by conjugating ligands to the liposomal surface, which interact selectively with receptors present on these cells. Several different types of ligands have been used including antibodies, antibody fragments and vitamins [99-101]. Many *in vitro* experiments have demonstrated highly specific binding to target cells. However, successful targeting applications *in vivo* are scarce (for a recent review see [99]). In the following, the major advances and strategies to improve liposome targeting are highlighted.

3.4.3 *Coupling to PEG-lipids and post-insertion*

An important advance was the development of coupling procedures that allow the attachment of ligands to the terminal end of PEG. This is currently the most widely employed strategy for the design of targeted liposomes since the presence of a PEG layer can increase circulation lifetime [102,103]. These procedures can be divided into two classes: procedures which either employ coupling to activated PEG-lipids that are incorporated into the liposomal membrane [102,104-106] or to PEG-lipids, which can subsequently be incorporated into liposomes [107-109]. An attractive strategy with regard to the latter is the possibility to insert PEG-lipid constructs into preformed liposomes [108,110-113]. The post-insertion relies on the spontaneous exchange of PEG-lipid conjugates from solution into the liposomal membrane at elevated temperatures. This approach allows the targeting characteristics of liposomes to be changed in a modular way.

3.4.4 *Immunotolerant antibody targeting ligands*

Attachment of whole antibodies to the liposome surface is the longest and most widely studied approach for liposome targeting [114]. The main problems associated with these so-called immunoliposomes have been the rapid recognition and clearance from the blood and immune responses, which drastically decreased circulation times of liposomes in case of repeated administration [103,115,116]. The immune responses were found to be stronger when the antibody was coupled to the terminal end of PEG [103]. These immune responses are an example of the immune potentiating role of liposomes (see Section 4) and can occur even though species-specific antibodies are used [115,116]. Therefore, the use of humanised antibodies or even fully human antibodies might alleviate this problem but may not fully solve it.

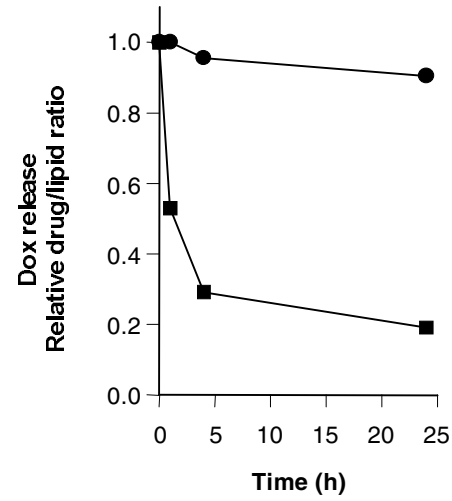

Figure 6. Kinetics of doxorubicin release from DSPC/chol and EPC/chol liposomes into the blood circulation following iv. injection into mice. DSPC/chol (●) EPC/chol (■). Large unilamellar liposomes, 100 nm in size, were prepared at a phospholipid to cholesterol ratio of 55:45 in the presence of a small amount of a non-exchangeable radiolabelled lipid (^{3}H-cholesteryl hexadecylether, ^{3}H-CHE). Doxorubicin was encapsulated at an initial drug-to-lipid ratio of 0.2 mg/mg using a pH gradient loading technique. Lipid levels in plasma were determined by assaying for ^{3}H-CHE and doxorubicin was quantified in extracted samples by fluorescence detection. This figure was reproduced with permission from Harasym *et al.* [76].
EPC: Egg phosphatidylcholine; Dox: Doxorubicin; DSPC:1,2-Distearoylphosphatidylcholine.

The most promising approach for immunotargeting of liposomes appears to be the use of small antibody fragments such as Fab′ and single chain antibody fragments (scFv) instead of whole antibodies [117]. These small targeting ligands have been shown to be cleared less rapidly from the circulation than the corresponding antibodies and are less immunogenic [118]. In addition, intratumoural distribution of PEG-immunoliposomes bearing anti-HER2/neu Fab′ fragments at the distal end of PEG chains has been observed [119]. Human scFv to cell-associated antigens can be readily selected through phage display and produced in large quantities. Lipid-tagged human scFv antibody fragments have been shown to successfully target liposomes *in vitro* [120,121]. *In vivo* data are lacking.

3.4.5 *Targeting to the tumour vasculature*

The problems associated with direct targeting of tumour cells have been discussed previously [99,122]. Most notably, problems include the high interstitial pressure inside tumours, which opposes the transport of liposomes towards the tumour core and the inhomogeneous distribution of vessels, which

Table 5. Liposomal drug formulations in preclinical development and in clinical trials.

