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Liposomal Nanomedicines: An Emerging Field

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ABSTRACT

Liposomal nanoparticles (LNs) encapsulating therapeutic agents, or liposomal nanomedicines (LNMs), represent one of the most advanced classes of drug delivery systems, with several currently on the market and many more in clinical trials. During the past 20 years, a variety of techniques have been developed for encapsulating both conventional drugs and the new genetic drugs (plasmid DNA-containing therapeutic genes, antisense oligonucleotides, and small, interfering RNA [siRNA]) within LNs encompassing a very specific set of properties: a diameter centered on 100 nm, a high drug-to-lipid ratio, excellent retention of the encapsulated drug, and a long (>6 hours) circulation lifetime. Particles with these properties tend to accumulate at sites of disease, such as tumors, where the endothelial layer is “leaky” and allows extravasation of particles with small diameters. Thus, LNs protect the drug during circulation, prevent it from reaching healthy tissues, and permit its accumulation at sites of disease. We will discuss recent advances in this field involving conventional anticancer drugs as well as gene-delivery, immunostimulatory, and gene-silencing applications involving the new genetic drugs. LNMs have the potential to offer new treatments in such areas as cancer therapy, vaccine development, and cholesterol management.

Keywords: Liposomal nanoparticles; drug delivery; enhanced permeation and retention; gene therapy; antisense oligonucleotides; siRNA.

INTRODUCTION

The 21st century has embraced the dawning of the nanotechnology era and has already seen explosive growth in this multidisciplinary field. Exciting advances can be anticipated in medicine for new treatments for a wide variety of diseases (Ebbesen and Jensen, 2006). Of particular interest is the field of drug delivery (Emerich and Thanos, 2007; Wagner, 2007), in which recent advances in the liposome and polymer fields have led to carrier systems capable of encapsulating therapeutic agents ranging from conventional drugs to the new genetic drugs (such as antisense oligonucleotides or small, interfering RNA [siRNA]; Semple et al., 2001; Fenske and Cullis, 2005; Zimmermann et al., 2006).

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Abbreviations: APC, antigen presenting cells; CHOP, chemotherapy treatment composed of cyclophosphamide, doxorubicin, vincristine, and prednisone; CpG, unmethylated CpG dinucleotides; DODAC, *N,N*-dioleoyl-*N,N*-dimethylammonium chloride; DODAP, 1,2-dioleoyl-3-(dimethylamino)propane; DODMA, 1,2-dioleoyloxy-*N,N*-dimethyl-3-aminopropane; DOPE, dioleoylphosphatidylethanolamine; dsRNA, double-strand RNA; LN, liposomal nanoparticle; LNM, liposomal nanomedicine; MLVs, multilamellar vesicles; NK, natural killer; ODNs, oligodeoxynucleotides; PEG, poly(ethylene glycol); PEGCerC₁₄, PEG-ceramide containing 14 carbon fatty acid; PEGCerC₂₀, PEG-ceramide containing 20 carbon fatty acid; PEG-S-DSG, PEG covalently bonded to distearoylglycerol; RISC, RNA-induced silencing complex; RNAi, RNA interference; SALP, stabilized antisense lipid particles; siRNA, small, interfering RNA; SNALP, stabilized nucleic acid lipid particles; SPLP, stabilized plasmid lipid particles; ssRNA, single-strand RNA; SVP, spontaneous vesicle formation; TLR, toll-like receptors.

In this article, we will focus on liposomal nanomedicines (LNMs) and describe how years of research and development have propelled liposomes into the emerging field of nanomedicines. We will also highlight the lessons we have learned along the way and describe our experiences with a variety of therapeutic agents, from traditional small-molecule drugs to genetic drugs. We will limit the discussion mostly to intravenous delivery of liposomal nanoparticles (LNs).

