Expert Opinion

Liposomal nanomedicines

David B Fenske† & Pieter R Cullis
†*University College of the Fraser Valley, Department of Chemistry, Abbotsford, BC, V2S 7M8, Canada*

Liposomal nanoparticles (LNs) encapsulating therapeutic agents, or liposomal nanomedicines, represent an advanced class of drug delivery systems, with several formulations presently on the market and many more in clinical trials. Over the past 20 years, a variety of techniques have been developed for encapsulating both conventional drugs (such as anticancer drugs and antibiotics) and the new genetic drugs (plasmid DNA containing therapeutic genes, antisense oligonucleotides and small interfering RNA) within LNs. If the LNs possess certain properties, they tend to accumulate at sites of disease, such as tumours, where the endothelial layer is 'leaky' and allows extravasation of particles with small diameters. These properties include a diameter centred on 100 nm, a high drug-to-lipid ratio, excellent retention of the encapsulated drug, and a long (> 6 h) circulation lifetime. These properties permit the LNs to protect their contents during circulation, prevent contact with healthy tissues, and accumulate at sites of disease. The authors discuss recent advances in this field involving conventional anti-cancer drugs, as well as applications involving gene delivery, stimulation of the immune system and silencing of unwanted gene expression. Liposomal nanomedicines have the potential to offer new treatments in such areas as cancer therapy, vaccine development and cholesterol management.

Keywords: antisense oligonucleotides, drug delivery, enhanced permeation and retention, gene therapy, liposomal nanoparticles, siRNA

Expert Opin. Drug Deliv. (2008) 5(1):25-44

1. Introduction

Over the past two decades, liposomes have evolved from simple model membrane systems into sophisticated carriers for a wide variety of therapeutic agents [1]. This review focuses on drug delivery systems (DDSs) that are composed of liposomal nanoparticles (LNs; those with a diameter centred ~ 100 nm) encapsulating therapeutic agents. The therapeutic agents can range from conventional drugs, such as anticancer drugs [2,3] and antibiotics [4], to the newer classes of genetic drugs, which includes bacterial plasmids carrying therapeutic genes, antisense oligonucleotides and small interfering RNAs (siRNA) [5-11]. As a result of the wide variety of potential applications, it is convenient to specify three types of liposomal nanomedicines (LNMs), based on the biological effect of the encapsulated agent; the first includes those containing conventional drugs [12-15], the second includes those containing immunostimulatory oligonucleotides [6-9], and the third includes those containing genetic drugs (such as plasmid DNA for gene therapy applications or siRNA for gene silencing applications) [10,16]. The authors examine each of these areas in turn, highlighting the most recent advances and attempting to anticipate future applications and advances. Other types of LNMs, such as those containing peptide and protein drugs [17-19], are beyond the scope of this review and are not discussed.

informa
healthcare

2. Why liposomal nanomedicines work: the enhanced permeability and retention effect

The possibility of using liposomes as carriers of therapeutic agents was recognised soon after their discovery in the 1960s [20,21], even though it took many years before the appropriate technologies were in place [5,22-24]. The goal of LNMs is to reduce side effects while maintaining or enhancing the efficacy of a given drug. The former is achieved by the avoidance of healthy tissues: LNMs are not taken up by healthy tissue as is the free drug. Normal tissues possess a continuous, non-fenestrated endothelium of the vasculature, with tight endothelial junctions (on the order of 5 nm) that prevent the extravasation of small liposomal carriers. The basal tissues also inhibit the extravasation of macromolecules [25]. The latter goal may be achieved (depending on the disease under study and the drug in question) by a form of targeting known as passive targeting, by which LNs preferentially accumulate at sites of disease. Several types of diseased tissues, particularly those found in cancer, possess vascular tissue that is porous and rather leaky. The endothelial cells often have large gaps between them that allow LNs with diameters up to 400 nm [26] to extravasate from the blood into the surrounding tissues, where they accumulate. This effect, now known as 'enhanced permeability and retention' or EPR [27-30], is a fortuitous result of tumour biology. Thus, if LNMs have adequate circulation lifetimes, they can accumulate at tumours where they can either be taken up by cells, or where the drug can slowly leak out and be taken up by the surrounding cells (see **Figure 1**). This explains why most of the LNMs in development today consist of drugs encapsulated within a conventional lipid bilayer (which may contain PEGylated lipids to increase circulation lifetime [31]), and yet have demonstrated impressive results. It is now thought that the EPR effect is universally observed in solid tumours [28].

3. Liposomal nanomedicines for conventional drugs

The use of LNs as DDSs began with attempts to encapsulate small 'conventional' therapeutic drugs. The earliest attempts involving passive entrapment of drugs resulted in formulations exhibiting low encapsulation levels and poor retention properties (see [5,12] for a brief discussion). The first LNs with therapeutic potential followed the development of methods that enabled the rapid production of stable, homogeneous populations of large unilamellar vesicles (LUVs; diameter = 100 nm), in particular the extrusion technique [24], and with the development of the 'remote loading' techniques whereby transmembrane pH gradients were used to drive the uptake and retention of weakly basic therapeutic drugs into preformed vesicles [12] (see **Figures 2** and **3**). Over the past 20 years, at least four different methods of remote loading have been developed. The first and most straightforward involves the formation of LUVs in an acidic medium (usually citrate buffer at pH 4), followed by exchange of the external medium with a neutral buffer (usually HEPES at pH 7.5) by gel exclusion chromatography (see **Figure 3A**). These vesicles, which exhibit a pH gradient (ΔpH) of ~ 3 units, are then incubated with a weakly basic drug such as doxorubicin. At pH 7.5, the doxorubicin exists as a mixture of both charged and neutral forms, with the key point being the recognition that the former is membrane impermeable, and the latter is membrane permeable. Thus, the neutral form of the drug can diffuse across the membrane into the vesicle interior (moving down a concentration gradient), where it is protonated by the acidic citrate buffer into the charged form, which is therefore trapped within the vesicle. As long as the buffer capacity and total quantity of external drug are properly balanced, systems can be designed where essentially complete uptake of the drug occurs, with excellent retention. Other factors that impact retention include the lipid composition (mixtures of saturated phospholipids and cholesterol have been found to be the best). These approaches led to the first LNs exhibiting high encapsulation levels and excellent drug retention, for drugs such as the antifungal agent amphotericin B [32,33], and for the anticancer drugs doxorubicin [34-37] and vincristine [3,38-40]. A variety of *in vivo* studies revealed that some LNs exhibited enhanced potency or greater efficacy, and others had a similar efficacy profile to the free drug but exhibited significantly fewer side effects. The latter situation was observed with the anticancer drug doxorubicin, for which cardiotoxic side effects (one of the factors limiting its long-term use in patients) were greatly reduced in encapsulated form. The former situation was observed with liposomal vincristine, which exhibited a greatly increased efficacy compared with the free drug [3]. Of particular importance in this regard was the observation of significantly enhanced vincristine efficacy as the retention properties of the LNs was improved (an observation not observed with doxorubicin). BDF1 mice with peritoneal ascitic P388 tumours were treated with DSPC/Chol LNs containing vincristine (D/L = 0.1 w/w) and an intravesicular pH of either 4 or 2 (300 mM citrate buffer). Due to the relatively lower pK values of vincristine (pK_1 = 5.0 and pK_2 = 7.4) relative to doxorubicin (pK = 8.6), the former drug is not retained as well as the latter at comparable intravesicular pH values. Hence studies were also performed at pH 2 where retention was improved due to the higher percentage of protonated membrane-impermeable drug. Although the pH 4 systems resulted in a significant increase in the percentage of mice surviving at longer periods post-injection, relative to free drug, all of the mice had perished by day 35. In contrast, 100% of the mice treated with the pH 2 formulation were still alive at day 60 (when the experiment was terminated). In subsequent studies, it was found that replacing DSPC with sphingomyelin gave LNs with excellent retention characteristics and improved

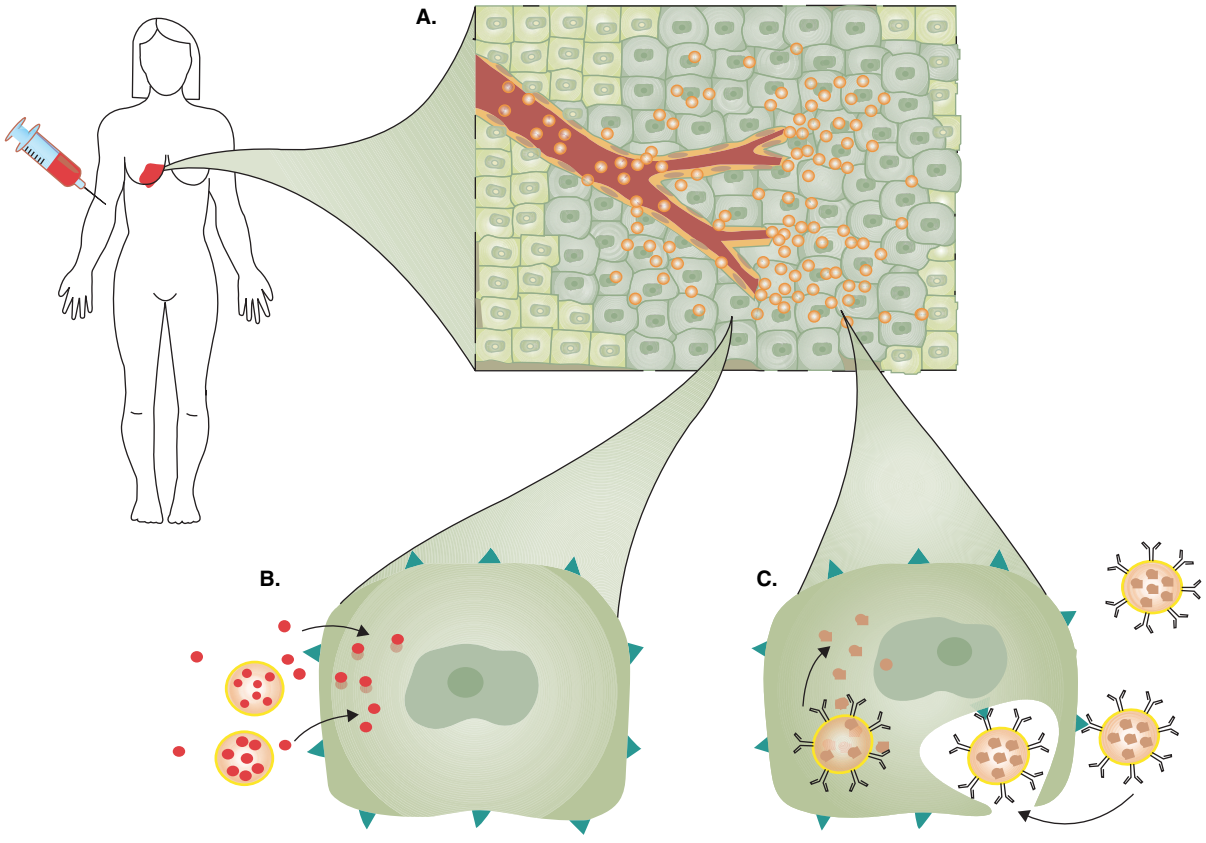

Figure 1. A schematic figure depicting the accumulation of LNs in breast cancer tissue through the EPR effect. A. LNs containing an encapsulated drug extravasate through gaps in the endothelial cells and accumulate in the darker tumour tissue, but not the lighter normal tissue. **B.** In passive targeting, as observed with many formulations of conventional anticancer drugs, the drug is released from LNs in the extracellular space and taken up into the tumour cell. **C.** LNs that contain targeting ligands (such as antibody fragments), or LNs containing encapsulated genetic drugs (SPLPs, SALPs, SNALPs), are taken up via binding to cell surface receptors, which result in internalisation of the particles via the endosomal pathway. Some of the encapsulated material escapes to its intracellular site of action (cytoplasm or nucleus).

Reprinted from [1] with permission.