Drug/proprietary name	Company	Therapeutic indication
All-trans retinoic acid Annamycin™ Nystatin™ /Nyotran™	Aronex Pharmaceuticals www.aronex.com	Cancer, leukaemia Cancer Systemic fungal infections
Prostaglandin E_1/Liprostin™	Endovasc Ltd. www.endovasc.com	Critical limb ischaemia
Vincristine™ /Onco-TCS™	INEX pharmaceuticals www.inexpharm.com	Cancer
Amikacin™ /Mikasome™	Nexstar/Gilead www.gilead.com	Bacterial infections
1-Octadecyl-2-methyl-phosphatidylcholine/ELL-12	The Liposome Company/Elan www.lipo.com	Cancer

exhibit increased permeability [122]. As a consequence extravasated liposomes are heterogeneously distributed throughout the tumour and are mainly located in perivascular regions [123,124]. Moreover, tumour cells are heterogeneous with respect to their phenotype. Expression of the target epitope on all malignant cells is therefore very unlikely. Tumour cells can also lose their antigens by mutation.

Endothelial cells lining the vasculature represent an easily accessible target. There is no necessity for extravasation and tumour tissue penetration and, unlike tumour cells, endothelial cells are genetically stable. Tumour cells cannot grow or spread without blood supply depending, like normal cells, on a constant supply of nutrients and oxygen. Depriving the blood supply will result in tumour cell death [125,126]. *In vivo* phage-display panning has identified small peptides that selectively home to tumour vasculature [127-129]. The feasibility of killing tumour cells by attacking their blood supply was demonstrated by several investigators utilising tumour vasculature-directed antibody or peptide drug conjugates [130-134].

4. Liposomes as immunological adjuvants and vaccines

4.1 Background information

Liposomes are immunological adjuvants, capable of augmenting immune responses to entrapped and surface-tethered antigens [135-140]. The basis for this immunopotentiating response lies in the natural targeting of liposomes to antigen-presenting cells (APC), most notably the macrophages of the MPS [139,140]. While the macrophages are the intended target in this particular application of liposomes, overcoming the rapid uptake of liposomes by macrophages was required for liposomal anticancer drugs in order to achieve increased accumulation of the drug at the sites of tumour growth as discussed in Sections 2.3.2 and 3.3. In continuation of the discussion on the immunological presentation of liposomal antigens, the involvement of dendritic cells should also be noted [140,141]. In addition, liposomes can be used as carriers of immunomodulators including macrophage activators such as muramyl peptides and cytokines with or without additional antigens (see Section 4.3).

To be effective, tumour vaccines and vaccines against intracellular pathogens have to be able to activate strong cellular immune responses, in particular a strong cytotoxic T-lymphocyte (CTL) response, to kill infected or malignant cells. However, (recombinant) protein and peptide antigens alone are frequently only slightly immunogenic and cannot elicit a CTL response [136,142]. This is due to the inability of these antigens to access the machinery of the major histocompatibility complex (MHC) class I antigen processing pathway. Exogenous antigen normally enters APCs through the endocytic route and cannot gain access to the cytoplasm of the cell, which is required for antigen presentation by MHC class I molecules to CTLs [140,143]. Association of antigen with liposomes allows antigen to gain access to both the MHC class I as well as the MHC class II pathway in APCs [140,143]. As a result, liposomal antigens can stimulate antibody production (humoral immune response) as well as cellular immune responses such as the activation of cytotoxic T-lymphocytes as well as helper T-cells [140].

4.2 Clinical applications

Currently, there are two liposome-based vaccines on the market, a vaccine against hepatitis A and one against influenza (**Table 6**) [144,145]. Both vaccines consist of the reconstituted membrane of influenza virus with its surface associated antigens and additional lipids such as EPC and phosphatidylethanolamine (PE). The surface-associated influenza antigens act as adjuvant in the hepatitis A vaccine. It should also be mentioned that there are two poultry vaccines commercially available in the US, one against Newcastle disease and the other against avian rheovirus, which consist of the killed viruses and non-phospolipid liposomes prepared from dioxyethylene cetyl ether (Ceteth-2), cholesterol and dicetylphosphate (Novasomes®) (Vineland Laboratories, Vineland, NJ, USA [301]) [146].

4.3 Future directions

The immunotherapy of cancer and infectious diseases is an

Table 6. Approved liposomal vaccines.

Product™	Company	Drug	Composition/size	Therapeutic indication
Epaxal Berna	Swiss Serum and Vaccine Institute, www.berna.org	Inactivated hepatitis A virus	Virosomes (influenza virus envelope phospholipids incorporating influenza virus surface antigens supplemented with EPC and PE), ~150 nm	Hepatitis A
Inflexal Berna V	Swiss Serum and Vaccine Institute, www.berna.org	Surface antigens of influenza virus (haemagglutinin and neuraminidase from influenza A and B)	Virosomes (influenza virus envelope phospholipids and EPC), ~150 nm	Influenza

Table 7. List of liposomal adjuvants and tumour vaccines in preclinical development and clinical trials.