OVERVIEW

Generally, liposomes have advantages over polymer-based nanoparticles for the formulation of therapeutics (Fenske et al., 2001). The lipid membrane structure, in most cases, mimics the most common structure found in nature—the lipid bilayer. The lipid bilayer provides a remarkable permeability barrier that defines an internal compartment and is capable of protecting the internal contents. Drugs encapsulated within this lipid bilayer are, therefore, protected from extra-liposomal reactions that could alter the effectiveness of the drug, such as enzymatic degradation or modification of the drug. The components that make up the lipid bilayer are mostly biocompatible. As we will discuss, a number of different components can be integrated into liposome membranes that control a number of key parameters, including drug retention, circulation longevity, and fusogenicity. The overall scheme in developing an LNM is to design an LN that exhibits an extended circulation lifetime (allowing the particle to accumulate at a site of interest) coupled with either an appropriate rate of drug retention (such that the drug can leak out of the particle to be taken up by the target cell) or the ability to be taken up by the target cell of interest so the payload can be delivered to the cell interior (see Figure 1).

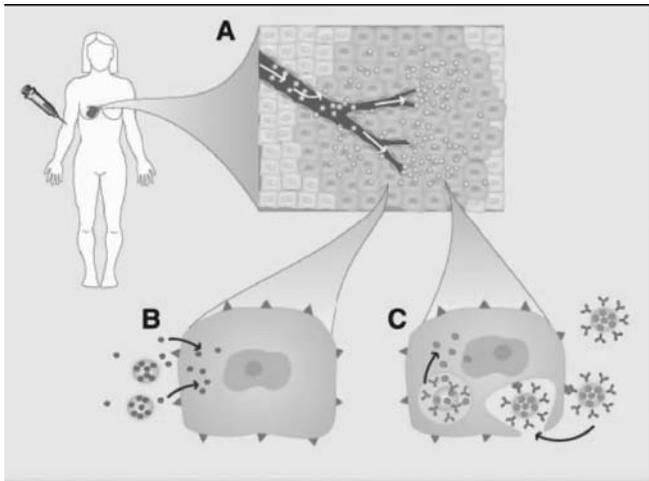


FIGURE 1.—A schematic figure depicting the accumulation of LNs in breast cancer tissue through the EPR effect. (a) LN containing an encapsulated drug extravasate through gaps in the endothelial cells and accumulating it in the darker tumor tissue but not the lighter normal tissue. (b) In passive targeting, as observed with many formulations of conventional anticancer drugs, the drug is released from LNs in the intracellular space and taken up into the tumor cell. (c) LNs that contain targeting ligands (such as antibody fragments), or LNs containing encapsulated genetic drugs (SPLP, SALP, SNALP), are taken up via binding to cell surface receptors, which results in internalization of the particles via the endosomal pathway. Some of the encapsulated material escapes to its intracellular site of action (cytoplasm or nucleus). EPR = enhanced permeation and retention; LN = liposomal nanoparticle; SALP, stabilized antisense lipid particles; SPLP = stabilized plasmid lipid particles; SNALP = stabilized nucleic acid lipid particles. Reprinted with permission from Allen and Cullis (2004).

Liposomes represent a highly flexible platform. Liposomes themselves range from multilamellar vesicles (MLVs) with diameters of several microns to small, unilamellar vesicles with diameters on the order of 20 nm (Fenske, 1993). For biomedical delivery applications, it has become clear that the particles with the greatest utility have diameters centered around 100 nm (large enough to carry significant payload, small enough to slip between leaky endothelial junctions), and these are now more commonly referred to as LNs. Recent advances have demonstrated that LN delivery systems can encapsulate and facilitate the delivery of therapeutic agents ranging from conventional small molecules to plasmid DNA containing therapeutic genes with sizes of several kbases. Between the late 1960s and the early 1990s, several successes in the liposomal field were achieved, all of which involved entrapment of weakly basic small molecules such as doxorubicin, vincristine, ciprofloxacin, or amphotericin B. These successes followed from a number of technological advances.

First, methods were discovered for producing liposomes of varying defined sizes, with narrow size distributions and lamellarity. For example, the extrusion technique, in which MLVs

are forced through polycarbonate filters with defined pore sizes under high pressure, allows generation of particles with diameters ranging from 50 nm to 400 nm (Mayer, Hope, et al., 1986). Second, it was observed that most drugs of interest were weakly basic amines that could be accumulated within liposomes in response to a transmembrane pH gradient (inside acidic; Cullis et al., 1997). Originally, the pH gradient was formed using a citrate buffer of pH 4 (Mayer, Bally, et al., 1986; Madden et al., 1990), but since then, other methods of generating transmembrane pH gradients have been developed that involve the use of ammonium gradients (Lasic et al., 1992; Lasic et al., 1995; Maurer-Spurej et al., 1999), or ion gradients in conjunction with ionophores (Fenske et al., 1998). These “first-generation” liposomes are by far the most clinically advanced of any nanoparticulate carrier system, with at least five drugs approved for clinical use and several more in clinical trials.

