15 Medical Applications of Lipid Nanoparticles

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CONTENTS

15.1 INTRODUCTION

Over the past three decades, nanoparticles have emerged as attractive vehicles for drug-delivery applications (Allen and Cullis 2004, Emerich and Thanos 2007, Faraji 2009, Fenske and Cullis 2008, Fenske et al. 2008, Maurer et al. 2001, Zhang 2007). There are now large bodies of literature describing nanoparticles based on lipids, inorganic materials, nanocrystals, nanotubes, dendrimers, and synthetic and natural polymers (Allen and Cullis 2004, Cadete 2012, Faraji 2009, Fenske and Cullis 2008, Liu 2008). Of these, nanoparticles based on lipids and polymers have shown the most promise, with lipid-based systems the clear leader in terms of sophistication and number of products in the clinic for systemic delivery of both small molecules and macromolecules. This review will first focus on lipid nanoparticle (LNP) drug-delivery systems encapsulating small molecule conventional drugs and then LNP-containing genetic drugs such as plasmids, antisense oligonucleotides (ODN), and small, interfering RNA (siRNA). This is followed by a section detailing recent advances in the last 5 years, followed by a summary of medical applications.

The potential of liposomal systems for transporting therapeutic agents has been recognized and exploited (Papahadjopoulos et al. 1976, Poste and Papahadjopoulos 1976, Poste et al. 1976) since Bangham's discovery in the 1960s that phospholipids dispersed in water spontaneously formed closed bilayer structures (Bangham 1968, Bangham et al. 1965). Liposomes have many advantages as drug-delivery systems: they are made of biodegradable components, their bilayer structure

provides a selective permeability barrier for encapsulated materials, and their size and lamellarity can be varied dramatically, from multilamellar vesicles (MLVs) with diameters in the tens of microns consisting of concentric bilayer rings in an onion skin configuration down to unilamellar vesicles with diameters in the 20–100 nm range. From the beginning, considerable efforts have been made to generate unilamellar vesicles. Sonication of MLVs gave rise to the smallest liposomal nanoparticles possible, the 20 nm small unilamellar vesicle (SUV) (Huang 1969). Unfortunately, this technique is not scalable; in addition these "limit-size" particles tend to fuse to form larger vesicles (Schullery et al. 1980). Other early approaches, such as ethanol-injection (Batzri and Korn 1973) or detergent-dialysis (Racker 1979), give rise to small vesicles but are difficult and time-consuming to perform and again lack scalability. Another decade would pass before the extrusion technique provided scalable and reproducible methods for generating stable populations of unilamellar vesicles (Hope 1985, Mayer et al. 1986a, Szoka et al. 1980) that exhibited appropriate size and retention characteristics (Fenske and Cullis 2008). The nearly simultaneous development of techniques for loading vesicles with weakly basic small molecule drugs in response to transmembrane pH gradients (Cullis et al. 1997a, Fenske and Cullis 2005, Madden et al. 1990, Mayer et al. 1986b, 1989, 1990a) enabled investigation of liposomal formulations of a variety of drugs and parameters required to achieve optimized therapeutic activity. The terminology used to describe these systems has varied over the years, from liposomal drug-delivery systems to liposomal nanoparticles and/or liposomal nanomedicines (LNMs) (Fenske and Cullis 2008), as the carriers were based on a lipid bilayer structure, but recent advances show that LNP of considerable utility can have hydrophobic cores surrounded by a lipid monolayer (Leung et al. 2012, Zhigaltsev et al. 2012), rendering the term "liposome" increasingly inaccurate.

The utility of LNP formulations of drugs rests, to a large extent, on the ability of small, long-circulating particles to preferentially accumulate at sites of disease, with reduced accumulation in many healthy tissues. This effect, known as the enhanced permeation and retention (EPR) effect (Maeda et al. 2000, Maeda 2001, Matsumura and Maeda 1986) and diagrammed in Figure 15.1, arises due to the leaky vasculature in the region of tumors and sites of inflammation and infection. In the case of tumors, this has been attributed to abnormalities in tumor neovasculature leading to extravasation of nanoparticles with sizes as large as 400 nm diameter (Yuan et al. 1995), resulting in local accumulation of LNP. Tissues such as muscle possess a continuous, nonfenestrated vascular endothelium, with tight junctions that prevent extravasation of particles as small as 5 nm (Sarin 2009, 2010). Encapsulation of drugs within LNP thereby prevents uptake by such tissues, leading to a reduction in toxic side effects. If LNP have adequate circulation lifetimes, they can therefore preferentially accumulate at tumors where they can either be taken up by target cells, or where the drug can slowly leak out and be taken up by the surrounding cells (see Figure 15.1). It is now thought that the EPR effect is universally observed in solid tumors (Maeda et al. 2000). Fortuitously, while LNP formulation generally also increases drug accumulation in fenestrated tissues such as the liver, there is no evidence of increased hepatotoxicity either in preclinical studies or in the very large cohort of patients who have received liposomal formulations of anticancer drugs such as Doxil.

15.2 GENERATION AND LOADING OF LNP SYSTEMS CONTAINING CONVENTIONAL DRUGS

Generating LNP requires either breaking down larger membrane systems ("top–down" approaches), or mixing lipid dissolved in organic solvent with water where the lipid condenses into LNP structures as the polarity is raised ("bottom–up" approaches). Two scalable procedures for generating LNP are extrusion (a top–down process) and in-line mixing (a bottom–up process) as discussed in the following sections. Most top–down processes start with lipids dried down from organic solvent in the form of a dry film or powder, which are subsequently dispersed in water by mechanical agitation such as vortex mixing. For bilayer forming lipids, this results in the spontaneous formation of

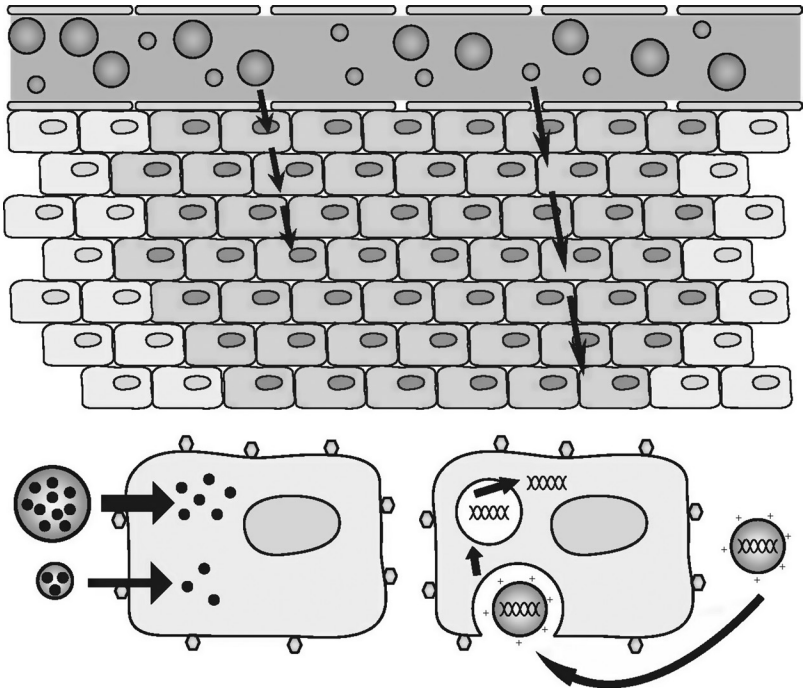

FIGURE 15.1 The accumulation of LNP at tumor tissue (shaded cells) is a result of the EPR effect. If LNP administered intravenously by injection have a sufficient circulation lifetime, they are able to extravasate through the leaky tumor vasculature resulting in LNP accumulation. Evidence now suggests that smaller LNP may be able to penetrate further into the tissue of certain tumor types. Drug release from the LNP may occur by several mechanisms: (1) encapsulated drug may slowly leak out of the LNP, regardless of whether the particle is taken up by endocytosis (*bottom left*), or (2) LNP encapsulating nucleic acids will contain a quantity of cationic lipid, some of which will be present on the particle surface. If the PEG-lipids can exchange off the LNP, this positive charge will be exposed and may result in binding to the cell surface with particle internalization (*bottom right*). Nucleic acid liberated from the particle may escape to the cytoplasm and manifest its effects.

MLVs with diameters on the order of microns (Bangham 1968, Hope 1985). MLVs can be excellent model systems for biophysical studies of membrane lipid structure but are of less utility for drug-delivery applications. Most "bottom–up" processes mix solutions of lipids in ethanol with aqueous media which can result in the formation of unilamellar vesicles as small as 20 nm diameter (Batzri and Korn 1973).