LN: Liposomal nanoparticle; SALP: Stabilised antisense-lipid particle; SNALP: Stabilised nucleic acid lipid particle; SPLP: Stabilised plasmid-lipid particle.

chemical stability (for internal pH = 4) [41]. Nevertheless, not all drugs showed this clear relationship between increased retention and efficacy. As discussed above, doxorubicin in LNs displays excellent retentive properties while maintaining similar efficacy to the free drug. Similarly, the retention of cisplatin within LNs was found to be excellent, with increased accumulation of the LNs at the tumour, but lower levels of available drug than obtained with free cisplatin [42]. This led to very low activity against tumours in a number of studies [43,44]. Clearly some drugs can be retained too well, to the point where their bioavailability is reduced.

Despite its successful application in several drug delivery systems [12,45], the pH-gradient approach using internal citrate buffer suffers several drawbacks: firstly, it does not permit the formation of vesicles with high drug-to-lipid ratios, and secondly, it does not provide adequate uptake of all weakly basic drugs. The first point is discussed below; an example of the second point is given by the antibiotic ciprofloxacin, a commercially successful, quinolone antibiotic widely used in the treatment of respiratory and urinary tract infections. Ciprofloxacin is a zwitterionic compound that is charged and soluble under acidic and alkaline conditions, but is neutral and poorly soluble in the physiological pH range – the precise external conditions used in the citrate loading technique. This results in encapsulation levels of < 20% when the drug is loaded using the standard citrate technique. However, high levels of encapsulation of ciprofloxacin can be achieved using an alternate ΔpH-loading method that is based on transmembrane gradients of ammonium sulfate [46,47] (see **Figure 3**). This approach involves forming LUVs in a solution of unbuffered ammonium sulfate (usually 300 mM), and exchanging the external solution for unbuffered saline (150 mM NaCl), thereby creating an ammonium sulfate gradient. As the internal ammonium

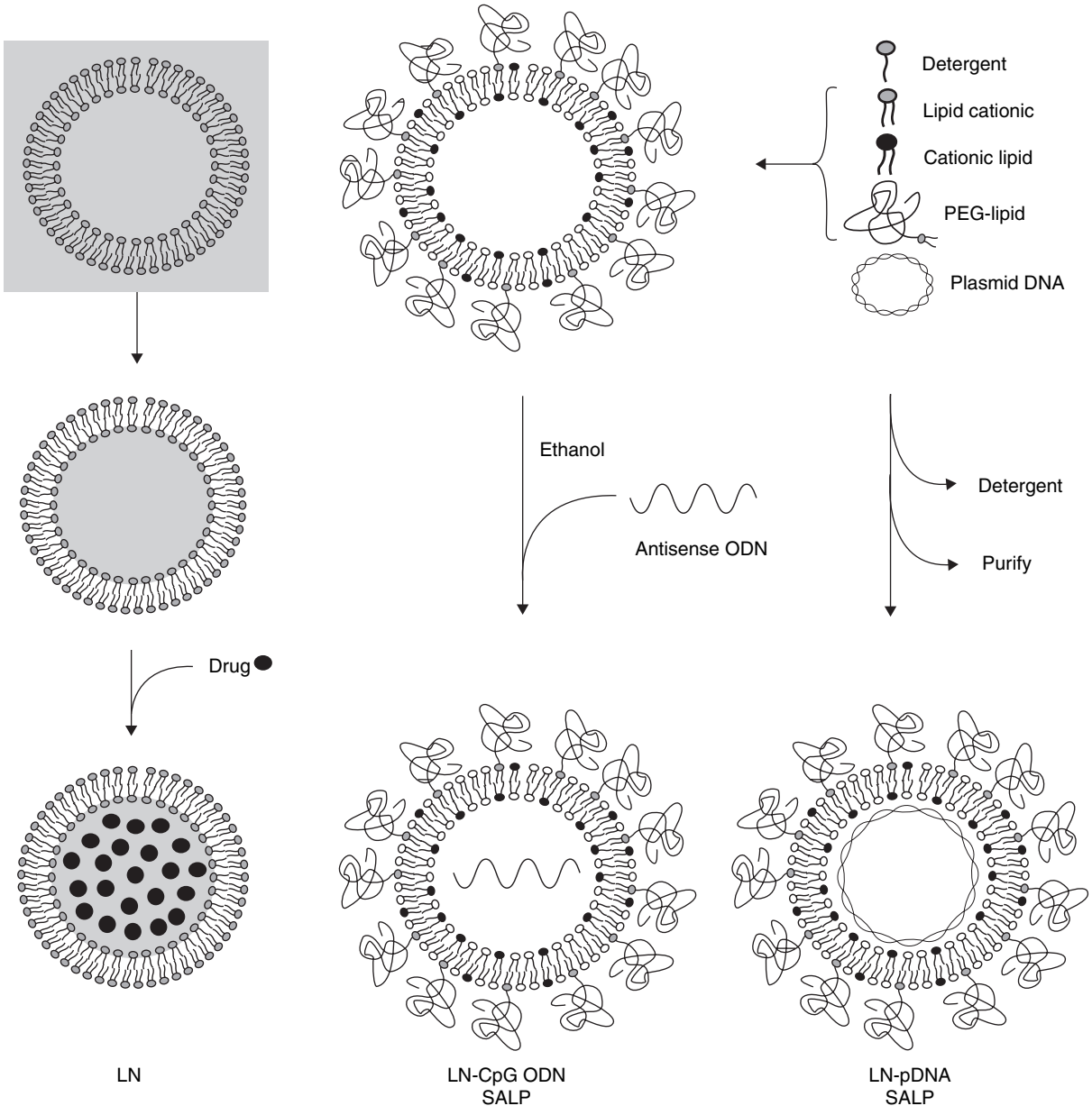

Figure 2. **Diagrammatic comparison of the differences in the methods used to form LNs encapsulating conventional drugs (left column), SALPs (centre column), and SPLPs (right column).** LNs with conventional drugs are formed by loading pre-formed vesicles with drug by means of transmembrane pH or ion gradients (see **Figure 3** for more details). SALPs – LNs encapsulating antisense ODNs – are similar in that the LNs are formed first, with subsequent uptake of ODNs occurring in the presence of ethanol, which destabilises the membrane allowing uptake to occur. The final particles are largely multilamellar and not unilamellar as indicated (see **Figure 7**). SPLPs – LNs encapsulating plasmid DNA – are formed by a detergent-dialysis procedure in which particle formation and plasmid encapsulation occur spontaneously, giving largely unilamellar particles (see **Figure 5**). Variations on these approaches are possible (for example, SALPs can be formed in a one-step process involving the removal of ethanol, and plasmid DNA can be encapsulated in pre-formed vesicles in the presence of ethanol). See text for further details.

LN: Liposomal nanoparticle; ODN: Oligodeoxynucleotide; SALP: Stabilised antisense-lipid particle; SPLP: Stabilised plasmid-lipid particle.

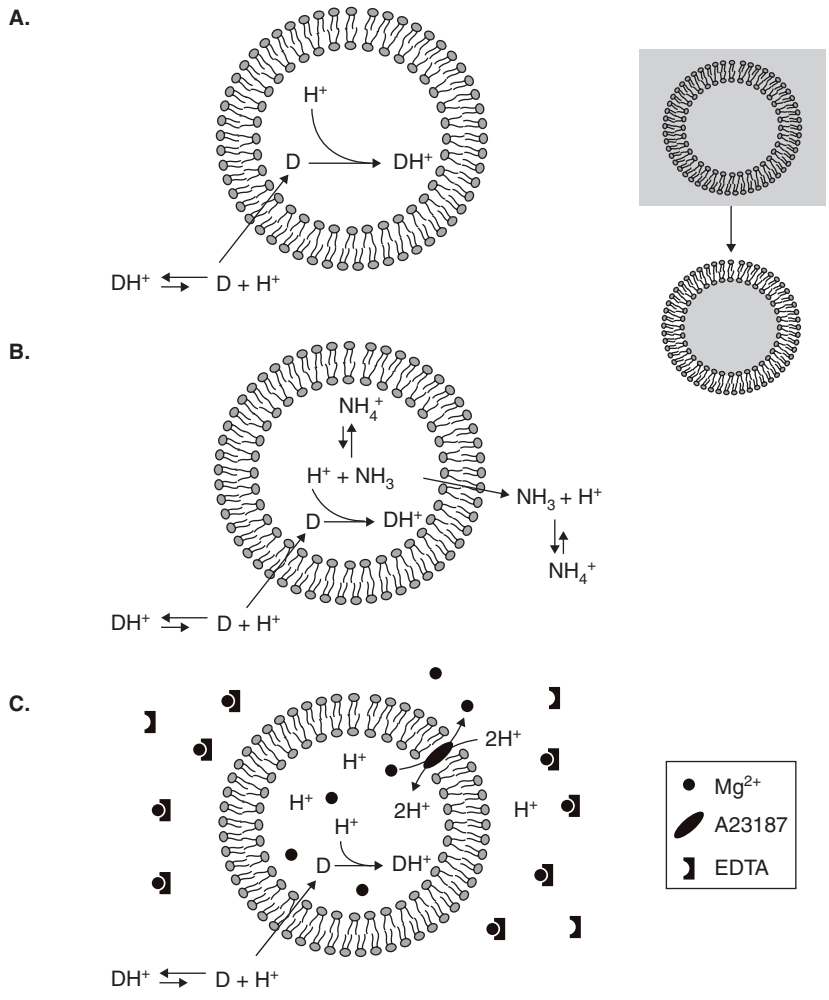

Figure 3. Diagrammatic representations of drug uptake in response to transmembrane pH gradients. Prior to drug loading, it is necessary to establish the primary pH gradient or the primary ion gradient that will generate a ΔpH. Lipid films or powders are initially hydrated and then extruded in the hydration buffer, giving rise to a LN in which both the internal and external solutions are the same (grey shading of upper frame of insert). The LNs are then passed down a gel exclusion column hydrated in a different external buffer, giving rise to LNs with a ΔpH or ion gradient (lower frame of insert). **A.** The standard pH gradient method, with internal citrate buffer (usually 300 mM pH 4) and external HEPES-buffered saline (usually pH 7.5). Many drugs form precipitates within these LNs, which provides an additional driving force for uptake (not shown). **B.** A second method for generating ΔpH involves transmembrane gradients of ammonium sulfate (initial internal concentration usually 300 mM), which creates an acidified vesicle interior as neutral ammonia leaks from the LN. Possible drug precipitation is not indicated. **C.** ΔpH can also be established by ionophores (such as A23187) in response to transmembrane gradients of divalent cations such as Mg^{2+} or Mn^{2+} (solid circles). A23187 couples the external transport of one divalent cation to the internal transport of two protons, resulting in acidification of the vesicle interior. An external chelator such as EDTA is required to bind the divalent cations as they are transported out of the LN. The internal solution is usually a sulfate salt of the cation of interest.

Reproduced from [5] with permission.

LN: Liposomal nanoparticle.

exists in an equilibrium with a small amount of neutral ammonia, the latter will diffuse out of the vesicle (down its concentration gradient), leaving behind its protons and thereby creating an unbuffered acidic interior with a pH ~ 2.7 – 2.8 [4,48]. If external drug is added to these vesicles, neutral drug will diffuse into the vesicle interior, consume a proton and become charged and, therefore, trapped. If continued drug uptake leads to consumption of the available protons, the diffusion of additional neutral ammonia from the vesicles will create more protons to drive drug uptake. This will continue until all the drug has been loaded, or until the internal proton supply is depleted, leaving a final internal pH that ranges between 3.1 – 5.1 [4,48]. The technique is ideal for ciprofloxacin as the drug is supplied as an HCl salt, and thus is acidic and soluble when dissolved in water. In addition, the amine

method results in higher uptake levels for other drugs such as doxorubicin. For 300 mM internal solutions, the highest drug-to-lipid ratios (mol:mol) easily achieved are ~ 0.2 for citrate and 0.4 for ammonium sulfate. A diagrammatic scheme of the method is given in **Figure 3B**.