From the late 1980s onward, the emergence of novel lipid classes, namely [poly(ethylene glycol) [PEG]-conjugated lipids and strongly cationic lipids (Felgner et al., 1987; Felgner and Ringold, 1989), allowed the design of LNs capable of efficient encapsulation of DNA- and RNA-based drugs (Semple et al., 2001; Fenske et al., 2002; Fenske and Cullis, 2005; Jeffs et al., 2005). Research in our laboratory as well as in others has demonstrated that all types of nucleic acids, including DNA for gene therapy (Wheeler et al., 1999), oligonucleotides for immunostimulatory therapy (Mui et al., 2001), ribozymes and siRNA for gene-silencing applications (Jeffs et al., 2005; Zimmermann et al., 2006), and polyplexes for gene therapy (Heyes et al., 2007), can be encapsulated in LNs. These particles exhibit several differences from LNs used for conventional drugs.

First, encapsulation requires the presence of both a cationic lipid (which interacts with negatively charged nucleic acids) and a PEG-lipid conjugate (which stabilizes the particles; Fenske et al., 2002). Second, encapsulation occurs either during particle formation or when the particles are destabilized by an agent such as ethanol (Maurer, Wong, et al., 2001). Finally, whereas LNs encapsulating plasmid DNA are often unilamellar in their bilayer structure (see Figure 2), certain LNs—specifically, those encapsulating antisense oligonucleotides—have a unique multilamellar structure, even though their diameters are on the order of 100 nm (Maurer, Wong, et al., 2001; see Figure 3).

Because of these differences in formation and structure, the nomenclature of these particles has been somewhat confusing: LNs containing plasmid DNA have been referred to as stabilized plasmid-lipid particles (SPLPs), those containing antisense oligonucleotides as stabilized antisense-lipid particles (SALPs) or LN-CpG ODNs (liposomal nanoparticles encapsulating oligodeoxynucleotides containing CpG motifs), and those containing siRNA as stabilized nucleic acid-lipid particles (SNALPs). Regardless, the potential to develop LNs for genetic medicines has been demonstrated for gene-expression applications (Fenske et al., 2001; Heyes et al., 2007), gene-silencing applications (Zimmermann et al., 2006), and immunostimulatory applications (de Jong et al., 2007; Wilson et al., 2007) involving several of these particles in various animal models.

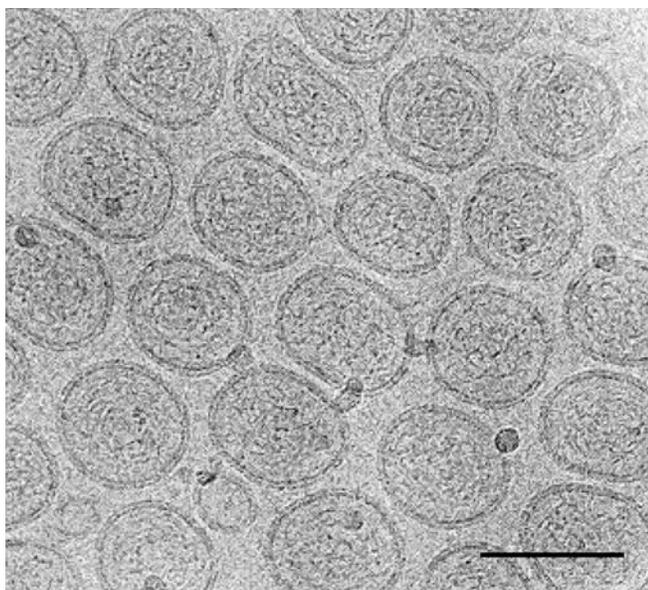


FIGURE 2.—Cryo-transmission electron micrographs of SPLPs prepared from DOPE:DODAC:PEG:CerC₂₀ (83:7:10 mol:mol:mol). The mean particle diameter is 70 nm. The bar indicates 100 nm. SPLP = stabilized plasmid-lipid particle; DODAC = *N,N*-dioleoyl-*N,N*-dimethylammonium chloride; DOPE = dioleoylphosphatidylethanolamine; PEG = poly(ethylene glycol). Reprinted with permission from Tam et al. (2000).