15.2.1 Extrusion

Extrusion is a conceptually simple technique that involves the use of high pressure to force preformed MLVs through polycarbonate filters of defined pore size. The MLVs are sheared into smaller membrane fragments that form closed unilamellar, and in some cases, bi- or multilamellar vesicles. In the case of filters with pore size of 100 nm, the vesicles produced are "large unilamellar vesicles" (LUV) with a diameter close to 100 nm (Figure 15.2a). The extruder is a stainless-steel barrel, where the MLVs are extruded under N_2 pressure (~500 psi or less) through two stacked polycarbonate filters up to 10 times to ensure size homogeneity. Filters are available with pore sizes of 0.05, 0.1, 0.2, 0.4, and 0.8 μm. Particles formed from the first three filter sizes have diameters ranging from approximately 60 to 200 nm (Mayer et al. 1986a, Patty and Frisken 2003); the sizes of particles formed from 0.4 and 0.8 μm are less well-defined. Vesicles 100 nm diameter and smaller are

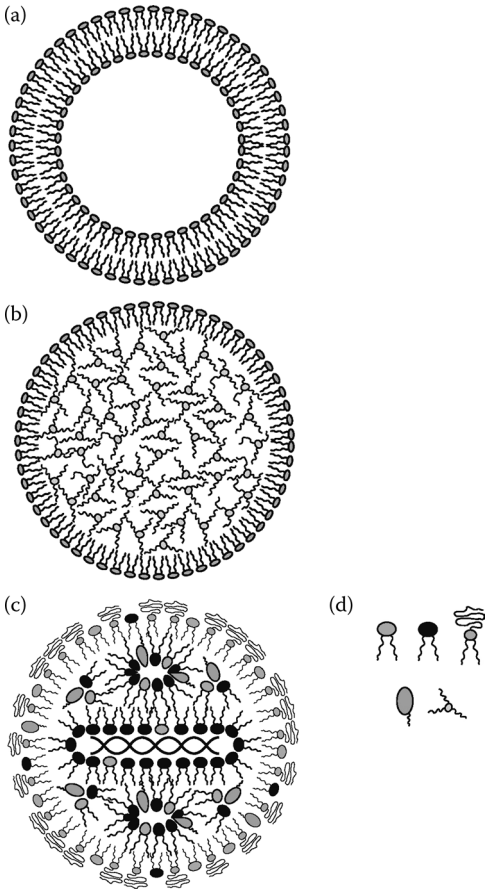

FIGURE 15.2 Diagrammatic representation of three types of LNP. (a) Bilayer LNP. When formed by extrusion, these LNP generally had a diameter of ~100 nm and were referred to as LUV. They are commonly made from phospholipids or phospholipid–cholesterol mixtures. When formed using a microfluidic approach, limit-size stable vesicles can be formed with diameters as small as 20 nm. (b) Prior to the development of the microfluidic approach using the SHM, emulsions of phospholipid and triacylglycerol were difficult to form. Using the SHM, limit-size LNP can be formed consisting of a hydrophobic triacylglycerol core surrounded by a phospholipid monolayer. The size of the particle can be varied considerably by varying the ratio of the two lipids. (c) In-line mixing methods, including microfluidics with the SHM, allow the generation of LNP siRNA with sizes varying from 20 to 100 nm. Although the structure is not well-defined, it would appear to involve hydrophobic inverted micelles in the core of the particle (consisting of cationic lipid surrounding the siRNA, and regular phospholipid and cholesterol surrounding water) surrounded by a monolayer of phospholipid. (d) Lipids used in these formulations include (L to R, top to bottom) phospholipid, cationic lipid, PEG-lipid, cholesterol, and triacylglycerol.

predominantly unilamellar, but above 100 nm and increasing proportion are bi- and multilamellar (Mayer et al. 1986a).

Many LNPs contain lipids with relatively high gel-to-liquid crystalline phase transition temperatures such as DSPC or sphingomyelin (SM), usually mixed with equimolar levels of cholesterol. For extrusion to proceed the lipids must be in the fluid or liquid-ordered states, thus the temperature must be maintained approximately 10°C above the gel-to-fluid phase transition temperature, T_m, of the phospholipid. The dry lipid mixture (lipid film or powder) is hydrated in buffer, subjected to freeze-thaw cycles to increase the trapped volume and separation between lamellae of the MLV systems (Hope 1985, Mayer et al. 1986a), and then extruded. An alternative procedure is to dissolve the lipids

in ethanol, and then inject the lipid–ethanol mixture, at a temperature above T_m, into a vortexed solution of the extrusion buffer, to give a final ethanol concentration of approximately 10%. This generates smaller vesicles to begin with, and the presence of 10% ethanol helps fluidize the membranes for easier extrusion. Following extrusion, the ethanol is removed by dialysis. The ethanol method greatly facilitates extrusion for some lipid mixtures, especially those containing saturated lipids and SM. For most applications to date, the LNP are prepared by extrusion with a diameter of 100 nm or less. Once they are formed, weak base drugs can be loaded using methods described in Section 15.2.3.

15.2.2 In-Line Mixing

In-line mixing processes for producing LNP are based on the Batzri and Korn (1973) method for forming liposomes, which relied on injection of lipid in ethanol into a rapidly stirred volume of water resulting in lipid precipitation and formation of closed LNP systems. The in-line approach achieves improved mixing by combining streams of lipid in ethanol and aqueous media in a specialized mixing compartment. A large variety of in-line static mixers exists and has the advantage of being readily scaled up to produce commercial quantities of material (Thakur 2003). Some have been applied to produce liposomal systems (see, e.g., US Patent 6855296, "Method and Apparatus for Liposome Production") using turbulent mixing induced between an organic phase containing the lipids and an aqueous phase. A T-tube in-line mixer has also been used to generate LNP systems where lipid in ethanol is pumped in one arm of the T tube and mixed with aqueous media flowing into the apposing arm by turbulent mixing (Jeffs et al. 2005).

In-line microfluidic mixers have also been applied to generate liposomal systems. Most approaches have used hydrodynamic flow focusing (Jahn et al. 2007, 2008, 2010, Karnik et al. 2008, Valencia et al. 2010) to generate vesicular LNP with diameters 50 nm or larger. Jahn et al used processes where lipid was dissolved in isopropyl alcohol (IPPA) and the IPPA stream was hydrodynamically focused between two aqueous streams. The vesicle size decreased as the fluid rate ratio (FRR) between the aqueous streams and the IPPA stream was increased, with the smallest vesicles being obtained for an FRR of 60:1 (Jahn et al. 2007) leading to very dilute liposomal dispersions.

This problem has been overcome by using the staggered herringbone micromixer (SHM) (Stroock et al. 2002), which leads to the formation of vesicular LNP with diameters as small as 20 nm at aqueous buffer-to-alcohol flow rate ratios as low as 3 (Zhigaltsev et al. 2012). The herringbone mixer provides an exponential increase in surface area between the two fluids with distance traveled, resulting in much faster diffusional mixing than the hydrodynamic flow focusing approach at equivalent flow rate ratios and correspondingly improved ability to generate limit-size systems at lower FRR. This microfluidics approach utilizes a plexiglass block manufactured with a staggered herringbone mixing channel, as shown in Figure 15.3. The ethanol stream (containing lipids) and the aqueous stream are introduced into the mixing device in separate channels by a syringe pump. The herringbone structures cause mixing by chaotic advection of these solutions over a millisecond timescale, and the rapid increase in polarity causes the lipids to precipitate out of the solution to form LNP with diameters ranging from 20 to 100 nm, depending on the lipid composition. A particular advantage of LNP formation using microfluidic mixing employing the SHM is the ability to form "limit-size" LNP such as SUV that are the smallest size possible compatible with the molecular composition. Until recently nonscalable techniques involving sonication (de Kruijff et al. 1975, 1976) or ethanol injection (Batzri and Korn 1973) have been the only methods of generating SUV.

15.2.3 Loading of Small Molecules into LNP in Response to Transmembrane pH Gradients

The primary method of loading small molecule drugs into LNP relies on the presence of a transmembrane pH gradient to drive accumulation of drugs that are weak bases (primary, secondary, or tertiary amines) into preformed LNP. More than 50% of known drugs are weak bases (O'Neil 2006)

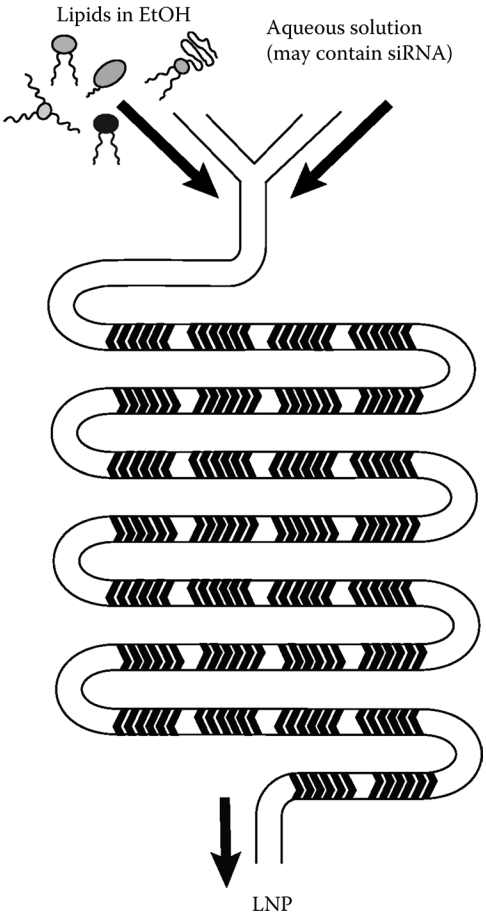

FIGURE 15.3 Schematic of LNP formulation using the staggered herringbone mixer (SHM). Lipids dissolved in ethanol and aqueous solution (which may contain siRNA) are introduced into the two inlets of the mixer using a syringe pump. When the two laminar streams meet the herringbone structures, chaotic advection occurs resulting in rapid mixing of the ethanol and aqueous fluids, with a corresponding rapid increase in the polarity of the solution (see also Figure 15.7). This results in rapid precipitation of lipids from solution (on the ms timescale) and spontaneous formation of LNP. Depending on the types of lipids present, or the presence of siRNA, LNP can be formed with any of the structures shown in Figure 15.2. The SHM channel is ~200 × 80 μm. The herringbone structures are ~30 μm high and 50 μm thick.