Drug (antigen, immunostimulatory compound)	Company
Tumour antigens encapsulated in liposomes (e.g., synthetic 25-amino acid sequence of the MUC-1 cancer mucin), liposomal IL-2	Biomira www.biomira.com
Muramyl peptides (macrophage activators) encapsulated/associated with(in) liposomes; antigen entrapped in Orasomes®, polymerised liposomes, which are used for oral vaccine delivery.	Endorex Corp. www.endorex.com
Immunostimulatory CpG oligonucleotides w/o tumour antigens encapsulated/associated with liposomes (OligoVax®)	INEX Pharmaceuticals www.inexpharm.com
ISCOM®: saponin-based adjuvant made into 40 nm particles by adding cholesterol and phospholipid	CSL Limited www.csl.com.au
Novasomes®: non-phospholipid vesicles (adjuvant platform)	Novavax, Inc. www.novavax.com

The list also includes lipid-based adjuvant systems such as ISCOMS® [153,154] and Novasomes® [155,156].

attractive application of liposomal systems. Active immunotherapy relies on the stimulation of the body's own defence mechanisms, its immune system, to recognise and destroy infected cells or cancer cells. Current approaches involve the use of liposomes in conjunction with immunostimulatory/modulatory compounds including muramyl peptides, oligonucleotides, pDNA and cytokines, most notably IL-2, as well as disease-specific antigens [137,139-141,147-151]. The immunostimulators act as activation signals attracting immune responses to infections or tumours. In combination with antigen these compounds can stimulate a targeted immune response to specific disease-associated antigens [152]. A list of liposomal adjuvants and vaccines in preclinical development and clinical trials is presented in Table 7. A compendium of adjuvants can be found on the Internet [142]. A further application of liposomal systems lies in gene therapy approaches for immunotherapy of cancer [157-159]. In fact immunogenetherapy is the most heavily pursued form of cancer gene therapy in current clinical trials (Section 5.3) [160,161]. Immunogenetherapy of cancer is aimed at enhancing the immunogenicity of tumours by introducing genes into tumour cells that encode foreign antigens such as HLA-B7 and E1A or immunostimulatory molecules including cytokines such as IL-2 and IFN-γ, which activate and attract immune effector cells. Companies running clinical trials using this form of cancer therapy include for example Vical [302] and Targeted Genetics [303].

Synthetic oligonucleotides are a novel class of immunomodulatory compounds and hold great promises for future developments of liposome-based tumour vaccines. In part, this can be also attributed to recent advances in the development of procedures that allow efficient encapsulation of oligonucleotides [37,162]. The origin of immunogenicity of these oligonucleotides also has direct consequences for gene therapy (see Section 5.1.3) [163,164]. They will therefore be briefly described.

The immune system has evolved a defence mechanism that is able to distinguish bacterial DNA from our own [165]. Bacterial DNA differs from mammalian DNA in the frequency and methylation of CpG dinucleotides. CpG sequences are much more abundant in bacterial DNA and most of them are unmethylated [166]. These immune stimulatory CpG motifs are able to activate both innate and acquired immune responses [165,167-169]. These immunestimulatory effects can be reproduced by synthetic oligonucleotides containing these CpG motifs. The immune effects of CpG dinucleotides depend strongly on the adjacent bases [167]. By mimicking bacterial DNA, oligonucleotides containing CpG motifs may function as danger signals activating innate immune defences including NK cells, macrophages, dendritic cells, as well as

the production of pro-inflammatory cytokines. Such oligonucleotides can also promote the induction of antigen-specific immune responses and redirect (rebalance) immune responses, apart form their ability to induce nonspecific immune activation. Therefore, CpG-containing oligonucleotides promise utility as a vaccine adjuvant and for the immunotherapy of allergies and cancer [167]. Companies with a focus on CpG-containing oligonucleotides include Coley Pharmaceuticals [304] and Inex Pharmaceuticals [305].

Immunostimulatory oligonucleotides can greatly benefit from encapsulation in liposomes. Techniques that allow the efficient entrapment of oligonucleotides in liposomes have been recently developed and are described in Section 5.2.1 [37,162]. Encapsulation protects oligonucleotides from degradation and can enhance their immune stimulatory potency (PRC, unpublished results). Furthermore, encapsulation can change the pattern of pro-inflammatory cytokines that are produced in response to entrapped oligonucleotides. This could be due to the different context in which these oligonucleotides are presented to immune cells or to the increased stability of encapsulated oligonucleotides. Finally, antigens can be attached to the liposome surface using the same coupling procedures as for conjugating targeting ligands (see Section 3.4.2). This results in co-delivery of oligonucleotides and antigen into the same immune cell.

5. Liposome-based carriers for genetic drugs

Genetic drugs such as plasmids containing therapeutic genes and antisense oligonucleotides have great potential for the treatment of human diseases such as cancer, genetic disorders and infections. The advantage of gene therapy over conventional therapy is the ability to regulate the function of specific genetic elements. This potentially results in greater specificity and reduced toxicity of genetic drugs over conventional drugs.

One of the problems associated with nucleotide-based molecules is the susceptibility to degradation by nucleases present in serum or the intracellular environment. For example, unprotected DNA is degraded in the circulation within minutes. Its plasma half-life after iv. injection into mice is about 5 - 10 min [170]. In addition, genetic drugs are large and highly charged molecules and are not readily taken up into cells. As a result, the development of an effective delivery system, which protects the DNA and delivers it into cells, is critical for clinical success of gene therapy.