Regardless of the type of drug being encapsulated or the nature of the carrier particle, we have discovered that the following underlying principles generally apply.

Formulation Is Key: The lipid bilayer can have a marked effect on the pharmacokinetics and biodistribution of LNs. For example, the presence of cholesterol leads to longer circulation lifetimes and increases drug retention. Likewise, LNs containing a version of sphingomyelin [SM] in which the native trans double bond has been reduced exhibit significantly improved drug-retention properties (Johnston et al., 2007). In addition to the formulation of the lipid bilayer, attention must be paid to the internal concentration of encapsulated drug. It is now becoming increasingly clear that regulating drug-release rates is a key parameter in achieving maximal efficacy of an LN system and that this can be achieved not only by varying the lipid composition but also by regulating the initial drug-to-lipid ratio (Johnston et al., 2006). Size, overall charge, and lamellarity are other important parameters that influence particle biodistribution.

Biology Has a Major Impact on the Design of LNs: It is important to consider the interactions of LNs with the whole system. This includes proteins and peptides, especially those of the immune system, as well as cell, tissue, and organ systems. Most noteworthy is the phenomenon known as enhanced permeation and retention, the preferential accumulation of long-circulating LNs at sites of disease via extravasation of the LNs through the leaky vasculature at the disease site (Allen and

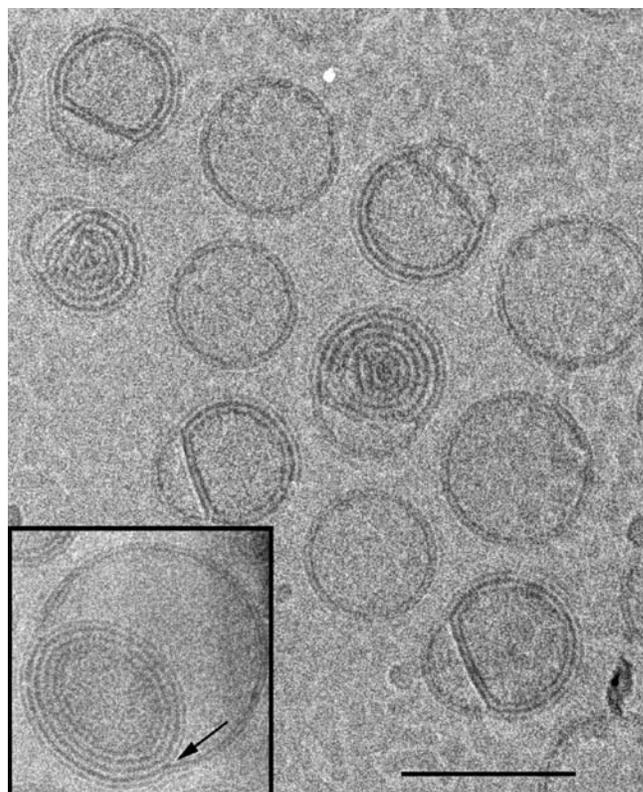


FIGURE 3.—Cryo-transmission electron micrographs of SALPs prepared from DSPC/Chol/PEG-CerC₁₄/DODAP (20:45:10:25 mol %), with an entrapped antisense-to-lipid weight ratio of 0.125 mg/mg. The unique multilamellar lipid bilayer structure can be seen in several of the particles. The inset is an expanded 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. DODAP = 1,2-dioleoyl-3-(dimethylamino)propane; SALP = stabilized antisense-lipid particle. Reprinted with permission from Maurer, Wong, et al. (2001).

Cullis, 2004). For this phenomenon to occur, the LNs have to be less than 100 nm in diameter and have circulation half-lives greater than 6 hours. For LNs carrying certain genetic drugs, particularly antisense oligodeoxynucleotides (ODNs) and siRNA, one must be aware of potential interactions with the immune system, as a strong immune response (Mui et al., 2001; de Jong et al., 2007) may or may not be desired, depending on the application.