and thus the pH-loading technique, often referred to as remote loading, is a platform technique (Cullis et al. 1997b, Fenske and Cullis 2005, 2008, Fenske et al. 1998, 2003, 2008, Harrigan et al. 1993a, Madden et al. 1990, Mayer et al. 1986b, 1990a,b, Zhigaltsev et al. 2010). It may be noted that, depending on the drug encapsulated, enhanced retention can be achieved due to precipitation of the drug in the LNP interior (Johnston et al. 2006). A related approach is to use metals or other agents inside the LNP to complex with accumulated drug (Abraham et al. 2002, 2004a,b, Cheung et al. 1998, Chiu et al. 2005, Taggar et al. 2006).

Many methods of achieving drug uptake in response to transmembrane pH gradients have been developed, and three of these are summarized in Figure 15.4. In all the methods, drug is introduced to an LUV formulation which has an acidic interior and an external pH of 7–8. At pH 7, a weakly basic drug with $pKa \sim 6–9$ will exist as a mixture of positively charged and neutral forms, with the former being membrane-impermeable and the latter membrane-permeable. The neutral form can diffuse across the LUV membrane to the acidic interior component,

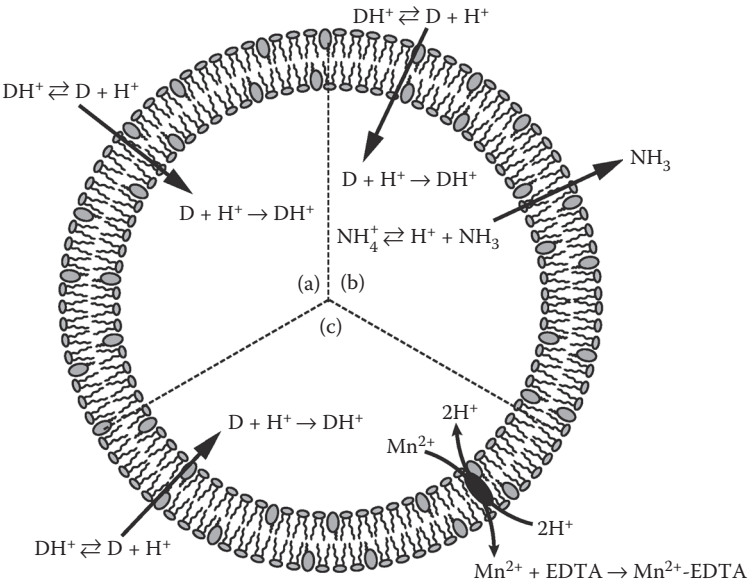

FIGURE 15.4 Schematic representation of drug uptake in response to transmembrane pH gradients. (a) The standard pH gradient method, in which the LNP contain an acidic interior. LNP are formed in 300 mM citrate pH 4, after which the external solution is exchanged for 20 mM HEPES 150 mM NaCl pH 7.5 by gel filtration or dialysis. The addition of external drug, which exists as a mixture of its charged and neutral forms, results in diffusion of the neutral, lipophilic form across the LNP bilayer into the particle interior, down its concentration gradient. Protonation of the drug converts it to the membrane-impermeable, charged form, and permits the continued uptake of the neutral form. (b) LNP formed in 300 mM ammonium sulfate acquires an acidic interior when the external solution is exchanged for 150 mM NaCl, due to the leakage of a small quantity of neutral ammonia to the external medium. This generates a pH gradient sufficient to drive drug uptake. (c) The ionophore method utilizes LNP possessing a primary ion gradient, which is used to generate a secondary pH gradient to drive drug uptake. LNP are formed in a 300 mM solution of a divalent cation (such as Mn^{2+} (pictured), although other ions, such as Mg^{2+} or Cu^{2+}, will also work), and then dialyzed against HEPES-buffered sucrose containing EDTA. Addition of the ionophore A23187 catalyzes the outward transport of divalent cation coupled to the inward transport of two protons, leading to rapid acidification of the LNP interior. External EDTA complexes the Mn^{2+} as it leaves the LNP, maintaining the outward flow of divalent cation down its concentration gradient.

where it is protonated and trapped. Conditions can be achieved where essentially all externally added drugs will be taken up, and will be retained as long as the pH gradient is maintained. The rate of drug leakage is strongly influenced by the composition of the lipid bilayer (usually mixtures of saturated phospholipids and cholesterol), the temperature, and the type of drug encapsulated.

The first and most straightforward method involves the formation of LUV in an acidic medium (e.g., citrate buffer at pH 4), followed by exchange of the external medium with a neutral buffer (e.g., HEPES at pH 7.5) by gel-exclusion chromatography or dialysis (see Figure 15.4a). These vesicles, which exhibit a pH gradient (ΔpH) of approximately 3 units, are then incubated with a weakly basic drug such as doxorubicin at an external pH of 7.5, where the drug exists as a mixture of both charged and neutral forms. Drug diffuses into the vesicle interior as described above where it is protonated and trapped by the acidic citrate buffer. These approaches led to the first LNP exhibiting high encapsulation levels and excellent drug retention for the anticancer drugs doxorubicin (Harrigan et al. 1993b, Mayer et al. 1986b, 1989, 1990b) and vincristine (Boman et al. 1993, 1994, Mayer et al. 1990a, 1993, Webb et al. 1995).

A popular alternative pH-loading method is to use a primary ion gradient to generate a secondary pH gradient, which then drives drug uptake. This technique usually relies on establishing transmembrane gradients of ammonium sulfate (Haran et al. 1993, Lasic et al. 1992, 1995) (Figure 15.4b). This approach involves forming LUV 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. The internal ammonium exists in an equilibrium with a small amount of neutral ammonia, allowing the latter to 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 (Hope and Wong 1995, Webb et al. 1998b). The addition of external drug to these vesicles results in diffusion of the neutral form into the vesicle interior, where each molecule will consume a proton and become charged and therefore trapped. Continued drug uptake consumes available protons, resulting in further loss of neutral ammonia from the vesicles, which frees more protons to drive further drug uptake. The method is equally effective using a range of alkylammonium salts (e.g., methylammonium sulfate, propylammonium sulfate, or amylammonium sulfate) to drive uptake (Maurer-Spurej et al. 1999).

An important observation first reported by Barenholz and coworkers was that certain drugs surpass their solubility product following accumulation into LNP in response to pH loading and form stable precipitates (Haran et al. 1993, Lasic et al. 1992, 1995). Doxorubicin precipitates and forms a crystalline gel in the vesicle interior that is characterized by fibrous bundles that are easily visualized by cryo-EM (Haran et al. 1993, Lasic et al. 1992, 1995). Other drugs, such as vincristine, form amorphous, poorly characterized precipitates (Johnston et al. 2006), while a few, such as ciprofloxacin, do not form large precipitates (Lasic et al. 1995, Maurer et al. 1998), even though intraliposomal concentrations as high as 300 mM can be achieved (Maurer et al. 1998). Under these conditions, the drug forms small stacks as shown by ^{1}H-NMR, but does not form large precipitates, even though its maximum solubility in buffer is on the order of 5 mM. These results explain the release rates obtained from *in vitro* leakage assays in which drug-loaded LUVs are incubated in the presence of serum or ammonium acetate, agents which destabilize the vesicles or the pH gradient, respectively. In the case of ciprofloxacin, nearly 80% of the drug loaded into ESM/Chol LUVs was lost within 30 min (Maurer et al. 1998). In contrast, greater than 95% of loaded doxorubicin remained encapsulated in DPPC/Chol LUVs after 24 h (Cheung et al. 1998). In the commercial formulation Doxil, in which doxorubicin is encapsulated within PEG-coated LUV, the *in vivo* half-time for release is 45 h (Gabizon et al. 1994).