This technique has been applied to a variety of drugs including doxorubicin [46,47,49,50], epirubicin [50], ciprofloxacin [4,49-51] and vincristine [50]. The method is equally effective using a range of alkylammonium salts (e.g., methylammonium sulfate, propylammonium sulfate or amylammonium sulfate) to drive uptake [50]. One of the key insights that first came from uptake studies using ammonium sulfate was the realisation that many drugs form stable precipitates within liposomes. Doxorubicin precipitates and forms a gel in the vesicle interior that is characterised by fibrous bundles that are easily visualised by cryo-EM [46,49,52]. Other drugs, such as vincristine, appear to form a more amorphous, poorly characterised precipitate [53], and a few, such as ciprofloxacin, do not precipitate [49,51]. Interestingly, ciprofloxacin can reach intraliposomal concentrations as high as 300 mM, and although the drug does form small stacks as shown by ^{1}H-NMR, it does not form large precipitates [51], even though its solubility in buffer cannot exceed 5 mM. As a result of the lack of precipitation and rapid exchange properties, ciprofloxacin can respond rapidly to changes in electrochemical equilibria, such as depletion of the pH gradient. This explains the observed rapid leakage of ciprofloxacin from LUVs in response to serum destabilisation or loss of pH gradient. In contrast, doxorubicin, which is known to form insoluble precipitates within LUVs in the presence of both citrate [52] and ammonium sulfate [46,49], is retained within vesicles in the presence of serum. Thus, in the presence of a pH gradient, both ciprofloxacin and doxorubicin are retained within their LNs, but if the gradient is lost, the former will leak out rapidly but the latter is retained. This is a clear illustration of how the physical state of encapsulated drugs will affect retention under differing conditions and, therefore, may impact efficacy. This important insight is dealt with in more detail in the following section.

The observation that improved remote-loading of ciprofloxacin and doxorubicin could be achieved using LNs based on ammonium sulfate gradients rather than sodium citrate buffers strongly suggested that further development of drug loading methodologies was warranted. This led the present authors' group to develop a new method of remote loading in which ionophores, responding to transmembrane ion gradients (involving Na^+, Mn^{2+} or Mg^{2+}), generate a secondary pH gradient that provides the driving force for drug uptake [54]. The process is illustrated in **Figure 3C**. A primary ion gradient is generated when LUVs formed by extrusion in $MnSO_4$ or $MgSO_4$ solutions are passed down a column equilibrated in a sucrose-containing buffer. Sulfate salts are routinely used rather than chloride salts to avoid dissipating pH gradients, which can occur via the formation of neutral HCl that can diffuse out of the vesicle. Likewise, sucrose is chosen as a component of the external buffer rather than saline, as chloride ions can interfere with some ionophores [55]. After establishing the primary ion gradient, the drug is added, followed by the ionophore A23187 and the chelator EDTA. The A23187 couples the outward flow of a single divalent cation to the inward flow of a pair of protons. The ionophore-mediated ion transport is electrically neutral and results in acidification of the vesicle interior, thereby creating a pH gradient that drives drug uptake. The EDTA chelates calcium and magnesium as they are transported out of the vesicles, and is required to drive drug uptake. The method results in high levels of encapsulation for the drugs ciprofloxacin and vincristine (80 – 90%), and excellent *in vivo* circulation and drug retention properties that are comparable to systems loaded by the citrate or amine methods [54]. Several examples that expand on many of the points already mentioned are discussed below.

The present authors' initial observations regarding the uptake of vincristine were extended by Zhigaltsev *et al.* [56], who examined the *in vitro* and *in vivo* loading and retention properties of three closely related vinca alkaloids (vincristine, vinblastine and vinorelbine) in LNs composed of SPM/Chol. A key result of this study was that drug retention could be significantly improved by increasing the drug-to-lipid ratio (from 0.1 to 0.3 wt:wt), but only for the ionophore (using Mg^{2+}) method of loading. Drug retention remained the same following an increase in drug-to-lipid ratio when the citrate method of loading was used. These differences in retention were attributed to putative differences in the intravesicular forms of the drugs in the two systems.

Similar results have been described in a recent study comparing the encapsulation of topotecan within DSPC/Chol LNs [14]. Of 4 different loading methods examined (citrate method, ammonium sulfate method, and two variations of the ionophore A23187 method (one using $MnCl_2$ and the other $MnSO_4$), only the ammonium sulfate and A23187-$MnSO_4$ methods exhibited high final drug-to-lipid ratios (ranging 0.2 – 0.3) and good retention (10-times better than citrate and $MnCl_2$ methods). In addition, as the drug-to-lipid ratio was increased from 0.1 to 0.2, drug retention was increased five-fold at 36 h at the higher ratio. Cryo-electron microscopy studies revealed the presence of topotecan precipitate within the two sulfate-containing systems. Together, these results demonstrate the improvements in drug retention that can be achieved using an appropriate drug-to-lipid ratio in combination with the correct counter-anion (in this case, sulfate).

Following from these studies, Semple *et al.* were able to optimise and characterise a SM/Chol (55:45) vinorelbine LN that possessed promising antitumour activity [15]. The ionophore method was chosen as vinorelbine is more lipophilic and membrane permeable than the other vinca

alkaloids and, therefore, more difficult to retain within a liposomal formulation. As noted above for other systems, increasing the drug-to-lipid ratio (from 0.1 to 0.3 wt:wt) led to increased retention. The vinorelbine formed electron-dense amorphous precipitates as revealed by cryo-electron microscopy. This system had the best *in vivo* retention, and exhibited significant antitumour activity in a subcutaneous murine B16 melanoma model after a single bolus injection. Similar efficacy was also noted using the same system carrying vinblastine. Finally, this LN possessed excellent storage stability (< 5% drug leakage, > 99% intact drug, and no changes in particle size after 1 year at 2 – 8°C).

Several key observations can be made on the basis of the studies discussed above. The first is that both the quantity of drug that can be encapsulated within a liposome, and the rate of release of that drug (either in the presence of serum or some other agent or *in vivo*), are very dependent on the specific method of drug loading. That this is observed in situations where the magnitude of the pH gradients that drive uptake are similar in magnitude points to the influence of the intravesicular counter-anions, for example, which can form precipitates with different drugs and thereby have a profound effect on the rate of release. Thus both citrate and sulfate anions form stable fibrous gel precipitates with doxorubicin, which accounts for the excellent retention observed with this drug. In contrast, drugs such as ciprofloxacin do not form precipitates in the presence of sulfate, and drugs such as vincristine form a more amorphous precipitate from which drug leakage may occur to a greater extent than observed with doxorubicin.

The second key observation is that, for systems loaded using the ionophore method, drug retention appears to increase with an increase in the drug-to-lipid ratio. As is discussed below, this is direct evidence for the formation of precipitates within the liposome. However, it also raises the question of the relationship between drug retention and efficacy. At first glance, it would appear that the higher the drug-to-lipid ratio, the better. It would be logical to assume that drug efficacy would be dependent on the amount of drug that is delivered to a site of disease. However, what is important is the bioavailability of that drug once it reaches the site of disease, as well as the biochemical method of attack on the cell. Certain drugs such as vincristine are cell-cycle specific and need to be present at a concentration greater than the minimum effective concentration for extended periods of time. Thus, if drug retention within a LN is too high, insufficient levels of the drug may be bioavailable, even though high concentrations of the carrier have accumulated in the tissue of interest (as appears to happen in the cisplatin-LN discussed earlier [42]). Conversely, if a drug is not adequately retained within a LN, it will not accumulate at a tumour site and will act like free drug. These considerations suggest that for different drugs and LNs, there should be an optimal rate of drug release that will give maximal efficacy.

To the present authors' knowledge, the first and only demonstration of therapeutically optimised rates of drug release have been reported in a recent paper by Johnston *et al.* [53]. Vincristine was loaded into SM/Chol LNs at drug-to-lipid ratios 0.025 – 0.7 (wt:wt) using the ionophore method. For each drug-to-lipid ratio, release rates were measured by either an *in vitro* assay using ammonium chloride or fetal bovine serum, or from *in vivo* pharmacokinetic studies. From the first-order release rates, half-lives of release $T_{1/2}$ were determined. These were 15 – 161 min *in vitro* and 6 – 65 h *in vivo*. A plot of $T_{1/2}$ versus drug-to-lipid ratio gave a straight line fit. The key results came from *in vivo* antitumour efficacy studies with LNs possessing drug-to-lipid ratios of 0.025, 0.05, 0.1 and 0.6, and $T_{1/2}$ values of 6.1 h, 8.7 h, 15.6 h and 117 h, respectively. All of the LNs were more efficacious than free drug, and caused a significant decrease in median tumour volume over a period of 30 days (**Figure** 4), but only the LNs with a drug-to-lipid ratio of 0.1 maintained that reduction up to 56 days; in all other cases tumour regrowth occurred. A plot of tumour growth delay versus $1/T_{1/2}$ clearly demonstrated the optimised efficacy observed for a specific release rate. It should be noted that the necessity to terminate the experiment at 56 days means that the actual delay in tumour growth (and therefore efficacy) was significantly higher than calculated. Furthermore, due to the small number of data points all that can be said is that the optimised efficacy occurs somewhere between a drug-to-lipid ratio of 0.1 and 0.6. Further work is necessary to fill in the curve to assess whether the optimised retention rate is narrow or fairly broad, and to see whether these observations apply to other drugs as well.

A fairly straightforward application of Fick's Law of diffusion allows for some insight into the mechanism behind the increased retention observed with increasing drug-to-lipid ratio. Fick's first law of diffusion states that the flux J (mol cm^{-2} s^{-1}) of a molecule M down a concentration gradient is proportional to the product of the diffusion coefficient D (cm^2 s^{-1}) and the concentration gradient dC/dx (mol cm^{-4}).

$$J_M = -D\frac{dC}{dx} \quad (1)$$

If the diffusion is occurring across a membrane of infinitesimal thickness (dx), this can also be expressed in terms of the rate of diffusion dM/dt (mole s^{-1}) of M across the membrane and the cross-sectional area A (cm^2) of the diffusion volume:

$$\frac{dM}{dt} = -DA\frac{dC}{dx} \quad (2)$$

In addition, the concentration gradient dC can be approximated by $C_1 - C_2$, and as the membrane thickness

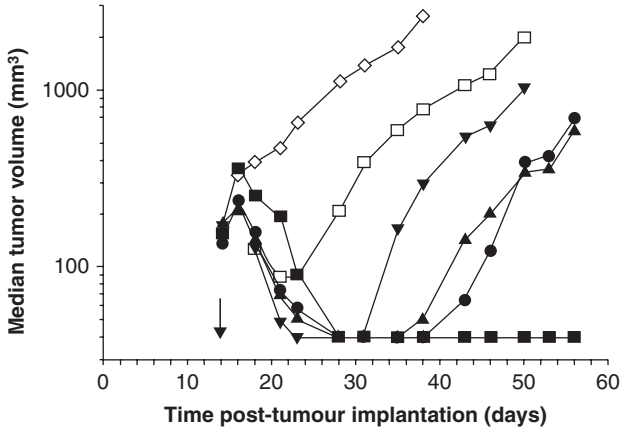

Figure 4. Therapeutic effects of LNs encapsulating vincristine with different drug-to-lipid ratios (and correspondingly different release rates) in the MX-1 human mammary xenograft model. MX-1 tumours were planted in the flanks of nude mice and allowed to grow for 14 days, at which time the animals were treated with a single intravenous dose of LN-vincristine (1.5 mg/kg) or the appropriate control. Empty vesicles were used to ensure each animal received the same lipid dose as the drug-to-lipid ratio was varied. Treatments: 300 mM sucrose (◇), free vincristine (□) or LN-vincristine with drug-to-lipid ratios (wt/wt) of 0.025 (▼; $T_{1/2}$ = 6.1 h), 0.05 (▲; $T_{1/2}$ = 8.7 h), 0.1 (■; $T_{1/2}$ = 15.6 h) and 0.6 (●; extrapolated $T_{1/2}$ = 117 h).
Reprinted from [53] with permission.
LN: Liposomal nanoparticle.