Efficacy Depends on Drug Type as Well as Release Rate: LNs can increase the therapeutic index of drugs by affecting the biodistribution of the drug—increasing the accumulation of the drug at sites of disease and reducing the accumulation of the drug in healthy tissue. This increased accumulation of the drug can act extracellularly as a local depot, or the LNs can facilitate the internalization of the drug, with the drug's being processed intracellularly. Regardless, increased accumulation of the drug at a disease site does not necessarily translate to an increase in efficacy. Doxorubicin is an example of a drug that has greatly

reduced side effects in encapsulated form but similar efficacy to the free drug (Abraham et al., 2005). Other drugs, such as vincristine and topotecan, exhibit greatly increased efficacy when encapsulated in LNs (Boman et al., 1994). The reasons for this will be discussed below, where we will give a few specific examples from our research to illustrate these fundamental principles.

LN FOR COVENTIONAL THERAPEUTICS

There are a number of examples of LNs for conventional small-molecule drugs (for reviews, see Hope and Wong, 1995; Cullis et al., 1997; Maurer, Fenske, et al., 2001; Fenske and Cullis, 2005; Semple et al., 2005). As an example, we will describe our research and development efforts with liposomal vincristine to illustrate the advantages of LNs.

Vincristine is a microtubule inhibitor that acts in the M phase of the cell cycle and is one of the most widely used anti-cancer drugs; its primary indication is for non-Hodgkin's lymphoma (NHL). Previous work has shown that the efficacy of LNs containing cell cycle-specific drugs is particularly sensitive to drug-release rates. In studies using the L1210 and P388 murine leukemia models or the A431 human squamous-cell carcinoma model, formulations of vincristine with progressively slower release rates converted the drug from being essentially inactive to producing 100% cures (Boman et al., 1993; Boman et al., 1994). This dependence on release rates is consistent with the fact that prolonged exposure of cells to cell cycle-specific agents results in greater cell killing in vitro and enhanced anti-tumor activity in vivo (Boman et al., 1993; Sarris et al., 2000; Semple et al., 2005; Johnston et al., 2006).

Clearly, it is desirable to increase drug retention, but can it be increased too far? A thought experiment will demonstrate this to be the case: if the drug leaks out of the LNs too quickly, the efficacy will be similar to that of the free drug, in which the concentration of drug at the tumor peaks early and rapidly tapers off, only briefly rising above the minimum concentration required to kill the cell. If the drug is retained too well and leaks out of the LNs very slowly, the concentration of drug at the tumor will always be below the threshold concentration. A leakage rate somewhere in the middle will allow the drug to be retained long enough for the LNs to accumulate at the tumor yet still be released at the tumor site at a rate sufficient to provide enough bio-available drug to inhibit tumor growth.

Drug retention is increased by a variety of factors: increasing acyl chain length and degree of saturation; the lipid composition of the LNs, including the presence of cholesterol and the use of SM (Webb et al., 1995) or dihydrosphingomyelin (Johnston et al., 2007) instead of phosphatidylcholine; and the use of more acidic internal environments in the liposome (Boman et al., 1994). Recently, it has been observed that increased drug retention is related to increased drug-lipid ratios (Johnston et al., 2006). Use of the ionophore method of drug loading (Fenske et al., 1998) allows for much higher drug-to-lipid ratios than the conventional citrate method.

Using this method, a series of LN-vincristine was prepared with a wide range of drug-to-lipid ratios, and drug leakage rates were measured both in vitro and in vivo. In both cases, the rate of drug release was found to decrease with an increase in the