A third way of driving ΔpH-loading is based on the use of ionophores, acting on transmembrane divalent cation gradients (involving Ca^{2+}, Mn^{2+}, or Mg^{2+}), to generate a secondary pH-gradient that provides the driving force for drug uptake (Fenske et al. 1998). The process is diagrammed in Figure 15.4c. A primary ion gradient is generated when LUV formed by extrusion in $MnSO_4$ or $MgSO_4$ solutions (usually 300 mM) are either dialyzed or passed down a column of Sephadex G-50 equilibrated in a sucrose-containing buffer. Chloride salts are avoided as they can dissipate pH gradients via formation of neutral HCl that can diffuse out of the vesicle. Likewise, sucrose is chosen for the external buffered solution rather than NaCl, as chloride ions can interfere with some ionophores (Wheeler et al. 1994). After establishing the primary ion gradient, drug is added, followed by the ionophore A23187 (which couples the outward flow of a divalent cation to the inward flow of a pair of protons) and the chelator ethylenediaminetetraacetic acid (EDTA). The ionophore-mediated ion transport results in acidification of the vesicle interior, thereby creating the pH gradient required for drug uptake. EDTA, which chelates calcium and magnesium as they are transported out of the vesicles, is also required for effective drug uptake. Initial studies involving the drugs ciprofloxacin and vincristine gave high levels of encapsulation (80–90%), and excellent *in vivo* circulation and drug-retention properties (Fenske et al. 1998).

For drugs that precipitate within liposomes, drug retention is improved with increasing drug-to-lipid ratios (Abraham et al. 2004a, Johnston et al. 2006, 2008, Semple et al. 2005, Zhigaltsev et al. 2005). This property has been observed with vincristine (Johnston et al. 2006, Zhigaltsev et al.

2005), vinorelbine and vinblastine (Zhigaltsev et al. 2005), topotecan (Abraham et al. 2004a), and doxorubicin (Johnston et al. 2008). It is therefore of interest that significantly higher drug-to-lipid ratios can be achieved using the ionophore method than with either the ammonium sulfate or citrate methods, allowing slower efflux rates to be achieved. Drug-to-lipid ratios as high as 0.4 (wt:wt) and 0.7 (wt:wt) have been achieved for LNP formulations of doxorubicin (Johnston et al. 2008) and vincristine (Johnston et al. 2006), respectively.

A final alternate method of drug loading involves uptake that is driven by the formation of drug-metal ion complexes. For example, doxorubin uptake can be driven by Mn^{2+}-gradients with the driving force the formation of Mn^{2+}-drug complexes which exhibit a different morphology by cryoEM than complexes formed with citrate or sulfate ion (Abraham et al. 2002, Cheung et al. 1998). This approach can result in significantly higher doxorubicin encapsulation levels than a pH gradient approach (Chiu et al. 2005). Other drugs may be encapsulated in a similar manner using different cations. For example, only Cu^{2+} was found to be effective for complex formation with topotecan in LNP in which pH gradients were absent (Taggar et al. 2006).

Other precipitating agents have also been employed to enhance drug retention within LNP. The formation of intraliposomal complexes involving complex anions such as the arylsulfonates have been used to increase the retention of ciprofloxacin and vinorelbine loaded by an ionophore-method (Zhigaltsev et al. 2006). The *in vivo* half-life of drug release was increased nearly fivefold (from ~6 h to 30 h) when the anion was present. Similar results were obtained for the drug irinotecan using the triethylammonium salts of a linear polyphosphate and sucrose octasulfate, along with a gradient method involving a sterically hindered amine (Drummond et al. 2006).

Finally, varying the lipid composition of the LNP can also lead to slower drug release rates. Most LNP systems loaded with weak-base drugs possess a lipid composition optimized for good retention properties, usually a phospholipid containing long chain saturated acyl chains, and near equimolar proportions of cholesterol to prevent formation of crystalline phospholipid phases which can lead to rapid leakage. LNP-encapsulating vincristine that are formed from SM:Chol possess better retention for vincristine than systems formed using DSPC:Chol (Boman et al. 1993, 1994, Webb et al. 1995), resulting in improved anticancer efficacy. Thus, the observation that LNP containing dihydrosphingomyelin (in which the native double bond is reduced) had drug release rates and circulation lifetimes that were increased approximately threefold and twofold, respectively, over LNP containing SM (Johnston et al. 2007) is of interest. Further enhancement of these parameters should be possible using higher drug-to-lipid ratios or one of the other approaches discussed above.

The emphasis placed on drug retention in the studies discussed above stems from the need to retain drug in LNP long enough for accumulation at tumor sites to occur. However, the fact that drug *must* leak out of the LNP to be effective suggests the existence of an optimal rate of drug release to maximize efficacy. This is particularly true for cell cycle-specific drugs such as vincristine where a long-term exposure to the drug may be expected to result in improved efficacy due to an increased probability of being present during the vulnerable part of the cell cycle. Obviously, if the rate of release is too slow, then drug concentrations never reach cytotoxic levels and will be ineffective. Currently, the only demonstration of therapeutically optimized rates of drug release has been reported by Johnston et al. (2006). The ionophore method was used to load vincristine into SM/Chol LNP at drug-to-lipid ratios ranging between 0.025 and 0.7 (wt:wt), after which drug release rates were measured and *in vivo* half-lives of release $T_{1/2}$ were determined (6–65 h). A linear relationship was observed between $T_{1/2}$ and the drug-to-lipid ratio. In a series of antitumor efficacy studies, a number of drug-to-lipid ratios were chosen giving $T_{1/2}$ values of 6.1, 8.7, 15.6, and 117 h. All of the LNP were more efficacious than free drug, and caused a significant decrease in median tumor volume over a period of 30 days, but only the LNP with $T_{1/2} = 15.6$ h maintained that reduction up to 56 days; in all other cases, tumor regrowth occurred. This important result demonstrates that an optimal rate of drug release exists, at least for certain drugs treating specific tumor types, and can be of crucial importance for treatment success.

15.3 GENERATION AND LOADING OF LNP SYSTEMS CONTAINING NUCLEIC ACID POLYMERS

15.3.1 LNP-Plasmid Systems

The successes of LNP in delivery of small molecule anticancer drugs led to efforts to encapsulate and deliver nucleic acids in a similar manner. The potential for lipid-based intracellular delivery of nucleic acids was demonstrated by Felgner's (Felgner and Ringold 1989, Felgner et al. 1987) pioneering studies on lipoplexes, effective *in vitro* transfection vectors composed of cationic vesicles complexed with plasmid DNA. However, as with other carriers for *in vitro* delivery of nucleic acid molecules, including polymer-based polyplexes, the lipoplexes have limited utility for systemic applications (Fenske et al. 2001). Both lipoplexes and polyplexes are large, micron-sized complexes that are cleared rapidly from the circulation, and possess a high positive charge that can lead to toxic side effects (Lv et al. 2006). *In vivo* delivery of genetic drugs required vectors that possessed the optimized EPR characteristics of LNP containing conventional drugs: small size, serum stability, and long circulation lifetimes. In addition, nucleic acids require eventual uptake of the LNP by the cell of interest to allow intracellular delivery of the particle contents (see Figure 15.1).

The first LNP formulation of plasmid employed a detergent-dialysis approach to produce LNP known as stabilized plasmid-lipid particles (SPLP) (Fenske et al. 2002, Leung et al. 2012, Maurer et al. 2001, Monck et al. 2000, Saravolac et al. 2000, Semple et al. 2001, Tam et al. 2000, Wheeler et al. 1999, Zhang et al. 1999). SPLP are small (~70 nm), monodisperse particles consisting of a single plasmid encapsulated in a unilamellar lipid vesicle composed of a fusogenic "helper" lipid (DOPE), a cationic lipid (originally dioleoyldimethylammonium chloride; DODAC), and a PEG-lipid. The PEG-lipid can be a PEG-ceramide containing either a 14- or 20-carbon saturated fatty acyl chain (PEG-CerC_{14} or PEG-CerC_{20}) (Wheeler et al. 1999, Zhang et al. 1999), or a PEG-diacylglycerol (usually PEG-distearoylglycerol) (Ambegia et al. 2005). The primary role of the cationic lipid is to interact with DNA to allow condensation of the large plasmids with concomitant encapsulation in the SPLP, whereas the role of the PEG-Cer is to allow formation of small nanoparticle systems. In the absence of a PEG-lipid, large aggregates are generated (Maurer et al. 2001). The different chain lengths dictate the rate at which the PEG-Cer lipids can dissociate from the nanoparticle; C_{14} acyl chains allow relatively rapid dissociation (halftimes of ~1 h) of PEG-lipid from the surface, whereas C_{20} acyl chains result in much longer residence times of days or longer (Wheeler et al. 1999). The utility of dissociable PEG-lipids lies in the fact that the presence of a PEG shield inhibits cell uptake and thus the use of a PEG lipid that dissociates aids first in allowing the generation of small particles but, after it dissociates, also allows interactions with target cells leading to endocytosis.

The cationic lipid used in the first SPLP formulation was the permanently positively charged lipid DODAC at relatively low levels (5–10 mol% of total lipid). In addition to allowing loading of plasmid into the LNP, cationic lipids also interact with anionic lipids of endosomal membranes to result in bilayer destabilization (Hafez and Cullis 2001, Hafez et al. 2000) and nucleic acid release. The DOPE component, which will also encourage formation of nonbilayer membrane destabilizing structure (Fenske and Cullis 1992, Gruner et al. 1988, Tilcock et al. 1982), could also play a role in the endosomal destabilization process.