is constant, dx can be grouped with the other constants to give a first-order rate constant k ($cm^3\ s^{-1}$):

$$\frac{dM}{dt} = -k(C_1 - C_2) \quad (3)$$

For a spherical membrane, with an internal volume V, both sides of the equation can be divided by V to express the rate of diffusion in terms of concentration. This also changes the rate constant k to one with units of s^{-1}. Furthermore, it can be assumed that C_1 (the internal concentration) >> C_2 (the external concentration) to give:

$$\frac{dC}{dt} = -kC_i \quad (4)$$

Equation 4 can then be integrated between time 0 and time t to give the familiar first-order rate equation below. For the specific case where we are considering the efflux of a drug D from a LN, the initial internal drug concentration can be expressed as [D_i (0)], and the internal drug concentration at time t as [D_i (t)]:

$$[D_i(t)] = [D_i(0)]e^{-kt} \quad (5)$$

From Equation 5 it is possible to see that $[D_i(t)]/[D_i(0)] = e^{-kt}$, and so it follows that the precentage of drug released over time should be independent of the initial internal drug concentration, depending only on the time over which release has occurred. However, this was not observed experimentally: the drug release expressed as a percentage of the initial internal drug concentration was highly dependent on the initial drug-to-lipid ratio. The fact that a plot of the $T_{1/2}$ of release versus initial drug-to-lipid ratio was linear strongly suggested that a large proportion of encapsulated drug was present as a precipitate. If all drug was soluble within the liposome, then the efflux rates should be proportional to the total amount of encapsulated drug (Equation 4) and the percentage of drug released over time should be independent of the initial interior drug concentration (Equation 5). $T_{1/2}$ would remain constant with drug-to-lipid ratio. However, if a sizeable proportion of the drug is precipitated, then only a small quantity of soluble drug will be present. The efflux rate will be proportional to the amount of drug in soluble form, which will remain constant until all the precipitate is dissolved. Thus, the half-life of release will be proportional to the total drug, most of which is in precipitated form. Under these conditions, the half-life of release will be directly proportional to the initial drug-to-lipid ratio, as was observed. The prediction of drug precipitation obtained from analysis of the release data was also confirmed by cryo-transmission electron micrographs, which showed the presence of an amorphous electron dense precipitate at higher drug-to-lipid ratios.

The most extensively studied vincristine-based LNs is the formulation developed by Inex Pharmaceuticals Corporation (now Tekmira Pharmaceuticals Corporation) known as Marqibo®, which consists of 100 nm LUVs composed of SPM:Chol (55:45), a drug-to-lipid ratio of 0.1 (wt:wt), and 300 mM internal citrate pH 4. As discussed above, this formulation exhibits long circulation lifetimes, greatly enhanced tumour accumulation and enhanced *in vivo* efficacy in appropriate animal tumour models. Although the loading methods are different, the drug-to-lipid ratio employed in these and subsequent human studies is close to the optimal value determined by Johnston *et al.* [53]. The key question is whether these *in vivo* benefits are reflected in human studies, and the answer so far appears to be very positive. A Phase II study was performed of liposomal vincristine in first line therapy in patients with untreated aggressive B-cell non-Hodgkin's lymphoma [57-60]. The standard treatment for this disease is the drug cocktail known as CHOP, which is composed of cyclophosphamide, doxorubicin, vincristine and prednisone. In this study, liposomal vincristine (Marqibo) was substituted for vincristine in the formulation known as lipo-CHOP, and the efficacy of CHOP versus CHOP + rituxan [61] versus lipo-CHOP + rituxan was examined. For these three treatments, complete response rates of 63% versus

76% versus 91% were obtained, with overall survival rates at 22 – 24 months of 57% versus 70% versus 98%, respectively. Clearly the vincristine LN results in significant improvements in the efficacy of this drug cocktail, and validates further trials of this and other LNMs in humans (many of which are presently in progress).

The fact that drug efficacy is highly sensitive to drug release rates, especially for drugs that only act at a certain point in the cell cycle (such as vincristine), demonstrates the importance of having a variety of methods available for regulating the rate of drug release. This highlights the need for developing new methods of drug encapsulation, and for investigating each existing technology for each drug of interest. Of particular interest at this time will be *in vivo* animal studies that fine-tune the relationship between drug retention rates and antitumour efficacy for different loading systems. Other advances in this area may come from alternate loading methods or approaches that will enhance drug retention or result in different types of intravesicular drug complexes. One promising area involves drug uptake that is driven by the formation of drug–metal complexes. For example, it has been shown that doxorubin uptake can be driven by Mn^{2+} gradients in the absence of pH gradients, with the driving force being the formation of Mn^{2+}–drug complexes that exhibit a different morphology by cryoEM than those complexes formed with citrate or sulfate ion [13,62]. This approach, when applied to two formulations of thermosensitive liposomes, resulted in significantly higher doxorubicin encapsulation levels than a pH gradient approach (drug-to-lipid ratios of 0.2 compared with 0.05, respectively) [63]. Other drugs may be encapsulated in a similar manner but not necessarily via Mn^{2+}. For example, topotecan can be encapsulated via the formation of complexes with copper in the absence of pH gradients – an effect that was not observed with other ions [64].

A similar approach for the enhancement of drug retention within LNs involves the formation of intraliposomal complexes involving complex anions such as hydroxybenzenesulfonate [65] or the triethylammonium salts of a linear polyphosphate and sucrose octasulfate [66]. The former approach was applied to the drugs ciprofloxacin and vinorelbine, and resulted in a substantial increase in the *in vivo* half-life of release of these drugs (30 h) relative to LNs containing $MgSO_4$ alone (6.4 h). Likewise, the latter approach allowed the generation of an irenotecan formulation with an *in vivo* half-life of drug release in the order of 57 h. Clearly these precipitating agents have the potential to greatly extend the release rates of many drugs.

Finally, it must not be forgotten that varying the lipid composition of the LNs can also extend drug release rates. Although much of the discussion has focused on drug-to-lipid ratios and drug precipitation within LN, it must be kept in mind that the systems under discussion already possess, in most cases, a lipid composition optimised for retention properties. In general, this includes a phospholipid containing highly saturated acyl chains, and high concentrations of cholesterol, both of which function to increase the order of the membrane. As discussed earlier, most vincristine LNs are formed from SM:Chol; replace the SM with DSPC and both retention and efficacy will drop dramatically [3]. One exciting recent development was the observation that LNs containing dihydrosphingomyelin (in which the native double bond is reduced) had drug release rates and circulation lifetimes that were increased approximately threefold and two-fold, respectively, over LNs containing sphingomyelin [67]. These improvements can be further enhanced by then using higher drug-to-lipid ratios or one of the other approaches discussed above.

The development of multiple loading methodologies has lead to recognition that LNMs can be developed that take advantage of combination treatment for diseases such as cancer. In principle, it should be possible to co-encapsulate two or more drugs within a single LN, with each drug present at an optimised concentration that will give rise to synergistic effects (where the efficacy of the combination drugs is greater than their additive effects alone). The rationale for co-encapsulation derives from the enhanced efficacy obtained from drug cocktails such as CHOP that are increasingly used in cancer chemotherapy. The challenges ahead and the general direction to pursue have been discussed [68], and several combination formulations have been developed with some very promising results. Doxorubicin and vincristine have been co-encapsulated within the same LNs using Mn^{2+} loading and ionophore-mediated pH gradient loading to drive the uptake of the two drugs, respectively [69]. Irenotecan and floxuridine have been co-encapsulated into a low-cholesterol formulation using a combination of copper complex formation and passive entrapment [70]. In a comprehensive study examining three different co-encapsulated drug pairs, Mayer *et al.* [71] demonstrated that synergistic, additive and antagonistic activity against tumour cells could be achieved *in vitro* simply by varying the encapsulated drug ratios, and that synergistic ratios could be maintained *in vivo* and lead to increased efficacy in preclinical tumour models. This suggests that combination therapy will play an important role in the next generation of LNs.

The encouraging results observed so far with anticancer LNMs in humans, coupled with the potential discussed above for further development of the technology, bodes well for significant improvements in the treatment of cancer and other human disease. However, despite the large number of formulations now in preclinical development and in clinical trials (of which a few are listed in **Table 1**), few LNMs have received approval for commercial development. Those that have are listed in **Table 2**, and represent several formulations of the anti-fungal agent amphotericin B and the anticancer drugs doxorubicin and daunorubicin.

Table 1. Selected liposomal drug formulations in preclinical development and in clinical trials.

Product	Company	Drug	Therapeutic indication
Marqibo® (liposomal vincristine)	Tekmira Pharmaceuticals [124] Hana Biosciences [125]	Vincristine	Cancer
INX-0125/Alocrest™ (sphingosomal vinorelbine)	Tekmira Pharmaceuticals [124] Hana Biosciences [125]	Vinorelbine	Cancer
INX-0076 (Sphingosomal topotecan)	Tekmira Pharmaceuticals [124] Hana Biosciences [125]	Topotecan	Cancer
Liposomal ciprofloxacin	Tekmira Pharmaceuticals [124] Aradigm Corporation [126]	Ciprofloxacin	Cystic fibrosis, inhalation anthrax
Liposomes	Tekmira Pharmaceuticals [124] Pfizer [127]		Atherosclerosis
INX-0167 (immune stimulatory oligonucleotides)	Tekmira Pharmaceuticals [124]	CpG-ODN	Vaccine development
Lipid-encapsulated siRNA products	Tekmira Pharmaceuticals [124] Alnylam Pharmaceuticals [128]	siRNA	Various
SPI-077 (liposomal cisplatin)	Alza [129]	Cisplatin	Cancer
Aroplatin™	Antigenics [130]	Diaminocyclohexane form of platinum	Cancer
ThermoDox™	Celsion Corporation [131] Duke University Medical Centre	Temperature-sensitive liposome encapsulated-doxorubicin	Cancer

ODN: Oligodeoxynucleotide.

Table 2. Approved liposomal and lipid-based drug formulations.

Product	Company	Drug	Therapeutic indication
AmBisome®	Gilead [132]	Amphotericin B	Systemic fungal infections
Abelcet®	Enzon Pharmaceuticals [133]	Amphotericin B	Systemic fungal infections
Amphocil®/Amphotec®	Samaritan Pharmaceuticals [134] Three Rivers Pharmaceuticals [135]	Amphotericin B and cholesteryl sulfate	Systemic fungal infections
Doxil®	Alza [129]	Doxorubicin	Cancer (metastatic ovarian cancer, AIDS-related Karposi's sarcoma)
DaunoXome®	Gilead [132] Diatos [136]	Daunorubicin	AIDS-related Karposi's sarcoma
Myocet®	Sopherion Therapeutics [137]	Doxorubicin	Metastatic breast cancer

4. Liposomal nanomedicines for genetic drugs

The sequencing of the human genome will open the floodgates of development of DNA-based drugs as an increasing number of genes involved in human diseases are identified. Already a great deal of research has been expended in the development of several new classes of genetic drugs, which include plasmid DNA carrying therapeutic genes for gene replacement applications, antisense oligonucleotides for immumostimulatory applications, and siRNAs for gene silencing applications, among others [72]. Much present effort is directed at development of LNMs composed of LNs capable of accessing distal disease sites following systemic (intravenous) administration. Numerous methods exist for effective *in vitro* delivery of nucleic acid molecules, such as the use of viral vectors, lipoplexes and lipopolyplexes; however, these all have limited use for systemic applications [73]. For *in vivo* applications, genetic drugs need to be encapsulated within LNs that possess the optimised *in vivo* characteristics of conventional drug carriers: small size, serum stability and long circulation lifetimes. Nucleic acids require protection from serum endonucleases, yet need to circulate for a time sufficient to allow accumulation at sites of disease (EPR effect). In addition, uptake of the particle by the cell of interest must occur so that intracellular delivery of the particles contents can occur (see **Figure 1**). Development of such carrier systems was long hampered by the technical challenges involved in

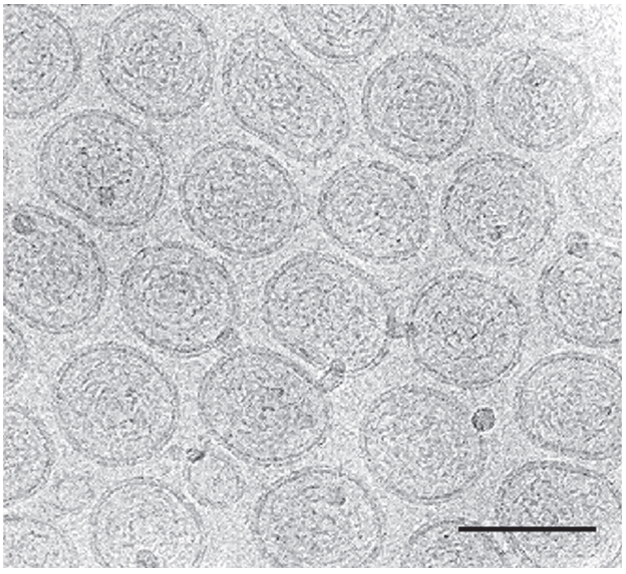

Figure 5. Cryo-transmission electron micrographs of SPLPs prepared from DOPE:DODAC:PEGCerC$_{20}$ (83:7:10 mol:mol:mol). The mean particle diameter is 70 nm. The bar indicates 100 nm.
Reprinted from [75] with permission.
SPLP: Stabilised plasmid-lipid particle.

encapsulating large, highly charged molecules within small vesicles. A diagrammatic comparison of the different methods used to form LNs encapsulating conventional and genetic drugs is shown in **Figure 2** (see text and figure legend for details).