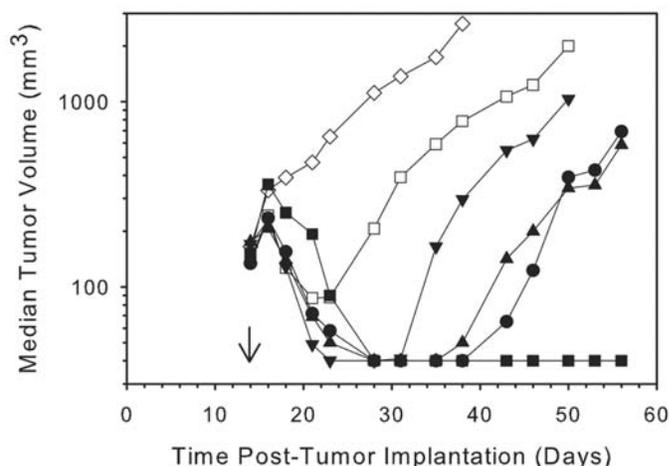


FIGURE 4.—Therapeutic effects of liposomal nanoparticles (LNs) encapsulating vincristine with different drug-to-lipid ratios (and correspondingly different release rates) in the MX-1 human mammary xenograft model. MX-1 tumors were planted in the flanks of nude mice and allowed to grow for 14 days, at 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 that each animal received the same lipid dose, as the drug-to-lipid ratio was varied. Treatments: 300 mM sucrose (\diamond), free vincristine (\square), or LN-vincristine with drug-to-lipid ratios (wt/wt) of 0.025 (\blacktriangledown , $T_{1/2} = 6.1$ hr), 0.05 (\blacktriangle , $T_{1/2} = 8.7$ hr), 0.1 (\blacksquare , $T_{1/2} = 15.6$ hr), and 0.6 (\bullet , extrapolated $T_{1/2} = 117$ hr). Reprinted with permission from Johnston et al. (2006).

concentration of encapsulated drug. In vivo efficacy studies using an MX-1 breast cancer model (see Figure 4) revealed an important observation: the formulation with the maximal efficacy had an intermediate half-life of drug release of approx. 15.7 hours, corresponding to a drug-to-lipid ratio of about 0.1 (mol:mol). Formulations with half-lives of release of 8.7 hours and 117 hours had greatly reduced efficacy. Fortunately, this drug-to-lipid ratio is very close to the value chosen for the formulation that has undergone the greatest degree of clinical testing for a liposomal version of this drug (Sarris et al., 2000; Rodriguez et al., 2002; Rodriguez et al., 2004).

This formulation has been giving excellent results in studies comparing the efficacy of conventional CHOP (chemotherapy treatment composed of cyclophosphamide, doxorubicin, vincristine, and prednisone) and CHOP in which the vincristine has been replaced by LN-vincristine (Rodriguez et al., 2002; Rodriguez et al., 2004). Thus, in a study focused on the treatment of non-Hodgkin's lymphoma in elderly patients, LN-vincristine CHOP + rituxan (Rodriguez et al., 2004) was compared to CHOP + rituxan (Coiffier et al., 2002), and the overall response for the former study was 93% compared to 83% for the latter. The overall survival at 22 to 24 months was 98% for the former treatment and 70% for the latter. Also of significance was the fact that the treatment was as well tolerated by elderly patients as it was by younger patients. Clearly, these results bode well for this formulation, and several clinical trials are set to begin in 2007. Other cell-cycle-specific drugs for which enhanced efficacy has been observed in animal studies and that are entering clinical trials in the near future include topotecan and vinorelbine.

LN FOR DELIVERY OF GENETIC DRUGS

LNs for Plasmid DNA-carrying Reporter Genes

Genetic drugs act at the level of gene expression and include DNA vectors for gene therapy, antisense oligonucleotides, ribozymes and siRNA for gene-silencing applications, and antisense oligonucleotides for immunostimulatory applications. These drugs have tremendous potential. When administered intravenously, however, genetic drugs exhibit inherent sensitivity to rapid inactivation by nucleases, renal and dose-limiting hemodynamic toxicities, and rapid elimination from the circulation. Therefore, they represent a class of drugs that could potentially benefit from liposome delivery systems to improve their pharmacokinetic properties and overall effectiveness.

The formulation of LNs for genetic drugs represented an interesting challenge: there was a need to produce LNs of well-defined sizes (similar to that of the conventional liposomal drugs), as size affects biodistribution and accumulation at disease sites; there was a need to efficiently encapsulate drugs within a lipid vesicle to protect the genetic drugs from nucleases present in plasma and to mask their inherent toxicities; and, once they have accumulated at the disease site, there was a need to deliver the drugs intracellularly to subcellular sites where they would become active.