SPLP are formed by codissolving the lipids and DNA in a solution of the detergent octylglucopyranoside (OGP) and dialyzing this mixture against a HEPES buffer (Saravolac et al. 2000, Wheeler et al. 1999, Zhang et al. 1999). The transfection potency of the SPLP, particularly *in vitro*, is sensitive to the cationic lipid content and the choice of PEG-lipid. The final encapsulation efficiency is highly dependent on the ionic strength, concentration of multivalent ions, and cationic lipid content. SPLP protect plasmid DNA from DNaseI and serum nucleases (Wheeler et al. 1999), possess extended circulation half-lives (6–7 h) (Tam et al. 2000), and have been shown to accumulate in distal tumor sites with subsequent gene expression in distal mouse tumor models following intravenous injection (Fenske et al. 2001). This gene expression is 100–1000 times greater than observed in any other

tissue. The particles formulated using PEG-diacylglycerols possess similar circulation lifetimes and levels of gene expression in distal tumor models (Ambegia et al. 2005).

Jeffs et al. (2005) used a T-tube in-line mixer to encapsulate plasmid DNA within LNP. Briefly, lipids (DSPC, cholesterol, an ionizable cationic lipid, and a PEG-lipid) were dissolved in 90% ethanol, the DNA was dissolved in a pH 4 citrate buffer, and both solutions were mixed by pumping through apposing arms of the T-tube mixer. The LNP formed were found to have similar properties to the SPLP formed by detergent-dialysis: relatively monodisperse vesicle populations with particle sizes <200 nm, circulation half-lives of 13 h, and tumor accumulation and gene expression similar to SPLP (Jeffs et al. 2005). The LNP DNA systems formed were somewhat more heterogeneous than those formed by detergent-dialysis but possessed the advantage of scalability. An interesting advance was reported by Heyes et al. (2007), who found that the T-tube in-line mixer could be used to encapsulate polyplexes formed from DNA and either poly(ethyleneimine) (PEI) or poly-L-lysine. The resulting polyplex-SPLP (pSPLP) were similar in size and circulation characteristics to regular SPLP, but exhibited a sixfold-enhancement in gene expression in a Neuro-2a model. Polyplexes are macromolecular complexes formed between DNA and a suitable polyanion, which function as transfection vectors *in vitro* (Fenske et al. 2001).

15.3.2 LNP Formulations of ODN and siRNA

Substantial efforts have been made to devise methods to encapsulate smaller genetic drugs such as antisense oligodeoxynucleotides (ODN) and siRNA. As both ODN and siRNA are short (~20 mer) nucleic acid polymers, the encapsulation processes worked out for ODN also apply, with minor modifications, to siRNA. Initial efforts had the same objective as noted above for plasmid delivery, namely, the development of small systems with a relatively neutral exterior to allow long circulation lifetimes that exhibited high encapsulation efficiencies. A key advance was the development of ionizable cationic lipids with apparent *pKa*'s in the region of 7 or lower. This allowed encapsulation at lower pH values (e.g., pH 4.0) where the cationic lipid is charged but results in a relatively neutral surface at physiological pH values. The ionizable cationic lipid 1,2-dioleoyldimethyaminopropane was the first lipid employed in these studies (Semple et al. 2001). Dioleoyldimethylaminopropane was originally referred to as AL1 (Bailey and Cullis 1994) or DODAP (Semple et al. 2001) and is referred to here as $DODMA_{est}$ (ester linkage to acyl chains) to emphasize structural continuities (see Figure 15.5). An ether version of $DODMA_{est}$ (usually referred to as DODMA) with enhanced chemical stability was synthesized as a precursor to DODMA-CN (Mok et al. 1999). $DODMA_{est}$ and DODMA possess *pKa*'s of 6.8 (Semple et al. 2010) and are thus well suited to satisfy the demands of positive charge at low pH for ODN loading and relative neutrality at physiological pH to result in low LNP surface charge, longer circulation lifetimes, and reduced toxicity.

The original ODN encapsulation methods involved the use of approximately 40% ethanol in the pH 4 lipid hydration buffer. ODN could be added at the time of hydration, leading to encapsulation efficiencies as high as 80% (Semple et al. 2001), or could be added subsequently to preformed vesicles formed in the presence of 40% ethanol, leading to ODN encapsulation efficiencies of approximately 60% (Maurer et al. 2001). It was found that a PEG lipid such as PEG-Cer-C_{14} was required to prevent aggregation of particles during formulation and the presence of DSPC and cholesterol added to stability. A typical lipid mixture consisted of DSPC/Chol/$DODMA_{est}$/$PEGCerC_{14}$; 25/45/20/10; mol/mol). Ethanol was removed by dialysis against a buffer at pH 4, followed by further dialysis against HBS pH 7.6. These first ODN-containing LNP, known as stabilized antisense-lipid particles (SALP), were found to be a mixture of unilamellar and MLVs with a diameter of 80–140 nm, and maximum ODN content of approximately 0.16 g ODN/g lipid, corresponding to approximately 2200 ODN per 100 nm LNP (Maurer et al. 2001).

These SALP possessed good EPR characteristics, with circulation half-lives ranging from 5–6 h for particles formed with $PEGCerC_{14}$ to 10–12 h for particles formed with $PEGCerC_{20}$ (Semple et al.

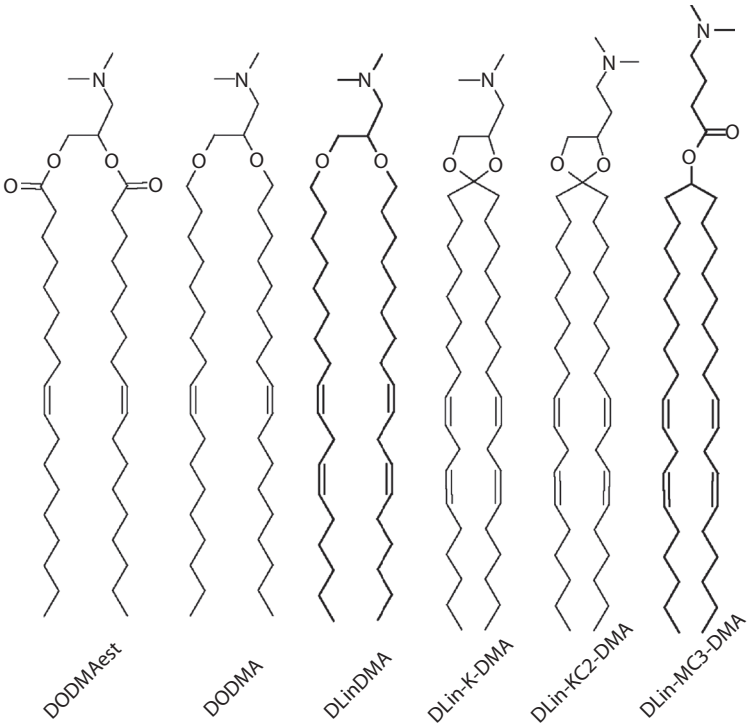

FIGURE 15.5 Chemical structures of ionizable cationic lipids used in the formulation of nucleic acids, particularly LNP-siRNA. The ability of LNP to silence the FVII gene in mice increases with cationic lipid type going from DODMAest (the only lipid with ester linkages) to DLin-MC3-DMA (see Figure 15.8). Distinguishing features are the presence of ether linkages in DLinDMA, and ketal linkages in DLin-K-DMA and DLin-KC2-DMA. DLin-MC3-DMA, with three methylene groups in the headgroup, was the most active lipid identified in a recent study surveying 56 novel lipids. (Adapted from Jayaraman, M. et al. 2012. *Angew. Chem. Int. Ed Engl.,* 51(34), 8529–8533.)

2001). As with SPLP, they possess the combination of high entrapment efficiencies, small size, and extended circulation lifetimes necessary for effective *in vivo* delivery of their contents.

The in-line T-tube mixing technique has also been applied to encapsulation of siRNA again using DSPC, DODMA, cholesterol, and a PEG-lipid (Heyes et al. 2005) and the effects of varying the unsaturation of the cationic lipid investigated on gene-silencing potency *in vitro*. It was found that more unsaturated versions such as dilinoleoyl-DMA (DLinDMA) (Figure 15.5) exhibited the most potent gene-silencing properties, in agreement with the proposal that cationic lipids combine with anionic lipids following endocytosis to form nonbilayer structures resulting in destabilization of the endosomal membrane and release of the nucleic acid polymer (Hafez and Cullis 2001, Hafez et al. 2001). The gene-silencing properties of DLinDMA-containing LNP (known as stabilized nucleic acid lipid particles, or SNALP) were also investigated *in vivo*, demonstrating good gene-silencing properties in hepatocytes following intravenous administration at dose levels as low as 1 mg siRNA/kg body weight (Geisbert et al. 2006, Morrissey et al. 2005, Zimmermann et al. 2006).