4.1 Encapsulation of nucleic acids

Recently, several different approaches have proven fruitful in generating LNs capable of systemic delivery of small nucleic acids or plasmid DNA. The first of these was designed specifically for plasmid DNA (carrying a marker gene such as luciferase or a therapeutic gene) and employs a detergent-dialysis approach for the formation of liposomal DNA carriers known as stabilised plasmid-lipid particles (SPLPs) [74-77]. The formation of these particles is outlined in **Figure 2**, and representative cryo-electron micrographs are shown in **Figure 5**. SPLPs are small (~ 70 nm), monodisperse particles consisting of a single plasmid encapsulated in a unilamellar lipid vesicle composed of a fusogenic lipid (DOPE), a cationic lipid (usually DODAC), and PEG-ceramide containing a 20 carbon saturated fatty acyl chain (PEG-CerC$_{20}$). The cationic lipid plays two essential roles: primarily it interacts with DNA and aids in the condensation of the large plasmids so that encapsulation can occur. Later, after the particles have accumulated at disease sites and some PEG-Cer has diffused away from the particle, the cationic lipid may aid in interactions with cells leading to endocytosis. The method involves co-dissolving the lipids and DNA in a solution of the detergent octylglucopyranoside prepared in HEPES-buffered saline, and dialysing this mixture against HEPES-buffered saline containing 140 – 150 mM NaCl. If higher concentrations of cationic lipid are desired than the usual 8 mol%, multivalent ions such as citrate must also be present [78]. After removal of unencapsulated DNA, the particles are purified by centrifugation. SPLPs protect plasmid DNA from DNaseI and serum nucleases [74], possess extended circulation half-lives (6 – 7 h) [75], and have been shown to accumulate in distal tumour sites (to concentrations in excess of 1000 plasmids per tumour cell) with subsequent gene expression in distal mouse tumour models following intravenous injection (**Figure 6**) [73]. This gene expression is 100- to 1000-times greater than observed in any other tissue. The particles can also be formulated using PEG-diacylglycerols with similar circulation lifetimes and levels of gene expression in distal tumour models [16].

The second method involves entrapping smaller polynucleotides (usually antisense oligonucleotides), via electrostatic interactions with an ionisable aminolipid (DODAP), within pre-formed ethanol-destabilised cationic liposomes [79-81]. This gives rise to particles known as stabilised antisense-lipid particles (SALPs) (**Figures 2** and **7**). Initially, an ethanolic liposome solution is formed by addition of lipids (DSPC/Chol/PEGCerC$_{14}$/DODAP) dissolved in ethanol to an aqueous buffer with subsequent extrusion [80], or by addition of ethanol to preformed vesicles of the same composition [79]. The addition of oligonucleotide to the ethanolic liposome solution (following acidification with citrate buffer [pH 4] to protonate the cationic lipid), drives the formation of multilamellar liposomes (as well as some unilamellar and bilamellar vesicles), that trap oligonucleotides between the bilayers. The end result is a multilamellar vesicle with a small diameter (70 – 120 nm), capable of entrapping up to ~ 2200 oligonucleotide molecules per 100 nm liposome [79]. A cryo-electron micrograph illustrating this multilamellar structure is shown in **Figure 7**. The SALPs exhibit extended circulation half-lives, ranging 5 – 6 h for particles formed with PEGCerC$_{14}$ to 10 – 12 h for particles formed with PEGCerC$_{20}$ [80]. As with SPLPs, they possess the combination of high entrapment efficiencies, small size and extended circulation lifetimes necessary for effective *in vivo* delivery of antisense drugs.

Both of the methods described above are suitable for the formation of small preparations of SPLPs or SALPs [77]. Larger-scale preparations suitable for animal studies are feasible [76], but technically more demanding, with encapsulation efficiencies and final yields extremely sensitive to minor variations in such parameters as the ionic strength of the formulation buffer. However, even the larger-scale methods are inadequate for preclinical and clinical studies. An important step forward was, thus, realised with the development of a simple and fully scalable method for the formation of SPLPs by spontaneous vesicle formation [82]. This method is related to that used in the formation of

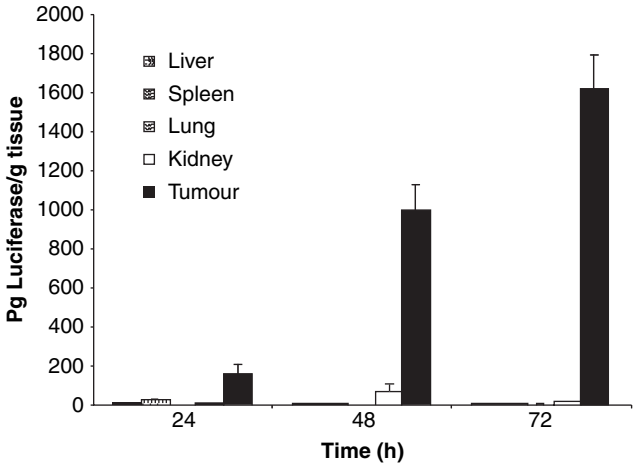

Figure 6. SPLPs (encapsulating plasmid DNA with the gene for luciferase) give rise to tumour-specific gene expression following intravenous injection in neuro-2A tumour-bearing mice. Tumours were grown on the hind flank of A/J mice following injection of 10^6 cells. Note that essentially all of the gene expression is in the tumour tissue, with minimal levels of expression in liver, spleen, lung and kidney.
Reprinted from [73] with permission.
SPLP: Stabilised plasmid-lipid particle.

SALPs as it involves the formation of particles in the presence of ethanol. Briefly, the DNA is dissolved in a Tris-EDTA-citrate buffer, and the lipids in 90% ethanol. The lipids are DSPC, Chol, DODMA (a more stable titratable cationic lipid with a pK_a = 6.8), and PEG-S-DSG (which replaces the PEG-Cer). Both solutions are heated to 37°C, and mixed using a peristaltic pump with dual pump heads. This results in spontaneous vesicle formation (SVF); further dilution steps with a saline citrate buffer stabilise the SPLPs, and free plasmid is then removed followed by concentration by tangential flow ultrafiltration. These SPLPs have similar properties to those formed by detergent-dialysis: monodisperse vesicle populations with particle sizes < 200 nm, DNA encapsulation efficiencies > 80%, circulation half-lives of 13 h, and tumour accumulation and gene expression similar to regular SPLPs. There are some differences: whereas SPLPs prepared by detergent dialysis are predominantly unilamellar, the SVF-SPLPs are a mixture of unilamellar, bilamellar and multilamellar vesicles. Significantly, the method was equally successful with preparations ranging from < 1 mg of DNA to those approaching 5 g, and thus is suitable for both basic research as well as preclinical or clinical studies.

Despite the clear successes that have been observed with SPLPs, specifically the avoidance of first-pass organs with particle accumulation and gene expression at distal tumour sites, it is not yet known whether the observed levels of gene expression will be adequate in therapeutic applications. A key area of research will, thus, involve increasing the transfection potency of SPLPs. Several relevant parameters have already been elucidated that affect transfection levels *in vitro*, such as increasing the content of cationic lipid [78], or post-insertion of a cationic PEG-lipid into pre-formed SPLPs [83,84]. Recent studies involving siRNA have shown that the degree of unsaturation of the cationic lipid has a profound effect on the intracellular delivery of nucleic acids to cells *in vitro* [85]. Unfortunately, many of these approaches are not of use *in vivo*, and others have yet to be investigated. However, in a recent study by Heyes *et al.* [86] it was reported that the SVF-SPLPs methodology could be used to encapsulate polyplexes of DNA with either poly(ethyleneimine) (PEI) or poly-L-lysine, giving rise to polyplex-SPLPs with size and circulation characteristics similar to regular SPLPs. Polyplexes are macromolecular complexes formed between DNA and a suitable polyanion, which function as excellent transfection vectors *in vitro* [73]. The polyplex-SPLPs containing PEI gave rise to a sixfold enhancement in gene expression in a Neuro-2a model relative to SPLPs. Thus the pharmacological properties of plasmid DNA can be changed by formation of polyplexes and encapsulation within an SPLP, highlighting the need for further research into formulation methods involving these and other polyanions.

4.2 Gene silencing via RNA interference: encapsulation of siRNA within stabilised nucleic acid lipid particles

One of the strengths of the SVF technique stems from its effectiveness at encapsulating a variety of different nucleic acid molecules: both DNA and RNA, ranging from small fragments to large plasmids. Recently, the focus of research involving this approach has shifted from plasmid DNA to a new, exciting class of drugs known as siRNAs. When the LNs encapsulate siRNA the particles are commonly known as stabilised nucleic acid lipid particles (SNALPs). siRNAs are short double-stranded RNA effector molecules involved in the mechanism of post-translational gene silencing known as RNA interference [87-90]. When dsRNA molecules are introduced into a cell, they are recognised and cleaved by the enzyme Dicer (a member of the RNaseIII family of dsRNA-specific ribonucleases), resulting in 19 – 23 bp dsRNA duplexes. These are incorporated into the multi-protein complex, RNA-induced silencing complex, where the antisense strand is used to guide the complex to recognise and cleave target mRNA. The end result is inhibition of gene expression, the desired endpoint of another class of genetic drugs known as antisense oligodeoxynucleotides (ODNs), which inhibit gene expression by virtue of their ability to bind to specific mRNA sequences and interfere with protein synthesis [91]. Although antisense ODNs have shown promise in the downregulation of certain genes (see discussion in [5]), siRNAs appear to be much more potent at silencing gene expression [88], and thus have received more attention in this regard. However, as discussed

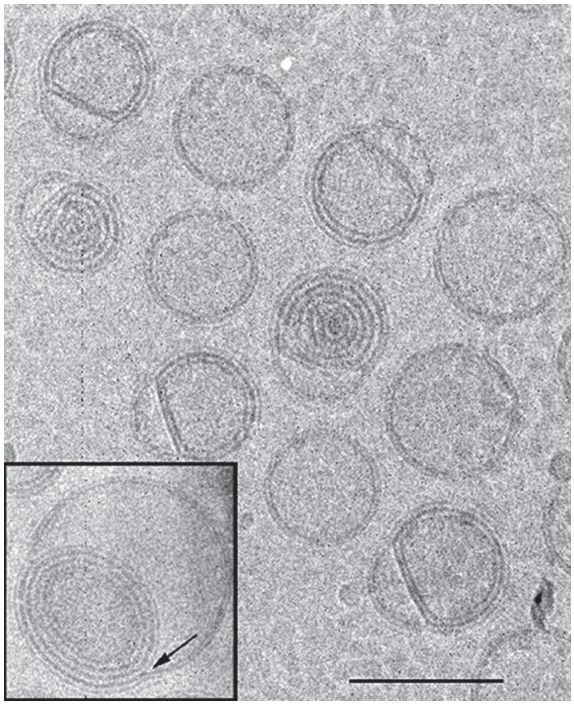

Figure 7. Cryo-transmission electron micrographs of SALPs prepared from DSPC/Chol/PEG-CerC_{14}/DODAP (20:45:10:25 mol %), with an entrapped antisense-to-lipid weight ratio of 0.125 mg/mg. The unique multilamellar lipid bilayer structure can be seen in several of the particles. The inset is an expanded view of a SALP particle showing two initially separate membranes forced into close apposition by bound oligonucleotides (indicated by the arrow). The bar indicates 100 nm.
Reprinted from [79].
SALP: Stabilised antisense-lipid particle.

below, the encapsulation of antisense ODNs in SALPs may prove beneficial in totally different applications involving the stimulation of the immune system.