Currently, the most efficient delivery systems for gene transfer are genetically engineered viruses including retroviruses, adenoviruses and adeno-associated virus (AAV) [161,171]. However, they can generate a strong immune response and are not suited to systemic delivery [172]. A comprehensive discussion of the limitations and risks associated with viral vectors can be found in Mountain [171] along with a comparison of viral and non-viral delivery systems. A recent report on viral and non-viral gene therapy companies can be found in Jain [173].

Of the > 530 clinical trial protocols involving gene transfer, > 60% are directed at cancer [160]. A primary determinant of survival in most cancers is access to and efficacy in metastatic disease sites. Therefore, for gene therapy to be effective, systemic application will generally be required.

5.1 Cationic liposome/pDNA complexes

Lipoplexes were first developed in 1987 and have been widely used for gene transfer *in vitro* as well as *in vivo* [174-178]. About 13% of all current gene therapy trials involve lipoplexes [160]. Lipoplexes are simple to prepare and efficiently transfect cells *in vitro* and are carriers of choice for transfecting cells *in vitro*. This is reflected in the wide variety of commercially available cationic liposome formulations (see Table 2 in Sorgi and Huang [179]).

5.1.1 *Preparation and structure of lipoplexes*

The large majority of cationic liposome formulations consist of a cationic lipid mixed with DOPE at a 1:1 molar ratio. The preparation procedure is simple. The cationic liposomes, usually vesicles with diameters ≤ 100 nm, are mixed with DNA in a dilute solution. The lipoplexes form spontaneously due to electrostatic charge interactions. The major parameters determining the final product are the charge ratio, the ionic strength of the solution and the overall concentration of the reactants [180]. Lipoplexes tend to be large and heterogeneous (100 nm - > 1 μm) at neutral or slightly positive charge ratio and become smaller (< 200 nm) at +/- charge ratios above three [180]. Low ionic strength, rapid mixing and low overall concentrations of reactants (≤ 1 mM lipid) also contribute to a decrease in size. Lipoplexes are always prepared with a slightly positive surface charge (+/- charge ratio 1.5 - 3) to allow for interaction with negatively charged cell surfaces, thus increasing cellular uptake.

Lipoplexes exhibit a large variety of different structures including clusters of aggregated liposomes with flat double-bilayer diaphragms in the areas of contact (**Figure 7**, top), liposomes coated with DNA and (aggregated) multilamellar structures, where DNA is sandwiched between lipid bilayers [34,181,182]. The latter structures can be flat stacks of bilayers or liposomes, which frequently exhibit non-concentric bilayer segments on their outer surface (**Figure 7**, bottom). Lipoplexes are thermodynamically unstable and display a tendency to grow into larger aggregates over time, which may undergo further structural rearrangements [183].

5.1.2 *In vitro transfection*

Lipoplexes efficiently transfect a wide variety of different cell lines *in vitro*. The transfection efficiency is highly dependent on the cell line, the type of cationic lipid (liposome formulation), the ratio of DNA to liposomes used (+/- charge ratio) and the presence of serum in the media [174,177,184,185]. For *in vitro* transfection the charge of the lipoplexes is slightly positive (+/- charge ratio 1.5 - 3) to increase cellular uptake. At charge ratios > 3 the lipoplex system can exhibit significant toxicity.

Recent advances have been made in elucidating the mechanism whereby the lipid components of lipoplexes facilitate escape of pDNA from lysosomal degradation. The main route of entry of lipoplexes into cells is by endocytosis [60,186]. Once the lipoplexes are taken up into the cell they are transported to the lysosomes for degradation. In order for gene expression to occur DNA has to escape the endocytic pathway and redistribute into the nucleus.

The lipid components of the lipoplexes play a major role in the release of pDNA from endocytic compartments [15,187]. The cationic lipid component can directly disrupt the endosomal membrane, causing the release of the plasmid into the cytoplasm. This appears to be related to the ability of cationic lipids to form non-bilayer H_{II} phase structures in combination with anionic lipids present in the endosomal membrane [15]. Most of the lipofectin formulations require a 'helper lipid' such as DOPE for optimal activity. DOPE, in isolation, adopts the inverted hexagonal phase (H_{II}) and as a consequence can further increase the membrane-destabilising activity of cationic lipids by facilitating the formation of non-bilayer structures.

5.1.3 *In vivo transfection*

Positively charged lipoplexes are rapidly cleared from the circulation with half-lives in the order of minutes [176,188]. The bulk of the plasma clearance after iv. injection is due to uptake by endothelial cells in the lung and to a lesser extent the fixed macrophages in the liver and spleen [176,178,189-192]. Transgene expression is highest in the lungs with the level of gene expression in the order of ng luciferase/mg protein [176,188,189,192].

The clearance and expression profile of intravenously injected lipoplexes limits their application *in vivo* to transfection of the lungs and to direct injection into tumours. Although gene expression and appropriate physiological responses were observed in humans following regional administration, in particular in cystic fibrosis patients, the efficiency of gene transfer is still too low to show a real clinical benefit [193,194].