The ability to silence genes in the liver has led to a number of proof-of-concept studies to demonstrate the potential of LNP siRNA systems to treat liver diseases. For example, LNP siRNA systems to silence apolipoprotein B (apoB) have been shown to reduce circulating LDL levels in nonhuman primates (Zimmermann et al. 2006). In this work, siRNA that targeted apoB mRNA was developed, which possessed cross-reactivity to mouse, human, and monkey genes. When formulated in LNP siRNA containing DLinDMA, and administered systemically to cynomolgus monkeys,

a striking dose-dependent reduction of liver apo-B mRNA levels was observed. A single 2.5 mg dose of siRNA in the LNP resulted in a 90% reduction of liver apoB mRNA by 48 h, and the effect persisted up to 11 days. This resulted in reductions in blood apoB-100, cholesterol, and LDL levels of approximately 78%, 62%, and 82%, respectively, over the same time period. The results were superior to those achieved by cholesterol-lowering drugs (such as the HMG-CoA reductase inhibitors). HDL levels were unaffected.

LNP siRNA systems have also been found to effectively reduce HBV DNA levels in mice with replicating hepatitis B virus (Morrissey et al. 2005) and have also been shown to protect against infection by the Zaire species of Ebola virus in guinea pigs (Geisbert et al. 2006). The Ebola virus causes a severe and often lethal hemorrhagic fever for which there is no effective treatment. siRNA targeting the polymerase gene of the virus was encapsulated in LNP formed by in-line ethanol dilution and was administered shortly before or after a lethal viral challenge. Even when administered an hour *after* the challenge, the LNP siRNA completely protected the guinea pigs against viremia and death.

15.4 RECENT ADVANCES IN LNP FOR DELIVERY OF CONVENTIONAL DRUGS AND siRNA

Two areas where significant advances have been made over the last 5 years are in the generation of LNP by microfluidic mixing (using the SHM), resulting in the production of limit-size LNP, and improvements in the cationic lipid components of LNP systems for delivery of siRNA. One of the advantages of LNP formation via microfluidics is the ability to form structurally different limit-size LNP capable of transporting very different therapeutic agents. Thus, LNP formed from DOPC or DOPC/cholesterol have a bilayer structure, with diameters of ~20 and ~40 nm, respectively, while LNP formed from mixtures of DOPC and triolein consist of a PC monolayer surrounding a triolein core (Figure 15.2b) (Zhigaltsev et al. 2012). Given the small internal volume of limit-size bilayer LNP, they were assessed for their potential as drug-delivery vehicles by attempting to load doxorubicin by a pH-gradient method, and assess its retention within the particles. Using the ammonium sulfate method (Figure 15.4b) (Haran et al. 1993, Lasic et al. 1992, 1995), uptake of doxorubicin was observed, with a final drug-to-lipid ratio of ~0.2 (very close to the maximum ratio expected for LNP with such small internal volumes), and good retention properties (Zhigaltsev et al. 2012). TEM images of the particles revealed drug precipitates within the LNP. The sizes determined by TEM following drug uptake were the same as the original particles, on the order of 22 nm (Zhigaltsev et al. 2012).

In the case of DOPC/triolein LNP, the particle diameter was very sensitive to the DOPC to triolein ratio, with excellent agreement between predicted and experimental sizes assuming that the triolein is in an internal hydrophobic core and the DOPC is in a monolayer surrounding the core. LNP could be formed with diameters between 20 and 70 nm by varying the DOPC/triolein ratio from 60/40 (mol/mol) to 17/83 (mol/mol). This represents the first report of stable emulsions with a lipid core below 50 nm diameter. Previous emulsions formed by sonication were considerably larger and more heterogeneous (Atkinson and Small 1986). The microfluidics approach opens the door to formulating a new class of limit-size LNP with nonpolar drugs in the core.

The structure of LNP formulations of siRNA formulated by SHM technology was also found to have novel solid core characteristics. Leung et al. (2012) utilized a combination of cryo-transmission electron microscopy, ^{31}P-NMR, membrane fusion assays, light scattering, density measurements, and molecular modeling to arrive at the first structural description of these systems (with composition DLin-KC2-DMA/DSPC/Chol/PEG-lipid (40/11.5/47.5/1 mol/mol)). Cryo-EM revealed an electron dense core, RNase studies demonstrated that the siRNA was protected from degradation, and a surface charge/fusion assay revealed that at high siRNA content, the entire cationic lipid was within the LNP complexed to siRNA. These experimental results, in combination with molecular modeling simulations, indicated that the LNP have a nanostructured core (Figures 15.2c, 15.6, and 15.7) in which internal lipid is organized in inverted micellar structures of varying size, some of which contain siRNA. This structure also offers insight regarding the mechanism of formation of LNP siRNA

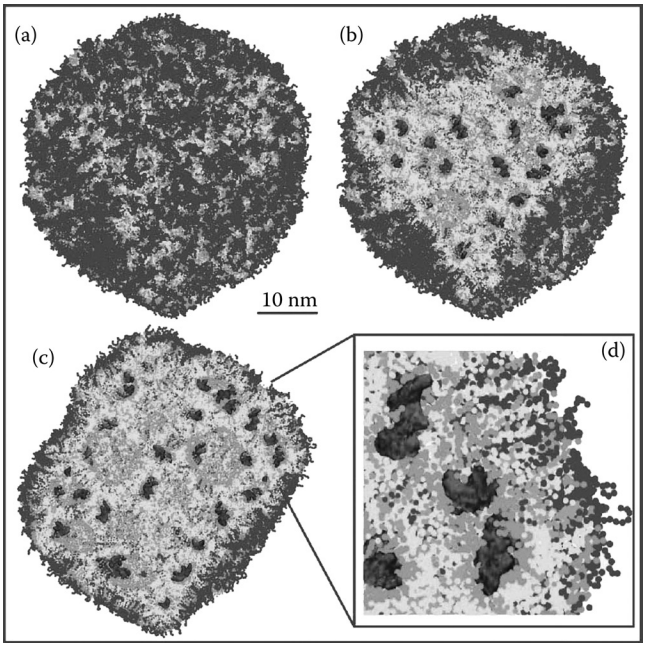

FIGURE 15.6 (**See color insert.**) Molecular modeling simulation of LNP-siRNA, predicting that the particles contain irregular water-filled cavities separated by bilayer membranes, with nucleic acids bound to internal membrane surfaces. Shown are side (a), cross-sectional (b,c), and zoom-in (d) views. The cationic lipid DLin-KC2-DMA is shown in yellow, cholesterol in pink, DSPC in grey, lipid polar moiety in cyan, PEG-lipid in violet, and duplex DNA in red. Water is not shown. The lipid composition was DLin-KC2-DMA/DSPC/Cholesterol/PEG-lipid (4:1:4:1 mol/mol), and a DNA/Lipid ratio of 0.05 (wt/wt).

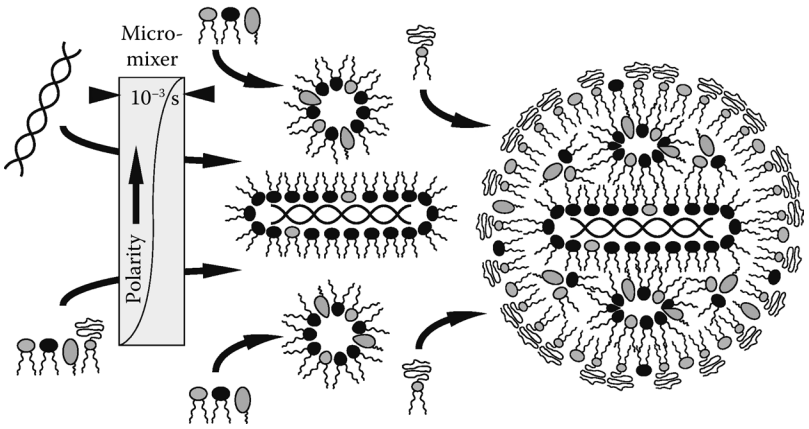

FIGURE 15.7 Formation of LNP-siRNA by microfluidic mixing. The mixing of ethanol–lipid mixtures with siRNA in aqueous medium in the SHM (see Figure 15.3) results in a rapid increase in the polarity of the medium, with precipitation of lipid with formation of siRNA-cationic lipid-inverted micelles and lipid–water-inverted micelles. As these structures aggregate, they are coated by PEG-lipids which form most of the outer monolayer of the LNP.

systems and how encapsulation efficiencies approaching 100% can be achieved. In particular, as the cationic lipid in ethanol is mixed with siRNA in water, the formation of hydrophobic nucleates of siRNA surrounded by cationic lipids would be expected, which would then be stabilized by a monolayer coating of more polar lipids such as PEG-lipids as they fall out of solution due to the rapidly increasing polarity of the medium (see Figure 15.7).

The potential utility of the smallest limit-size LNP systems is illustrated by recent studies showing that size plays a key role in determining particle accumulation in different tumor types (Cabral et al. 2011, Sarin 2009). Cabral et al. (2011) studied the accumulation of polymeric micelles with diameters ranging from 30 to 100 nm in tumors that were either highly or poorly vascularized. The 30 nm particles demonstrated dramatically improved ability to accumulate at the poorly vascularized tumors, with concomitant improvements in anticancer potency.