Several recent studies highlight the therapeutic potential of SNALPs and RNA interference in the treatment of a variety of diseases. The effect of siRNA targeted to hepatitis B virus has been examined in mice carrying replicating HBV. SNALPs administered by intravenous injection gave rise to significant and long-lasting reductions in HBV DNA levels – an effect that was maintained for up to 6 weeks with weekly dosing. In another study, the ability of siRNA to protect against infection by the Zaire species of Ebola virus was examined using guinea pigs [92]. The Ebola virus causes a severe and often lethal haemorrhagic fever for which there is no effective human treatment. The efficacy of siRNA targeting the polymerase gene of the virus was compared when delivered in polyplexes with PEI, and in SNALPs. In each case, the siRNA LNs was administered shortly before or after a lethal viral challenge. Although the siRNA-PEI polyplexes provided some protection against viral infection when administered shortly before the challenge, the SNALP LN was found to completely protect the guinea pigs against viremia and death when administered an hour after the challenge. Although there was some question of an immune response contributing to the results, it would appear that RNA interfernece played a significant role in the observed antiviral effects.

An elegant and relevant example of RNA interference gene silencing, from the point of view of the treatment of human disease, comes from a recent paper by Zimmermann *et al.* [10]. This paper describes a new approach for the treatment of high serum cholesterol levels, based on the silencing of the liver *apoB* gene in non-human primates. Intial studies in mice allowed for the development and testing of an siRNA that targeted apoB mRNA (with cross-reactivity to mouse, human and monkey genes), was effective *in vitro* in gene-silencing studies, and did not possess immunostimulatory activity. The siRNA was formulated in SNALPs, and evaluated for pharmacokinetics, efficacy and safety in cynomolgus monkeys. Although the circulation half-life was shorter (72 min) than other liposomal DDSs, the key result was a striking dose-dependent reduction of liver apo-B mRNA levels following systemic administration of siRNA-SNALPs. For example, a single 2.5 mg dose of siRNA in SNALPs resulted in a reduction of liver apoB mRNA of 90% by 48 h – an effect which persisted up to 11 days (**Figure 8**). This led to maximum reductions in blood apoB-100, cholesterol and low-density lipoprotein levels of ~ 78, 62 and 82%, respectively, which were also observed over the same time period. No reductions in high-density lipoprotein levels were observed. These results were found to exceed the results obtained with presently approved cholesterol-lowering drugs (such as the HMG-CoA reductase inhibitors). Although the termination of the study at 11 days did not allow for full evaluation of the time course of RNA interference-mediated effects, the positive results were buoyed further by the absence of any observed toxicities.

4.3 Enhanced immunostimulation via encapsulation of CpG-oligodeoxynucleotides

A final application of liposomal DDSs containing nucleic acids involves those encapsulating small oligonucleotides: these can give rise to dramatic immunostimulatory properties, with applications ranging from vaccine adjuvants to enhancing antibody-dependent cellular cytotoxicity. The ability of bacterial and viral DNA to stimulate an innate immune response, with release of inflammatory cytokines and interferons, is well known [93-95]. This has also been observed with dsRNA [96], ssRNA [97] and siRNA [98]. The strong response to bacterial DNA results from the recognition of unmethylated CpG dinucleotides in a particular base context – mammalian DNA has a lower frequency of CpG sequences and they are usually methylated [99]. This recognition, and that of the other nucleic acids, is mediated by a class of innate pattern recognition receptors, known as

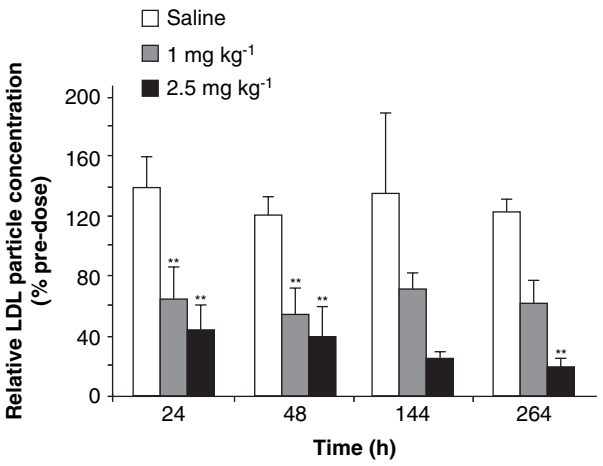

Figure 8. Effect of RNA interference-mediated silencing of APOB mRNA in non-human primates on the levels of circulating LDL. Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg kg^{-1} SNALP-siApoB-2, and measured for LDL. The results are expressed as a percentage of pre-dose values and are expressed as mean ± standard deviation. Data points marked with astericks are statistically significant compared with saline-treated animals ($^{*}p < 0.05$, $^{**}p < 0.005$).

Reprinted from [10] with permission.

LDL: Low-density lipoprotein; SNALP: Stabilised nucleic acid lipid particle.

the Toll-like receptors (TLRs) [95,96,99-101]. Of the ten TLRs that have now been identified, two (TLR3 and TLR9) are responsible for the nucleic acid response [96,99,100], and a third (TLR7) appears to mediate the immune response to siRNA [102].

For the applications under discussion, an immune response is not only desirable but essential. For others, such as the gene silencing effects of siRNA, an immune response can lead to toxicity issues and off-target gene effects associated with the inflammatory response [98,103]. Fortunately, appropriate minor chemical modifications of the siRNA have been able to produce non-inflammatory siRNA without disrupting their gene-silencing ability [104]. Although antisense oligonucleotides were originally designed to bind to mRNA and reduce the production of specific proteins, their success rate *in vivo* was variable, and it soon became clear that siRNAs were far more effective gene-silencing agents. However, small ODNs possessing unmethylated CpG dinucleotides within specific sequence contexts (CpG motifs) were quickly recognised for their immune stimulating abilities, and in this context the power of encapsulating ODNs within LNs readily becomes apparent.

Antisense oligonucleotides are rapidly degraded by serum nucleases, but their stability can be greatly enhanced by replacing non-bridging oxygen atoms with sulfur atoms to form a phosphorothioate backbone (PS ODN) [105,106]. Although the phosphodiester ODNs were found to have a greater stimulatory effect on NK cells *in vitro* [106], they usually elicit little or no immune response *in vivo* due to rapid degradation. However, PS ODNs show promise as immune adjuvants, as they significantly improve the immune response to co-administered antigens, stimulating a strong T-helper 1 response [107,108], and can also protect against a variety of microbial pathogens [109,110]. Unfortunately, they are also cleared rapidly from the circulation and are susceptible to serum nucleases. Thus improvements in efficacy will require improving their circulation lifetimes and directing the ODNs to macrophages and other cells of the immune system. Several recent studies have demonstrated that this can be achieved by encapsulating antisense ODNs in SALPs or similar systems. Gursel *et al.* [111] encapsulated immunostimulatory ODNs within a variety of liposomes using an approach where unilamellar vesicles and ODNs were lyophilised together followed by rehydration and extrusion to give final vesicles with diameters < 150 nm. The encapsulation occurs during the rehydration step. High encapsulation levels were obtained for sterically stabilised cationic liposomes (SSCLs) formed from DC-Chol, DOPE and PEG-PE. The SSCLs were able to stimulate macrophages to express a transfected luciferase gene to a much greater extent than free ODNs, and were taken up to a greater extent by macrophages, dendritic cells and B cells. ODNs administered *in vivo* in SSCLs gave rise to increased cytokine production, ranging 5- to 10-times higher than obtained with free ODN. Likewise, encapsulation of ODNs in SSCL was found to greatly enhance the ability of ODNs to act as immune adjuvants. Co-encapsulation of protein antigen (OVA) with CpG ODNs increased the production of IgG_{2a} and interferon more than sixfold over CpG ODNs administered with free OVA. Finally, ODNs administered in SSCL gave rise to extended protection (by 2 weeks) to BABL/c mice subjected to a pathogen challenge. Thus, in all cases, encapsulation within an appropriate carrier system greatly enhanced the immunostimulatory properties of the CpG ODN.

Similar results have been obtained by Mui *et al.* [6] using the SALPs LNs described earlier. They observed that PS ODNs administered in SALPs to mice gave rise to greatly increased levels of IL-12, IL-6, IFN-γ, monocyte chemoattractant protein-1 and TNF-α, relative to the same dose of free PS ODN. This response indicated an immune response that was T-helper cell 1 biased. Furthermore, phosphodiester ODN, which was inactive in its free form, gave a greater response in SALPs than the PS ODN. Thus both PS and phosphodiester ODNs could be used as immune adjuvants when administered in SALPs (often referred to as LN-CpG ODNs). If these LNs are to be used as immune adjuvants (e.g., in vaccines), it will be necessary to know which route of administration gives rise to a maximal response. A recent study has demonstrated that LN-CpG ODNs give rise to similar immunostimulatory effects (as monitored by levels of cellular activation and the

cytolytic activity of immune cells in the blood and spleen compartments) following intravenous or subcutaneous injection, despite large differences in particle pharmacokinetics and biodistribution [8]. Of particular interest is the recent observation that LN-CpG ODNs were able to adjuvanate adaptive immune responses against co-administered tumour-associated antigens, inducing effective anti-tumour activity in a number of murine tumour models [9]. No such effect was observed with free CpG ODNs. This confirmation of the ability of LN-CpG ODNs to enhance the immune response against specific tumours is a very significant result, and highlights again the benefit of the encapsulation of therapeutic agents within a LNM vector.

Finally, it should be noted that siRNA can also elicit a strong immune response when encapsulated in various SNALP formulations [98], and putative immunostimulatory sequences have been identified. Although this has allowed the design of siRNA exhibiting maximal gene silencing ability with minimal immune activation [98,104], it should be possible to use siRNA as an immune activator, in a manner similar to antisense CpG ODNs, if so desired. It would be interesting to compare the relative levels of immune stimulation that can be achieved from siRNA versus CpG ODNs.

5. Conclusion

LNs are a versatile class of drug carrier particles capable of delivering conventional and genetic drugs to diseased tissue that exhibits leaky vasculature (tumours and sites of inflammation) and, therefore, allows exploitation of the EPR effect. For both classes of drugs, the LNs should have a high drug-to-lipid ratio, a low drug leakage rate, a diameter of about 100 nm and exhibit an extended circulation lifetime (half-life > 6 h). For conventional drugs, enhanced efficacy can be expected for cell-cycle-specific drugs where leakage occurs at an optimised rate that will depend on several factors including target cell growth rate. Several products are already on the market and preclinical studies and clinical trials are planned for many others. For genetic drugs, maximal effects will require increasing particle uptake and enhancing the intracellular delivery of LN contents. Although still at the research stage, many exciting developments have been made in applications involving gene delivery, immune stimulation and gene silencing.