Lipoplexes formed with DOPE are unstable in blood (serum) [184,85]. The stability can be increased by incorporation of cholesterol. This results in increased transfection potency *in vivo* but leads to reduced transfection activity *in vitro* [185,188,190]. It should be noted that lipoplexes are also toxic [195]. The observed toxicities are mainly related to the cationic lipid. Transient immune responses to the bacterially derived pDNA can compromise transfection following repeat injection when the time period between subsequent injections is too short (CpG motifs, see Section 4.3) [163,164].

5.2 Plasmid DNA encapsulated in sterically stabilised liposomes

The development of procedures that allow efficient encapsulation of pDNA and oligonucleotides in liposomes has been a major advance towards systemic delivery of such drugs. The characteristics and limitations of these systems are discussed in the following and contrasted to lipoplexes.

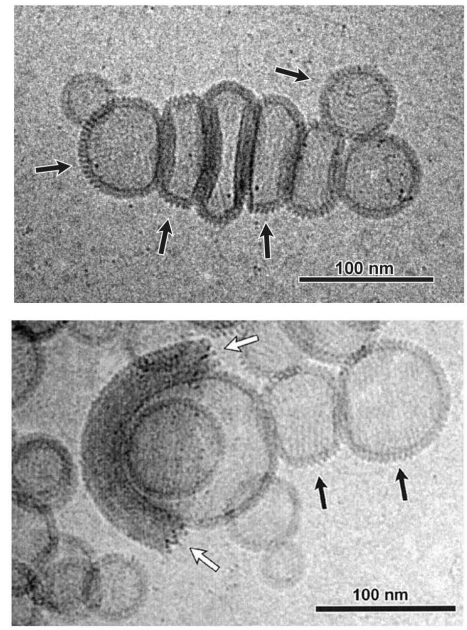

Figure 7. Structural diversity of lipoplexes. Lipoplexes can form a whole range of different structures which include, among others, aggregated liposomes (top) and bilayer stacks (bottom). Reproduced with permission from Huebner *et al.* [34].

5.2.1 *Encapsulation and physicochemical characteristics*

The design features for a lipid-based delivery system that preferentially accesses such disease sites as tumours and sites of infection are clear from studies on liposomal systems containing conventional drugs (see Section 3). From these studies it follows that the carrier containing genetic drugs should be a small, neutral and highly serum-stable particle that is not readily recognised by the fixed and free macrophages of the MPS.

Efficient entrapment requires the interaction of the lipid components with the plasmid. However, the example of the lipoplexes shows that the strong electrostatic interaction between pDNA and cationic liposomes usually leads to formation of highly aggregated structures. Control of these interactions is a prerequisite for the use of cationic liposomes for encapsulation. Previous studies have shown that incorporation of PEG-lipid conjugates into the liposomal membrane can prevent Ca^{2+}-induced aggregation and fusion of negatively charged liposomes [27]. In addition, a PEG-coating is required to obtain long circulation lifetimes. In the following, two liposomal carrier systems which rely on PEG-ceramide as the regulatory component are described.

Figure 8A demonstrates that pDNA can be efficiently entrapped in DOPE/DODAC/PEG-ceramide (84:6:10