Recent studies have also identified ionizable cationic lipids that, when formulated into LNP siRNA systems, lead to substantially improved *in vivo* gene silencing in hepatocytes following intravenous administration. A "rational design" approach was taken (Semple et al. 2010) based on the putative mechanism whereby cationic lipids assist in the intracellular delivery of nucleic acids by inducing nonbilayer structures following uptake into endosomes (Hafez and Cullis 2001, 2004, Hafez et al. 2000, 2001). Briefly, following endocytosis, the cationic lipid becomes progressively more positively charged in the acidic endosomal compartment, and interacts with anionic lipids such as the major endosomal acidic lipid, lyso-bis phosphatidic acid (LBPA). Cationic lipids and anionic lipids form ion pairs that prefer hexagonal-phase lipid packing, which is associated with membrane fusion and bilayer destabilization. In this picture, lipid structural parameters that enhance the ability of the cationic lipid to induce nonbilayer structure, or to optimize the pH at which they become positively charged, could lead to improvements in nucleic acid delivery.

Semple et al. (2010) screened LNP siRNA systems containing a large number of ionizable lipids for their ability to inhibit (as described by the ED_{50}, the dose required to achieve 50% gene silencing) production of Factor VII by hepatocytes following intravenous injection in mice (see Figures 15.5 and 15.8). Lipid *pKa* values in LNP were measured using a fluorescence assay, and the relative bilayer destabilizing ability were measured by ^{31}P NMR and differential scanning calorimetry by determining the L_α to H_{II} phase transition temperature of mixtures of cationic lipid with the negatively charged lipid phosphatidylserine. The most promising lipid, DLin-KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane), which incorporated a ketal linker in the headgroup, had an ED_{50} that was 10-fold lower than DLinDMA, and an L_α to H_{II} phase transition temperature that was 7°C lower; its pKa value (6.7) was similar. Incorporation of this lipid into an LNP-siRNA

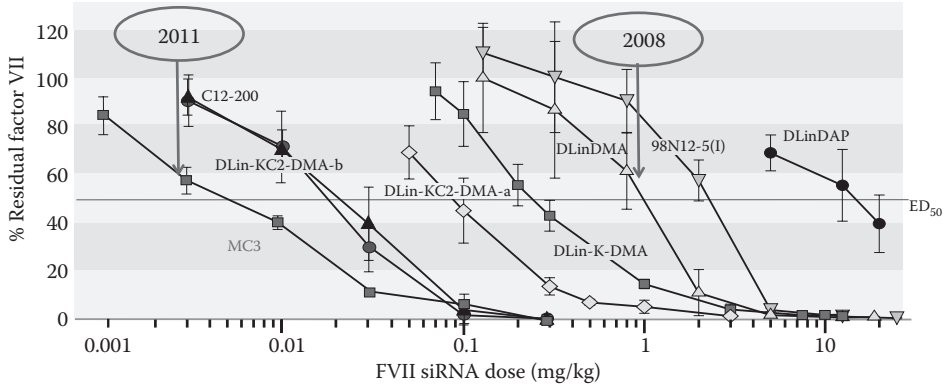

FIGURE 15.8 (**See color insert.**) Effectiveness of different cationic lipids in LNP in achieving 50% silencing of the FVII gene of hepatocytes in mice (ED_{50} = effective siRNA dose that results in 50% gene silencing). A dramatic improvement in cationic lipid effectiveness has been achieved in just 3 years (compare results with some of the structures shown in Figure 15.5).

formulation resulted in nearly 100-fold reduction in ED_{50} in the factor VII model as compared to using DLinDMA (Figures 15.5 and 15.8). Following the same design philosophy, but focusing more closely on optimizing the pKa, a further group of lipids were tested, leading to identification of heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLinMC3-DMA): LNP-siRNA formulated with this lipid displayed a further 10-fold improvement in potency, resulting in 50% gene silencing at a dose level of 0.005 mg siRNA/kg body weight (Jayaraman et al. 2012). This is the current "gold standard" for potency and has resulted in the remarkable potency of LNP containing DLinMC3-DMA in the clinic (see Section 15.5).

Recent studies have also shown that LNP can target antigen-presenting cells (APC), such as primary bone cell macrophages and dendritic cells. Four cationic lipids in LNP were tested for their ability to silence the GAPDH gene both *in vitro* and *in vivo* (in spleen and peritoneal macrophages and dendritic cells), and in both cases DLin-KC2-DMA was the most effective in producing a significant siRNA-mediated effect (Basha et al. 2011). Interestingly, increasing the size of the LNP from 80 nm up to 360 nm resulted in enhanced delivery to APC cells and less to the liver, most likely due to reduced access of LNP through the liver fenestrae, which are estimated to have sizes in the range of 100–150 nm. Other studies have shown that LNP-siRNA systems can be used to silence the androgen receptor, a validated oncogene driving the progression of prostate cancer (Lee et al. 2012). The effect was observed *in vitro* and *in vivo* in xenograft prostate cancer cell lines. The gene silencing observed *in vivo* with xenograft distal tumors resulted in reduced serum PSA levels following intravenous injection.

15.5 MEDICAL APPLICATIONS OF LNP

15.5.1 LNP Encapsulating Anticancer Drugs

At least seven formulations of LNP containing therapeutic agents, sometimes referred to as LNMs, are currently on the market in North America (Table 15.1). All of these involve conventional anticancer drugs (e.g., doxorubicin, daunorubicin, and vincristine) or antifungal agents (amphotericin B). Two of the most successful products are Doxil®, a formulation of doxorubicin encapsulated in PEG-coated

TABLE 15.1
Approved Liposomal and Lipid-Based Drug Formulations

Product	Company	Encapsulated Drug	Therapeutic Indication
AmBisome®	Gilead www.gilead.com	Amphotericin B	Systemic fungal infections
Abelcet®	Sigma-Tau Pharmaceuticals www.sigmatau.com	Amphotericin B	Systemic fungal infections
Depocyt®	Sigma-Tau Pharmaceuticals http://www.sigmatau.com	Cytarabine	Lymphomatous meningitis
Doxil®	Janssen Biotech www.janssenbiotech.com	Doxorubicin	Cancer (metastatic ovarian cancer, multiple myeloma)
DaunoXome®	Galen Ltd http://www.galen.co.uk	Daunorubicin	AIDS-related Karposi's sarcoma
Myocet®	Sopherion Therapeutics www.sopherion.com	Doxorubicin	Metastatic breast cancer
Marqibo®	Talon Therapeutics http://www.talontx.com	Vincristine	Acute lymphoblastic leukemia

LNP, and AmbiSome, a liposomal formulation of amphotericin B. Doxil is a formulation of doxorubicin within a carrier composed of fully hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PEG-DSPE. The drug is loaded using the ammonium sulfate method (Lasic et al. 1992), and has a circulation half-life of 55 h in humans. As with other LNP formulations of doxorubicin (Abraham et al. 2005, Kanter et al. 1993), treatment with Doxil resulted in similar levels of efficacy as the free drug, with a significant reduction in side effects, particularly cardiotoxicity (O'Brien et al. 2004). In clinical trials, the drug has shown promise in the treatment of breast cancer and Karposi's Sarcoma (James et al. 1994, O'Brien et al. 2004), and is currently approved for the treatment of ovarian cancer and multiple myeloma. AmBisome® is one of several LNP-formulations of amphotericin B, the gold-standard drug in the treatment of systemic fungal infections, also known as liposomal amphotericin B (L-AmB). It is composed of amphotericin B complexed with hydrogenated soy phosphatidylcholine, distearoylphosphatidylglycerol, and cholesterol. Other clinically approved formulations include amphotericin B lipid complex (ABELCET) (Janoff et al. 1988), composed of amphotericin B complexed with dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, and amphotericin B colloidal dispersion (ABCD), and formed from amphotericin B complexed with cholesteryl sulfate (Kleinberg 2006). All three formulations have similar efficacy to the free drug, but exhibit a significant reduction in renal toxicity (Barrett et al. 2003, Herbrecht et al. 2003, Tiphine et al. 1999, Wong-Beringer et al. 1998). There are also indications of reduced mortality for the lipid formulations (Barrett et al. 2003), and slight differences in efficacy between the LNP-formulations favoring ABELCET in some cases (Bassetti et al. 2011). The most recently approved drug is Marqibo®, a formulation of vincristine encapsulated within an SM–cholesterol LNP (Boman et al. 1993, 1994, Mayer et al. 1993, Webb et al. 1995, 1998a), which was approved in August 2012 for treatment of acute lymphoblastic leukemia at second or greater relapse. LNP formulations of at least five other conventional drugs are in preclinical development and/or clinical trials (Table 15.2).