6. Expert opinion

The results summarised in this review focus on LNs and, thus, represent only a small portion of the present research applying nanotechnology to drug delivery. Nevertheless, several key principles are becoming increasingly clear that should apply to all drug carriers, including those whose structure is based on polymers or agents other than lipids. The first insight is a general one: the most successful DDSs, at least in the short term, will take advantage of the EPR effect. The holy grail of drug delivery has been the desire to target drugs to specific diseased tissues and avoid healthy tissues in the process. For diseases such as cancer, this has been achieved by virtue of the leaky vasculature that occurs in the diseased state. Thus it makes strategic sense to focus on the development of drugs that are effective against diseases where the EPR effect can be used. In the longer term, it is anticipated that it will be possible to specifically target LNs to other tissues by attachment of targeting information to the liposome surface. Numerous attempts have been made in this regard, by attachment to the liposome surface of antibodies, antibody fragments, or small molecules specific for certain cell surface proteins or receptors [112-120]. However, so far, this approach has not yet led to clinical formulations, and is still in the stage of preclinical research. The second general insight is specific for smaller, conventional drugs: although encapsulation within a LN will almost assuredly lead to a reduction in side effects (due to avoidance of healthy tissue), increases in efficacy will most likely be observed for cell-cycle specific drugs (such as vincristine). For these drugs, an optimised LN with maximal efficacy will exhibit the following properties: a small size (on the order of 100 nm) that permits extravasation and accumulation at the site of disease, and a drug release rate that maximises the concentration at the tissue over a period of time corresponding to one or more cell cycles. Thus a leakage rate that is too fast or too slow will not allow drug accumulation to occur. Regulating the leakage rate can be achieved by choosing an appropriate initial drug-to-lipid ratio in conjunction with an appropriate loading method, encapsulating specific ions or polymers that form intravesicular complexes, and/or using specific lipids in the LNs formulation (such as SM and cholesterol). What will be important here will be determining optimal release rates for different tumours or tissues and designing LNs with those characteristics. Also exciting in this area is the idea of co-encapsulating several drugs within a single LN and looking for synergistic effects in cancer therapy. Evaluations of LN anticancer systems should involve multiple preclinical animal models, rather than just a single model. Different LN–drug combinations will be more or less effective against different models depending on cell line sensitivities to the drug at hand. Thus a wider initial screening may be a better predictor of success in clinical trials. The third general insight comes from studies with genetic drugs. For these large molecules, drug leakage and uptake will not occur due to their size and charge characteristics. What must occur is the uptake of the LNs and intracellular delivery of the drug to the cytoplasm or nucleus of the cell prior to degradation in endosomes and lysosomes. For these formulations, any approach that leads to increased uptake and release from endosomes following endocytosis is likely to increase their efficacy. Tissue-specific targeting is bound

to be important here. The lower pH values observed in endosomes may make pH-sensitive lipids or polymers particularly effective in this regard, and much research in this direction has already been conducted [121]. For approaches involving enhanced gene expression, a key area of research will involve increasing the transfection potency of SPLPs, either by increasing the uptake of the particles, directing the plasmid to the nucleus once uptake has occurred, or modifying the plasmid vector so that cytoplasmic expression can be achieved [122,123]. Although research involving genetic drugs is still in its infancy, we anticipate profound advances in this field and preclinical if not clinical applications in the near term. For applications involving immune stimulation (such as vaccine development, or cancer treatment), it would be interesting to compare the relative levels of immune stimulation that can be achieved from siRNA versus CpG ODNs, and compare their ultimate effectiveness. Finally, the excellent results obtained for serum cholesterol reduction in non-human primates bodes well for applications of this technology to human cholesterol management in the near future, and other gene silencing applications in the not-too-distant future. We anticipate that LNMs will become highly significant in the treatment of many human diseases over the next few decades.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. Science 2004;303:1818-22

•• **A concise review on recent advances in the field of drug delivery.**

2. Abraham SA, Waterhouse DN, Mayer LD, et al. The liposomal formulation of doxorubicin. Methods Enzymol 2005;391:71-97

3. Boman NL, Masin D, Mayer LD, et al. Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. Cancer Res 1994;54:2830-3

•• **One of the first papers to demonstrate how, for a cell-cycle specific drug, efficacy could be increased dramatically by increasing drug retention in liposomal systems.**

4. Hope MJ, Wong KF. Liposomal formulation of ciprofloxacin. In: Shek PN, editor. Liposomes in Biomedical Applications. Harwood Academic Publishers, London; 1995:121-34

5. Fenske DB, Cullis PR. Entrapment of small molecules and nucleic acid-based drugs in liposomes. Methods Enzymol 2005;391:7-40

6. Mui B, Raney SG, Semple SC, Hope MJ. Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles. J Pharmacol Exp Ther 2001;298:1185-92

7. Vollmer J, Janosch A, Laucht M, et al. Highly immunostimulatory CpG-free oligodeoxynucleotides for activation of human leukocytes. Antisense Nucleic Acid Drug Dev 2002;12:165-75

8. Wilson KD, Raney SG, Sekirov L, et al. Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles. Int Immunopharmacol 2007;7:1064-75

9. De Jong S, Chikh G, Sekirov L, et al. Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN. Cancer Immunol Immunother 2007;56:1251-64

10. Zimmermann TS, Lee AC, Akinc A, et al. RNAi-mediated gene silencing in non-human primates. Nature 2006;441:111-4

•• **A striking illustration of the ability of RNA interference to achieve gene silencing, and for its potential in the treatment of human diseases, in this case atherosclerosis.**

11. Chakraborty C. Potentiality of small interfering RNAs (siRNA) as recent therapeutic targets for gene-silencing. Curr Drug Targets 2007;8:469-82

12. Cullis PR, Hope MJ, Bally MB, et al. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim Biophys Acta 1997;1331:187-211

•• **Comprehensive summary of the influence of pH gradients on transbilayer transport phenomena.**

13. Abraham SA, Edwards K, Karlsson G, et al. Formation of transition metal–doxorubicin complexes inside liposomes. Biochim Biophys Acta 2002;1565:41-54

14. Abraham SA, Edwards K, Karlsson G, et al. An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes. J Control Rel 2004;96:449-61

15. Semple SC, Leone R, Wang J, et al. Optimization and characterization of a sphingomyelin/cholesterol liposome formulation of vinorelbine with promising antitumor activity. J Pharm Sci 2005;94:1024-38

16. Ambegia E, Ansell S, Cullis P, et al. Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. Biochim Biophys Acta 2005;1669:155-63

17. Liang MT, Davies NM, Toth I. Encapsulation of lipopeptides within liposomes: effect of number of lipid chains, chain length and method of liposome preparation. Int J Pharm 2005;301:247-54

18. Huang YY, Wang CH. Pulmonary delivery of insulin by liposomal carriers. J Control Release 2006;113:9-14

19. Vyas SP, Rawat M, Rawat A, et al. Pegylated protein encapsulated multivesicular liposomes: a novel approach for sustained release of interferon alpha. Drug Dev Ind Pharm 2006;32:699-707

20. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965;13:238-52

•• **The paper describing the original discovery of liposomes.**

21. Bangham AD. Membrane models with phospholipids. Prog Biophys Mol Biol 1968;18:29-95

22. Bangham AD. Liposomes: realizing their promise. Hosp Pract (Off Ed) 1992;27:51-6, 61-2

23. Bangham AD. Surrogate cells or Trojan horses. The discovery of liposomes. Bioessays 1995;17:1081-8

24. Mayer LD, Hope MJ, Cullis PR. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim Biophys Acta 1986;858:161-8

• **Paper describing the extrusion technique.**

25. Hofheinz RD, Gnad-Vogt SU, Beyer U, Hochhaus A. Liposomal encapsulated anti-cancer drugs. Anticancer Drugs 2005;16:691-707

26. Yuan F, Dellian M, Fukumura D, et al. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res 1995;55:3752-6

• **Demonstrates that LNs with diameters up to 400 nm are capable of the EPR effect.**

27. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 1986;46:6387-92

28. Maeda H, Wu J, Sawa T, et al. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Rel 2000;65:271-84

• **Excellent description of the EPR effect.**

29. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzyme Regul 2001;41:189-207

30. Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. J Control Rel 2001;74:47-61

31. Allen TM. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol Sci 1994;15:215-20

•• **Original paper describing the increase in circulation lifetime that can be achieved by grafting PEG to the liposome surface.**

32. Janoff AS, Boni LT, Popescu MC, et al. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc Natl Acad Sci USA 1988;85:6122-6

33. Madden TD, Janoff AS, Cullis PR. Incorporation of amphotericin B into large unilamellar vesicles composed of phosphatidylcholine and phosphatidylglycerol. Chem Phys Lipids 1990;52:189-98

34. Mayer LD, Bally MB, Cullis PR. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. Biochim Biophys Acta 1986;857:123-6

35. Mayer LD, Tai LC, Bally MB, et al. Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. Biochim Biophys Acta 1990;1025:143-51

36. Mayer LD, Tai LC, Ko DS, et al. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res 1989;49:5922-30

37. Harrigan PR, Wong KF, Redelmeier TE, et al. Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients. Biochim Biophys Acta 1993;1149:329-38

38. Mayer LD, Bally MB, Loughrey H, et al. Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors. Cancer Res 1990;50:575-9

39. Mayer LD, Nayar R, Thies RL, et al. Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother Pharmacol 1993;33:17-24

40. Boman NL, Mayer LD, Cullis PR. Optimization of the retention properties of vincristine in liposomal systems. Biochim Biophys Acta 1993;1152:253-8

41. Webb MS, Harasym TO, Masin D, et al. Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br J Cancer 1995;72:896-904

•• **Highlights the importance of lipid composition in regulating drug retention and efficacy. For vincristine, the use of sphingomyelin (rather than other long-chain phospholipids such as distearoylphosphatidylcholine) leads to significant improvements in drug retention and efficacy.**

42. Zamboni WC, Gervais AC, Egorin MJ, et al. Systemic and tumor disposition of platinum after administration of cisplatin or STEALTH liposomal-cisplatin formulations (SPI-077 and SPI-077 B103) in a preclinical tumor model of melanoma. Cancer Chemother Pharmacol 2004;53:329-36

43. White SC, Lorigan P, Margison GP, et al. Phase II study of SPI-77 (sterically stabilised liposomal cisplatin) in advanced non-small-cell lung cancer. Br J Cancer 2006;95:822-8

44. Kim ES, Lu C, Khuri FR, et al. A Phase II study of STEALTH cisplatin (SPI-77) in patients with advanced non-small cell lung cancer. Lung Cancer 2001;34:427-32

45. Madden TD, Harrigan PR, Tai LC, et al. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem Phys Lipids 1990;53:37-46

46. Lasic DD, Frederik PM, Stuart MC, et al. Gelation of liposome interior. A novel method for drug encapsulation. FEBS Lett 1992;312:255-8

• **Early demonstration of drug precipitation within LNs.**

47. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta 1993;1151:201-15

48. Webb MS, Boman NL, Wiseman DJ, et al. Antibacterial efficacy against an in vivo Salmonella typhimurium infection model and pharmacokinetics of a liposomal ciprofloxacin formulation. Antimicrob Agents Chemother 1998;42:45-52

49. Lasic DD, Ceh B, Stuart MC, et al. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim Biophys Acta 1995;1239:145-56

50. Maurer-Spurej E, Wong KF, Maurer N, Fenske DB, Cullis PR. Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles exhibiting transmembrane pH gradients. Biochim Biophys Acta 1999;1416:1-10

51. Maurer N, Wong KF, Hope MJ, Cullis PR. Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR

study. Biochim Biophys Acta 1998;1374:9-20

• **Demonstrates ciprofloxacin solubility within LNs under conditions where precipitation would be predicted.**

52. Li X, Hirsh DJ, Cabral-Lilly D, et al. Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochim Biophys Acta 1998;1415:23-40

53. Johnston MJ, Semple SC, Klimuk SK, et al. Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations. Biochim Biophys Acta 2006;1758:55-64

•• **First paper to demonstrate that a specific rate of drug release can optimise drug efficacy.**

54. Fenske DB, Wong KF, Maurer E, et al. Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients. Biochim Biophys Acta 1998;1414:188-204

• **Ionophore method of drug loading.**

55. Wheeler JJ, Veiro JA, Cullis PR. Ionophore-mediated loading of Ca^{2+} into large unilamellar vesicles in response to transmembrane pH gradients. Mol Membr Biol 1994;11:151-7

56. Zhigaltsev IV, Maurer N, Akhong QF, et al. Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention. J Control Rel 2005;104:103-11

57. Sarris AH, Hagemeister F, Romaguera J, et al. Liposomal vincristine in relapsed non-Hodgkin's lymphomas: early results of an ongoing Phase II trial. Ann Oncol 2000;11:69-72