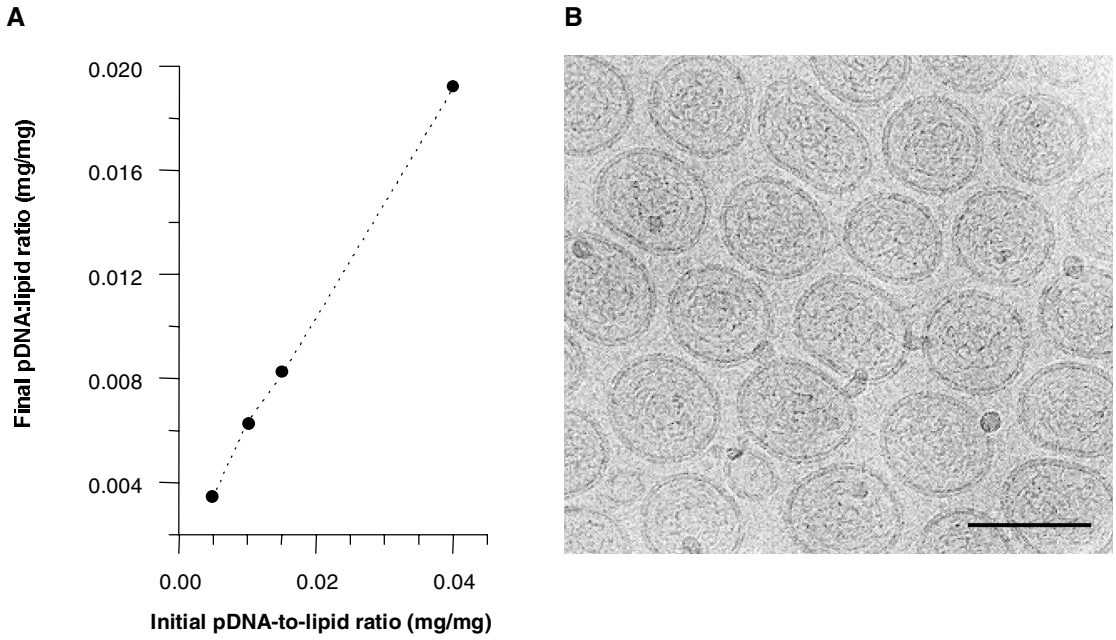

Figure 8. Encapsulation of plasmid DNA in small sterically stabilised liposomes (SPLP) using a detergent dialysis procedure. **A.** Entrapped pDNA-to-lipid ratio as a function of the initial pDNA-to-lipid ratio (mg/mg). The initial lipid concentration was 1 0mg/ml. **B.** Cryo-electron micrograph showing the structure of SPLP. The location of the plasmid is indicated by the striated pattern superimposed on the liposomes. The bar represents 100 nm. The latter was reproduced with permission from Tam *et al*. [195].

mol%) liposomes employing a detergent dialysis procedure [28]. This system will subsequently be referred to as SPLP (stabilised plasmid-lipid particles). Details about the mechanism of encapsulation can be found in [28]. The trapping efficiencies are a function of the relative amounts of cationic lipid and PEG-ceramide and the ionic strength of the medium [28,29,196]. The pDNA in SPLP is fully protected from degradation by DNAse I and serum nucleases in contrast to plasmid in cationic liposome/DNA complexes [28]. The cryo-EM picture in **Figure 8B** shows that these plasmid-lipid systems have the morphological features of large unilamellar liposomes (LUV). The encapsulated pDNA can be seen as a striated pattern superimposed on the liposomes. The average diameter from dynamic light scattering measurements is 70 nm.

A different approach uses ethanol in conjunction with preformed 100 nm LUVs. Preformed LUVs containing a cationic lipid and a PEG coating can be induced to entrap polynucleotides such as antisense oligonucleotides and pDNA in the presence of ethanol [37,162]. **Figure 9A** shows that oligonucleotides can be efficiently entrapped at high oligonucleotide-to-lipid ratios. The entrapment efficiency is plotted as a function of the initial oligonucleotide-to-lipid ratio. The maximum level of entrapment is 0.16 mg oligonucleotide per mg of lipid (0.023 mol/mol, negative-to-positive charge ratio = 1.5) and is reached at an initial ratio of 0.25 mg/mg. This corresponds to approximately 2200 oligonucleotide molecules per 100 nm liposome and demonstrates the high efficiency of this entrapment procedure. Entrapment efficiencies are about three orders of magnitude higher than obtained by passive encapsulation based on the trapped volume. The entrapment efficiency of pDNA is comparable to that of the SPLP system (**Figure 8A**). The interaction of the cationic liposomes with oligonucleotides leads to the formation of multilamellar liposomes ranging in size from 70 - 120 nm, only slightly bigger than the parent LUVs from which they originated (**Figure 9B**) [37].

5.2.2 Pharmacokinetics, tumour accumulation and tumour transfection of SPLP

The SPLP system is one of a few systems that have been directly compared to lipoplexes. The pharmacokinetics and biodistribution of the lipid as well as the pDNA was followed together with the levels of gene expression at a distal tumour site [195]. The results of this study are summarised below.

Figure10 A-C shows the pharmacokinetics and biodistribution of SPLP in tumour-bearing mice in comparison to

DODAC/DOPE lipoplexes followed by the lipid label (^{3}H-cholesterylhexadecylether). The clearance of SPLP from circulation can be described by a first order process with a half time of 6.4 ± 1.1 h (Figure 10A). Relatively low levels of uptake by the lung and liver have been observed (Figure 10B and C). Approximately 3% of the injected lipid dose accumulated at the tumour site. In contrast to SPLP, lipoplexes were rapidly cleared from circulation ($t_{1/2}$ << 15 min) and accumulated predominantly in the lung and liver. Less than 0.5% of the injected dose was found at the tumour site after 1h and decreased at later time points.

The levels of intact pDNA in the circulation and in tumour tissue following iv. injection of naked pDNA, lipoplexes and SPLP are shown in Figure 10 D and E. PDNA was analysed by Southern blot hybridisation and quantified by phosphor imaging analysis. For naked pDNA, less than 0.01% of the injected dose remained intact in the circulation after 15 min and no intact tumour-associated plasmid could be observed. Only a small fraction (< 0.2%) of the pDNA administered in the form of lipoplexes was still intact in circulation after 15 min and < 0.2% was found to be intact in tumour tissue after 1 h. In contrast, approximately 85% of the injected pDNA administered in the form of SPLP remained in intact form in the circulation at 15 min (Figure 11D). Progressively more intact pDNA accumulated at the tumour site over time with approximately 1.5% of the injected dose associated with the tumour after 24 h (Figure 11E).