Marqibo was developed by Inex Pharmaceuticals Corporation (now Tekmira), and licensed to Talon Therapeutics, Inc., and has recently been approved by the US FDA for treatment of acute lymphoblastic leukemia (ALL) at second or greater relapse. Marqibo consists of 100 nm SPM:Chol (55:45) LUVs, a drug-to-lipid ratio of 0.1 (wt:wt) (Webb et al. 1995), and 300 mM internal citrate at pH 4. This formulation exhibits long circulation lifetimes, greatly enhanced tumor accumulation, and enhanced *in vivo* efficacy in animal tumor models. The half-time for release of drug is 33 h (Webb et al. 1995), in the optimum range determined by Johnston et al. (2006). It is likely that the utility of Marqibo will go well beyond use as a single agent in the treatment of ALL as can be seen in the

TABLE 15.2
Selected Liposomal Drug Formulations in Clinical Trials

Product	Company	Encapsulated Drug	Therapeutic Indication
Alocrest® (Sphingosomal vinorelbine)	Talon Therapeutics http://www.talontx.com	Vinorelbine	Cancer
Brakiva™ (Sphingosomal topotecan)	Talon Therapeutics http://www.talontx.com	Topotecan	Cancer
Liposomal ciprofloxacin	Aradigm Corporation www.aradigm.com	Ciprofloxacin	Cystic fibrosis Inhalation anthrax
ThermoDox®	Celsion Corporation www.celsion.com	Doxorubicin	Cancer
Lipid-encapsulated siRNA products	Tekmira Pharmaceuticals www.tekmirapharm.com Alnylam Pharmaceuticals http://www.alnylam.com	siRNA	Various

results from a study entitled "Phase II Study of Liposomal Vincristine (VSLI) in First Line Therapy in Patients with Untreated Aggressive B-cell non-Hodgkin's Lymphoma (NHL)" (Rodriguez et al. 2002, Sarris et al. 2000, 2002). The standard treatment for NHL is the drug cocktail known as CHOP, which is composed of cyclophosphamide, doxorubicin, vincristine, and prednisone, in combination with Rituxan. In this study, Marqibo was substituted for vincristine in the CHOP regimen (lipo-CHOP) and the efficacy of lipo-CHOP + rituxan versus historical results for CHOP + rituxan (Coiffier et al. 2002) was examined. Complete response rates of 91% versus 76% were obtained for these two treatments, and overall survival rates were 98% versus 70% at 22–24 months.

The fact that combinations of drugs, such as CHOP, are used to treat cancer has led to attempts to develop combination formulations of LNP in which two or more drugs are coencapsulated within a single particle (Ramsay et al. 2005). If each drug was present at an optimized concentration, synergistic effects may be expected where the efficacy of the combination drugs is greater than their additive effects alone. Several combination formulations have been developed with promising results, including doxorubicin and vincristine coencapsulated using Mn^{2+}-complex formation and ionophore-mediated pH gradient loading (Abraham et al. 2004b), and irinotecan and floxuridine coencapsulated using a combination of copper complex formation and passive entrapment (Tardi et al. 2007). Mayer et al. (2006) examined three different coencapsulated drug pairs (irinotecan/floxuridine, cytarabine/daunorubicin, and cisplatin/daunorubicin), and found that for each drug pair, synergistic, additive, and antagonistic activity against tumor cells could be achieved *in vitro* simply by varying the encapsulated drug ratios. Furthermore, the synergistic ratios could be maintained *in vivo* and resulted in increased efficacy in preclinical tumor models. One of these drug pairs, cytarabine/daunorubicin in a fixed 5:1 molar ratio, have been tested in clinical trials as LNP formulation CPX-351 (Dicko et al. 2010a,b, Feldman et al. 2011, 2012, Kim et al. 2011, Lim et al. 2010a,b). CPX-351 consists of LNP with diameters of about 100 nm, a possible bilamellar structure, and interactions between the drugs and the internal Cu(II) gluconate/triethylamine-based buffer system that help to maintain the fixed drug ratio (Dicko et al. 2010b). Preclinical studies show enhanced efficacy relative to the free drugs against leukemia cells (Kim et al. 2011, Lim et al. 2010a,b). In phase 1 and 2 clinical trials involving patients with acute myeloid leukemia and acute lymphoblastic leukemia, the percentage of complete responses was observed in several patient groups, including patients who had previously received both drugs, ranged from about 25–33% (Feldman et al. 2011, 2012). Both the preclinical data and published clinical trial results suggest an important role for combination therapy in the next generation of LNP.

15.5.2 LNP Encapsulating Genetic Drugs

As discussed above, LNP formulations have been developed for plasmid DNA coding for therapeutic genes for gene replacement applications and ODN and siRNA for gene-silencing applications (Fenske and Cullis 2008, Patil et al. 2005). These LNP possess the characteristics necessary for *in vivo* delivery: small size, serum stability, low surface charge, low toxicity, and uptake by target cells such as hepatocytes, tumor cells, or antigen-presenting cells following systemic (intravenous) administration. LNP formulations of plasmids (SPLP) remain at the preclinical stage, as do LPN formulations of antisense oligonucleotides (SALP), which have proven most useful for their immunostimulatory properties (Chikh et al. 2009, de Jong et al. 2007, Raney et al. 2008, Wilson et al. 2007, 2009). The situation for LNP formulations of siRNA is considerably different, where several LNP-siRNA formulations are in clinical development and showing remarkable clinical proof-of-principle (Table 15.2).

The lead clinical development candidates are LNP-siRNA formulations to treat hepatocellular carcinoma (HCC), hypercholesterolemia, and transthyretin (TTR)-induced amyloidosis. The LNP formulation to treat HCC contains siRNA to silence the oncogene kinesin spindle protein (KSP) and the angiogenic factor vascular endothelial growth factor (VEGF). Phase-I clinical trial results using LNP containing DLinDMA as the ionizable cationic lipid show encouraging results

(see http://www.alnylam.com/Programs-and-Pipeline/Partner-Programs/Liver-Cancer.php). The LNP-siRNA systems to treat hypercholesterolemia contain the more potent ionizable cationic lipid DLinMC3DMA and siRNA to silence the PCSK9 gene. PCSK9 protein acts to decrease the number of LDL receptors and thus silencing PCSK9 leads to higher LDL receptor levels and lower circulating LDL. Clinical trial results on human volunteers show up to 84% reduction in circulating PCSK9 following a single intravenous administration at 0.4 mg siRNA/kg body weight with commensurate reductions in circulating LDL levels (see http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/Hypercholestoralemia.php). Finally, LNP-siRNA systems to treat TTR-induced amyloidosis, an incurable disease currently only treatable by liver transplant, have yielded equally remarkable results. Clinical trial results, again in human volunteers, have shown ~95% knockdown in serum TTR levels following a single injection of LNP-siRNA (containing DLinMC3-DMA) at 0.5 mg siRNA/kg body weight (see http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/TTR-Amyloidosis.php) with no significant toxicity issues.

In summary, the medical applications of LNP systems containing both small molecule conventional drugs and genetic drugs such as siRNA are substantial and growing. For small molecule drugs such as anticancer drugs essentially all can benefit from the reduced toxicity/enhanced potency profiles that LNP technology allows. This is likely to be augmented by recent advances in the formulation of LNP systems in the 20–50 nm diameter range, which will allow greater penetration into target tissues such as tumors. For genetic drugs such as siRNA, therapeutic applications that involve modulating gene expression in the liver (hepatocytes) are available now, leading us to expect that LNP-siRNA systems to treat a wide range of liver disorders will gain clinical acceptance. It is also to be expected that the range of tissues that will become amenable to LNP-siRNA therapeutics will be significantly extended in the near future as systems giving optimized gene silencing in a variety of cell types (immune, tumor, endothelial, bone marrow, and many others) are developed.

ABBREVIATIONS

APC	Antigen-presenting cells
CHOP	Chemotherapy treatment composed of cyclophosphamide, doxorubicin, vincristine, and prednisone
CpG	Unmethylated CpG dinucleotides
DLinDMA	1,2-dilinoleyloxy-3-*N,N*-dimethylaminopropane
DLinKDMA	1,2-dilinoleyloxy-keto-*N,N*-dimethyl-3-aminopropane
DLin-KC2-DMA	1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
DLin-MC3-DMA	(6*Z*,9*Z*,28*Z*,31*Z*)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate
DODAC	*N,N*-Dioleyl-*N,N*-dimethylammonium chloride
DODAP	1,2-dioleoyl-3-(dimethylamino)propane
DODMA	1,2-dioleyloxy-*N,N*-dimethyl-3-aminopropane
EPR	Enhanced permeation and retention
LNP	Lipid nanoparticle
LNM	Liposomal nanomedicine
MLVs	Multilamellar vesicles
ODNs	Oligodeoxynucleotides
PEG	poly(ethylene glycol)
$PEGCerC_{20}$	PEG-ceramide containing 20 carbon fatty acid
$PEGCerC_{14}$	PEG-ceramide containing 14 carbon fatty acid
RNAi	RNA interference
SALP	Stabilized antisense lipid particles
siRNA	small, interfering RNAs

SM	sphingomyelin
SNALP	Stabilized nucleic acid lipid particles
SPLP	Stabilized plasmid lipid particles
SUVs	Small unilamellar vesicles

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