58. Rodriguez MA, Sarris A, East K, et al. A Phase II study of liposomal vincristine in CHOP with rituximab for elderly patients with untreated aggressive B-cell non-Hodgkin's lymphoma (NHL). Proc Am Soc Clin Oncol 2002;21:Abstract 1132

59. Rodriguez MA, Dang NH, Fayad L, et al. Sphingosomal vincristine in CHOP is a promising new treatment for elderly, as well as poor prognosis patients with aggressive non-Hodgkin's lymphoma (NHL): follow-up results of a Phase II study. J Clin Oncol 2004;22:8080

• **Describes results of clinical trials with LNs encapsulating vincristine.**

60. Boehlke L, Winter JN. Sphingomyelin/cholesterol liposomal vincristine: a new formulation for an old drug. Expert Opin Biol Ther 2006;6:409-15

61. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235-42

62. Cheung BC, Sun TH, Leenhouts JM, Cullis PR. Loading of doxorubicin into liposomes by forming Mn^{2+}–drug complexes. Biochim Biophys Acta 1998;1414:205-16

• **First paper to describe the uptake of doxorubicin into LNs by complex formation in the absence of a pH gradient.**

63. Chiu GN, Abraham SA, Ickenstein LM, et al. Encapsulation of doxorubicin into thermosensitive liposomes via complexation with the transition metal manganese. J Control Rel 2005;104:271-88

64. Taggar AS, Alnajim J, Anantha M, et al. Copper-topotecan complexation mediates drug accumulation into liposomes. J Control Rel 2006;114:78-88

65. Zhigaltsev IV, Maurer N, Edwards K, et al. Formation of drug–arylsulfonate complexes inside liposomes: a novel approach to improve drug retention. J Control Rel 2006;110:378-86

66. Drummond DC, Noble CO, Guo Z, et al. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res 2006;66:3271-7

67. Johnston MJ, Semple SC, Klimuk SK, et al. Characterization of the drug retention and pharmacokinetic properties of liposomal nanoparticles containing dihydrosphingomyelin. Biochim Biophys Acta 2007;1768:1121-7

68. Ramsay EC, Dos Santos N, Dragowska WH, Laskin JJ, Bally MB. The formulation of lipid-based nanotechnologies for the delivery of fixed dose anticancer drug combinations. Curr Drug Deliv 2005;2:341-51

69. Abraham SA, McKenzie C, Masin D, et al. In vitro and in vivo characterization of doxorubicin and vincristine coencapsulated within liposomes through use of transition metal ion complexation and pH gradient loading. Clin Cancer Res 2004;10:728-38

70. Tardi PG, Gallagher RC, Johnstone S, et al. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. Biochim Biophys Acta 2007;1768:678-87

71. Mayer LD, Harasym TO, Tardi PG, et al. Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice. Mol Cancer Ther 2006;5:1854-63

72. Patil SD, Rhodes DG, Burgess DJ. DNA-based therapeutics and DNA delivery systems: a comprehensive review. AAPS J 2005;7:E61-77

73. Fenske DB, Maclachlan I, Cullis PR. Long-circulating vectors for the systemic delivery of genes. Curr Opin Mol Ther 2001;3:153-8

74. Wheeler JJ, Palmer L, Ossanlou M, et al. Stabilized plasmid-lipid particles: construction and characterization. Gene Ther 1999;6:271-81

• **Original paper describing SPLPs.**

75. Tam P, Monck M, Lee D, et al. Stabilized plasmid-lipid particles for systemic gene therapy. Gene Ther 2000;7:1867-74

76. Fenske DB, Maclachlan I, Cullis PR. Stabilized plasmid-lipid particles: a systemic gene therapy vector. Methods Enzymol 2002;346:36-71

•• **Comprehensive review on SPLPs.**

77. Fenske DB, Maurer N, Cullis PR. Encapsulation of weakly-basic drugs, antisense oligonucleotides, and plasmid DNA within large unilamellar vesicles for drug delivery applications. In: Torchilin VP, Weissig V, editors. Liposomes: A Practical Approach. Oxford: Oxford University Press, 2003. p. 167-91

78. Zhang YP, Sekirov L, Saravolac EG, et al. Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. Gene Ther 1999;6:1438-47

79. Maurer N, Wong KF, Stark H, et al. Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. Biophys J 2001;80:2310-26

80. Semple SC, Klimuk SK, Harasym TO, et al. Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel

small multilamellar vesicle structures. Biochim Biophys Acta 2001;1510:152-66

•• **One of the first papers describing SALPs.**

81. Stuart DD, Semple SC, Allen TM. High efficiency entrapment of antisense oligonucleotides in liposomes. Methods Enzymol 2004;387:171-88

82. Jeffs LB, Palmer LR, Ambegia EG, et al. A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. Pharm Res 2005;22:362-72

• **Methodology that improves the ease of formation of SPLPs, and permits the preparation of quantities suitable for preclinical research.**

83. Fenske DB, Palmer LR, Chen T, et al. Cationic poly(ethyleneglycol) lipids incorporated into pre-formed vesicles enhance binding and uptake to BHK cells. Biochim Biophys Acta 2001;1512:259-72

84. Palmer LR, Chen T, Lam AM, et al. Transfection properties of stabilized plasmid-lipid particles containing cationic PEG lipids. Biochim Biophys Acta 2003;1611:204-16

85. Heyes J, Palmer L, Bremner K, Maclachlan I. Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. J Control Rel 2005;107:276-87

86. Heyes J, Palmer L, Chan K, et al. Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA. Mol Ther 2007;15:713-20

87. Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 2007;59:75-86

88. Kong Y, Ruan L, Ma L, et al. RNA interference as a novel and powerful tool in immunopharmacological research. Int Immunopharmacol 2007;7:417-26

89. Masiero M, Nardo G, Indraccolo S, Favaro E. RNA interference: implications for cancer treatment. Mol Aspects Med 2007;28:143-66

90. Sioud M. RNA interference and innate immunity. Adv Drug Deliv Rev 2007;59:153-63

91. Stein CA, Cohen JS. Oligodeoxynucleotides as inhibitors of gene expression: a review. Cancer Res 1988;48:2659-68

92. Geisbert TW, Hensley LE, Kagan E, et al. Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. J Infect Dis 2006;193:1650-7

93. Heeg K, Sparwasser T, Lipford GB, et al. Bacterial DNA as an evolutionary conserved ligand signalling danger of infection to immune cells. Eur J Clin Microbiol Infect Dis 1998;17:464-9

94. Lipford GB, Heeg K, Wagner H. Bacterial DNA as immune cell activator. Trends Microbiol 1998;6:496-500

95. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med 2003;198:513-20

96. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8

97. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526-9

98. Judge AD, Sood V, Shaw JR, et al. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol 2005;23:457-62

99. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5

100. Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA 2001;98:9237-42

101. Kirschning CJ, Bauer S. Toll-like receptors: cellular signal transducers for exogenous molecular patterns causing immune responses. Int J Med Microbiol 2001;291:251-60

102. Hornung V, Guenthner-Biller M, Bourquin C, et al. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med 2005;11:263-70

103. Sioud M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. J Mol Biol 2005;348:1079-90

104. Judge AD, Bola G, Lee AC, Maclachlan I. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo Mol Ther 2006;13:494-505

105. Monteith DK, Henry SP, Howard RB, et al. Immune stimulation – a class effect of phosphorothioate oligodeoxynucleotides in rodents. Anticancer Drug Des 1997;12:421-32

106. Boggs RT, McGraw K, Condon T, et al. Characterization and modulation of immune stimulation by modified oligonucleotides. Antisense Nucleic Acid Drug Dev 1997;7:461-71

107. Roman M, Martin-Orozco E, Goodman JS, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997;3:849-54

108. Chu RS, Targoni OS, Krieg AM, et al. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997;186:1623-31

109. Elkins KL, Rhinehart-Jones TR, Stibitz S et al. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. J Immunol 1999;162:2291-8

110. Klinman DM, Conover J, Coban C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. Infect Immun 1999;67:5658-63

111. Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. J Immunol 2001;167:3324-8

112. Allen TM, Sapra P, Moase E, Moreira J, Iden D. Adventures in targeting. J Liposome Res 2002;12:5-12

113. Allen TM, Mumbengegwi DR, Charrois GJ. Anti-CD19-targeted liposomal doxorubicin improves the therapeutic efficacy in murine B-cell lymphoma and ameliorates the toxicity of liposomes with varying drug release rates. Clin Cancer Res 2005;11:3567-73

114. Cheng WW, Das D, Suresh M, Allen TM. Expression and purification of two anti-CD19 single chain Fv fragments for targeting of liposomes to CD19-expressing cells. Biochim Biophys Acta 2007;1768:21-9

115. Pastorino F, Brignole C, Marimpietri D, et al. Targeted liposomal c-myc

antisense oligodeoxynucleotides induce apoptosis and inhibit tumor growth and metastases in human melanoma models. Clin Cancer Res 2003;9:4595-605

116. Pastorino F, Brignole C, Di Paolo D, et al. Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy. Cancer Res 2006;66:10073-82
117. Sapra P, Allen TM. Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs. Cancer Res 2002;62:7190-4
118. Sapra P, Allen TM. Ligand-targeted liposomal anticancer drugs. Prog Lipid Res 2003;42:439-62
119. Sapra P, Moase EH, MA J, Allen TM. Improved therapeutic responses in a xenograft model of human B lymphoma (Namalwa) for liposomal vincristine versus liposomal doxorubicin targeted via anti-CD19 IgG2a or Fab' fragments. Clin Cancer Res 2004;10:1100-11
120. Sapra P, Tyagi P, Allen TM. Ligand-targeted liposomes for cancer treatment. Curr Drug Deliv 2005;2:369-81

• **Describes advances made in targeting LNs to cancer tissue.**

121. Chen T, McIntosh D, He Y, et al. Alkylated derivatives of poly(ethylacrylic acid) can be inserted into preformed liposomes and trigger pH-dependent intracellular delivery of liposomal contents. Mol Membr Biol 2004;21:385-93
122. Finn J, Lee AC, Maclachlan I, Cullis P. An enhanced autogene-based dual-promoter cytoplasmic expression system yields increased gene expression. Gene Ther 2004;11:276-83
123. Finn J, Maclachlan I, Cullis P. Factors limiting autogene-based cytoplasmic expression systems. FASEB J 2005;19:608-10
124. Tekmira Pharmaceuticals Available from: www.inexpharm.com [Accessed 5th December 2007]
125. Hana Biosciences Available from: www.hanabiosciences.com [Accessed 5th December 2007]
126. Aradigm Corporation Available from: www.aradigm.com [Accessed 5th December 2007]
127. Pfizer Available from: www.pfizer.com/home [Accessed 5th December 2007]
128. Alnylam Pharmaceuticals Available from: www.alnylam.com [Accessed 5th December 2007]
129. Alza Available from: www.alza.com [Accessed 5th December 2007]
130. Antigenics Available from: www.antigenics.com [Accessed 5th December 2007]
131. Celsion Corporation Available from: www.celsion.com [Accessed 5th December 2007]
132. Gilead Available from: www.gilead.com [Accessed 5th December 2007]
133. Enzon Pharmaceuticals Available from: www.enzon.com [Accessed December 2007]
134. Samaritan Pharmaceuticals Available from: www.samaritanpharma.com [Accessed December 2007]
135. Three Rivers Pharmaceuticals Available from: www.3riverspharma.com [Accessed December 2007]
136. Diatos Available from: www.diatos.com [Accessed December 2007]
137. Sopherion Therapeutics Available from: www.sopherion.com [Accessed December 2007]

Affiliation

David B Fenske[†1] & Pieter R Cullis[2] FRSC
[†]Author for correspondence
[1]University College of the Fraser Valley,
Department of Chemistry,
33844 King Road, Abbotsford,
BC, V2S 7M8, Canada
E-mail: david.fenske@ucfv.ca
[2]University of British Columbia,
Centre for Drug Research and Development,
Department of Biochemistry and
Molecular Biology,
Life Sciences Centre,
2350 Health Sciences Mall,
Vancouver, BC,
V6T 1Z3, Canada