The administration of SPLP results in reporter gene expression at the tumour site (Figure 10F). Injection of free plasmid or lipoplexes resulted in no detectable gene expression at the tumour site. However, transfection was observed in the lung, liver and spleen. SPLP on the other hand did not show detectable levels of gene expression in these organs.

The main limitation of the sterically stabilised SPLP system is the inefficient interaction with target cells, which results in low levels of uptake into cells and consequently low transfection levels.

5.3 Future directions

Lipid-based gene delivery systems are still at an early stage of development and there are many different ways in which the current generation vectors can be improved. Possible means of improvement are appropriate selection of the approach, production of more powerful expression systems and modifications of the carrier.

5.3.1 Appropriate selection of the approach

One possible way for improving the efficiency of gene transfer is the use of genes that amplify the biological response to gene expression or exhibit a bystander effect. This means that even though only a fraction of target cells is reached, gene expression in this limited number of cells has an effect on neighbouring cells or triggers a systemic response. These approaches include gene-directed enzyme pro-drug therapy (pro-drug activation through suicide genes), immunogene-therapy (cytokines, antigens, DNA vaccines) and gene therapy targeted to the tumour vasculature [157,160].

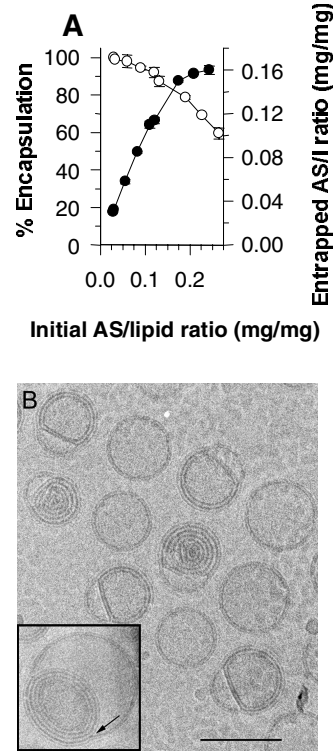

Figure 9. Encapsulation of oligonucleotides in small sterically stabilised liposomes using ethanol. A. Plot of the entrapment efficiency expressed as the entrapped oligonucleotide-to-lipid ratio (full circles) and percent entrapment (open circles) as a function of the initial oligonucleotide-to-lipid ratio. The ratios are given in w/w. **B.** Cryo-electron micrograph of DSPC/Chol/PEG-CerC14/DODAP (20:45:10:25 mol%) liposomes entrapping oligonucleotides. The inset is an expanded view of a multilamellar liposome showing two initially separate membranes forced into close apposition by bound oligonucleotides (indicated by the arrow). The entrapped antisense-to-lipid weight ratio was 0.125 mg/mg. The bar represents 100 nm. These figures were reproduced with permission from Maurer *et al*. [37].

5.3.2 Modification of the gene constructs

One strategy to increase levels of gene expression involves improving the plasmid design through the use of a cytoplasmic expression system. The advantage of a cytoplasmic expression system is that it bypasses the need for nuclear delivery of pDNA, a major obstacle in present day gene therapy. This can be achieved through the use of an autocatalytic cytoplasmic expression system using bacteriophage RNA polymerases. The feasibility and benefit of this approach was demonstrated by Gao and Huang [197] using a

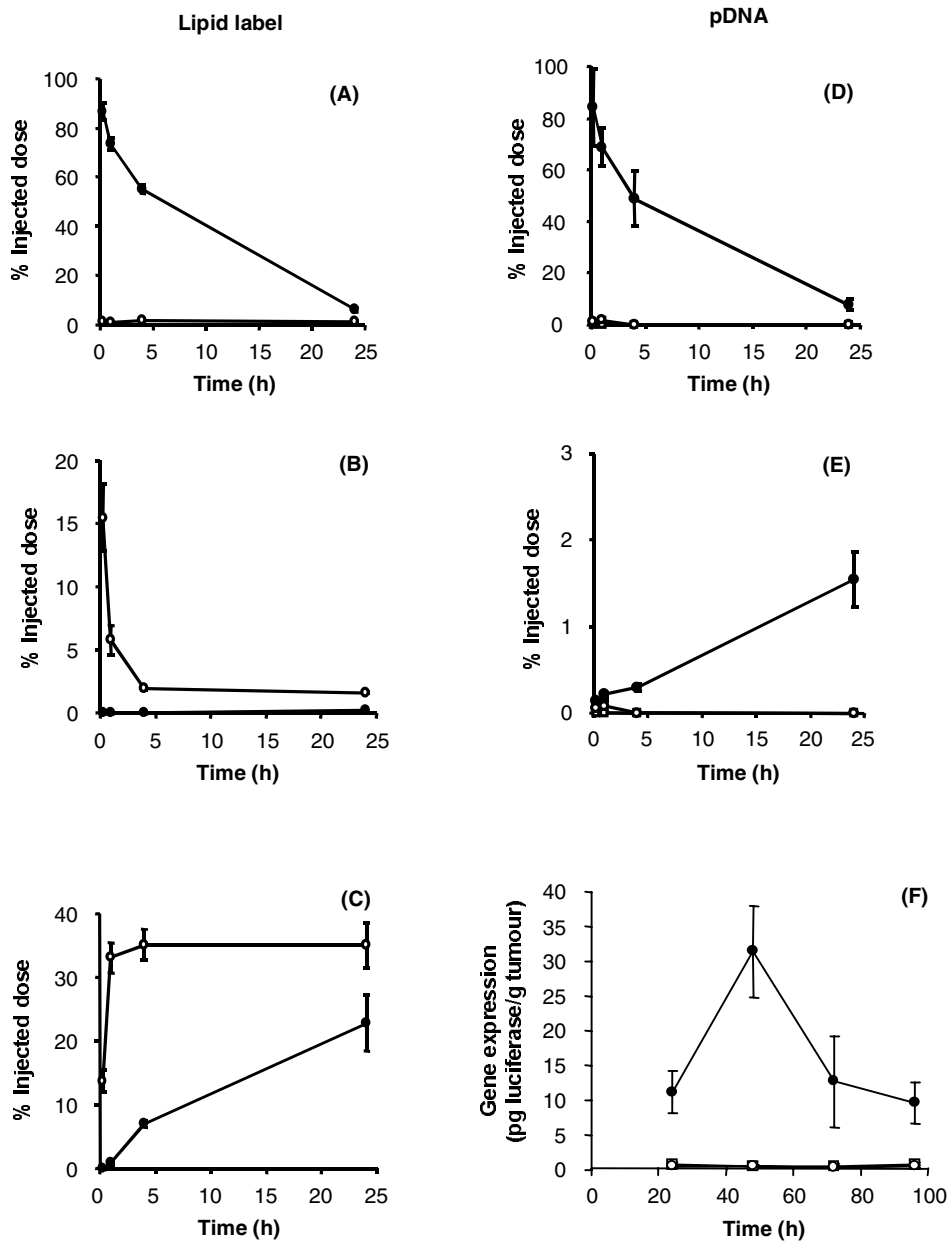

Figure 10. Pharmacokinetics, tissue distribution, tumour accumulation and transgene expression at a distal tumour site of SPLP and lipolpexes in tumour-bearing mice following iv. administration. Lipoplexes were prepared in 5% glucose by adding the plasmid pCMVluc to large unilamellar vesicles composed of DOPE:DODAC (1:1; mol:mol) to a final charge ratio (+/-) of 3.0. Lewis lung carcinoma cells were implanted s.c. in the hind flank of 6-week-old female C57BL/6 mice and the tumour allowed to grow to ~ 200 mg (12 - 14 days). **A - C** Pharmacokinetics and tissue distribution of SPLP and lipolpexes as reported by the radiolabelled marker lipid ^{3}H-CHE. The levels of lipoplexes (o) and SPLP (●) in the circulation, the lung and the liver are shown in panels **A**, **B** and **C**, respectively. The tumour accumulation of the lipid marker is shown in [195] and described in the text of this review. **D - F** Pharmacokinetics and tumour accumulation of plasmid DNA following iv. administration of naked plasmid, plasmid DNA-cationic liposome complexes and SPLP as reported by a Southern blot analysis (100 mg pDNA/mouse). The original Southern blot hybridisation gels can be found in [195]. The levels of intact plasmid resulting from iv. injection of naked pDNA (o), plasmid DNA-cationic liposome complexes (□) and SPLP (●) were quantified for plasma (panel **D**) and tumour tissue (panel **E**) by phosphor-imaging analysis and converted to mass quantities of pDNA by comparison to a standard curve made from known amounts of pDNA. Transgene expression at a distal tumour site **F**. These graphs were reproduced with permission from Tam *et al.* [195].

T7 promotor-driven plasmid complexed with cationic liposomes together with T7 RNA polymerase as well as a self-amplifying RNAP autogene with a nuclear promotor [197,198]. Other cytoplasmic expression systems are based on RNA viruses such as Semliki Forest and Sindbis virus [199,200]. Current lipid-based vectors could greatly benefit from the construction of self-amplifying genes with improved characteristics.

5.3.3 Modification of the carrier

One obvious strategy for improving the existing carrier systems is the incorporation of targeting ligands. In particular, the SPLP system would greatly benefit from increased uptake into cells. Strategies and problems associated with targeting of lipid-based gene carriers systems are presented in Gregoriadis and McCormack [201].

6. Summary and expert opinion

As summarised here, substantial progress has been made in the application of liposomal systems to drug delivery. Liposomal formulations of conventional drugs have clearly demonstrated therapeutic advantages if disease site targeting and optimised release characteristics are incorporated. It may be expected that many other conventional drugs will benefit from delivery in liposomes with similar design features. The design of liposomal systems containing genetic drugs for antisense therapy and gene therapy is becoming increasingly sophisticated. There is a clear clinical need for carriers that can deliver such drugs to a disease site and into a target cell following iv. injection and liposomes represent the leading technology in this area. Finally, liposomes are demonstrating remarkable potential as vaccine adjuvants and clinical utility should soon be demonstrated.

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