As we discovered for small-molecule LNs, the benefits of passive accumulation at sites of disease and inflammation are attained when LNs possess the following features: extended circulation lifetimes, high drug-to-lipid ratios, high encapsulation efficiencies, and vesicle diameters less than 100 nm. Early attempts to formulate genetic drugs resulted in lipid vesicles that had size and structural heterogeneity, low drug-to-lipid ratios, and low encapsulation efficiencies. What became clear from these early studies was that cationic lipids were a crucial component, as they facilitated interaction with the nucleic acid, charge neutralization, and condensation of large polyanionic macromolecules. In addition, the cationic lipids enhanced the interaction of the LNs with cells *in vitro* (Felgner et al., 1987; Felgner and Ringold, 1989; Felgner et al., 1995). We also discovered, however, that there were inherent toxicities associated with these cationic lipids and that excessive positive charge on the surface of the LNs dramatically reduced the circulation lifetime of these LNs. The solution to the latter problem came from studies on conventional liposomal delivery systems, from which it was well known that PEG lipids would be an essential component of any long-circulating LNs, as they sterically stabilized the LNs and greatly enhanced the circulation lifetime of the liposomes (Allen, 1994).

The 1990s saw the development of LN systems for DNA delivery, known as SPLPs, that fulfilled these requirements (Wheeler et al., 1999; Zhang et al., 1999; Fenske et al., 2002). SPLPs consist of plasmid DNA encapsulated within a lipid bilayer composed of dioleoylphosphatidylethanolamine (DOPE), a cationic lipid (usually *N,N*-dioleyl-*N,N*-dimethylammonium chloride [DODAC]), and PEG-ceramide (PEGCer). SPLPs are formed by a procedure in which mixtures of plasmid and lipid

are cosolubilized at a specific ionic strength by the detergent octyl-glucopyranoside, which is then removed by dialysis. The particles are purified by sucrose density gradient centrifugation. When conditions are optimized, high plasmid-encapsulation efficiencies are achieved (50% to 70%). The resulting purified SPLPs are small, monodisperse particles with a diameter of approximately 70 nm and a plasmid-to-lipid ratio of 62.5 $\mu\text{g}/\mu\text{mol}$, corresponding to one plasmid per SPLP (Wheeler et al., 1999; Tam et al., 2000). SPLPs provide protection of plasmid DNA from DNase I and serum nucleases and are highly stable in serum.

The pharmacokinetics, tumor accumulation, and transfection properties of SPLP composed of DOPE/DODAC/PEG-Cer (83:7:10) have been extensively characterized in several mouse tumor models. Following intravenous injection into mice bearing subcutaneous Lewis lung carcinomas, the circulation half-life of SPLP was 6.1 ± 1.1 hours and 7.1 ± 1.6 hours, as assessed by lipid and DNA markers, respectively (Tam et al., 2000). The accumulation of SPLPs at distal tumor sites and gene expression at those sites has been observed in mouse tumor models following intravenous injection (Tam et al., 2000; Fenske et al., 2001). Studies using the subcutaneous Lewis lung carcinoma revealed the presence of SPLPs in liver, plasma, and tumor at 24 hours after injection, with little found in the lung and spleen (Tam et al., 2000). Approximately 3% of the SPLP dose (~1,000 copies per cell) was found at the tumor at 24 hours. SPLPs gave rise to significant gene expression peaking at 48 hours at the tumor. SPLPs elicited no toxic side effects at dose levels as high as 100 μg DNA per mouse.

The accumulation of SPLP-associated plasmid DNA at a distal neuro-2a tumor site following intravenous injection is shown in Figure 5 (Fenske et al., 2001). The amount of intact plasmid delivered to the tumor is substantial, corresponding to >10% of the total injected dose per gram of tumor at 24 hours. This leads to significant levels of gene expression at the tumor at 24 hours, which reaches a maximum at 72 hours after injection. It is striking that the highest luciferase activity is located in the tumor, with other tissues' giving only low levels of transfection. At the later time points, the transfection levels in the tumor are two orders of magnitude greater than in other tissues. These results confirm that long-circulating LNs are capable of preferential disease-site targeting and gene transfer.

LNs for Antisense Oligonucleotide Drugs

Antisense oligonucleotides were designed to operate as gene-silencing agents. As their sequence matches a portion of a gene, they are capable of binding either to the gene itself or to the mRNA transcribed from that gene (Stein and Cohen, 1988). Either way, the binding of the ODNs should reduce the expression of that gene. Unfortunately, free ODNs are degraded in the circulation (even the more stable phosphorothioate variety have limited stability) and are not taken up readily into target cells; thus, they appeared as ideal candidates for LN encapsulation, and attempts were made to produce an effective delivery system.

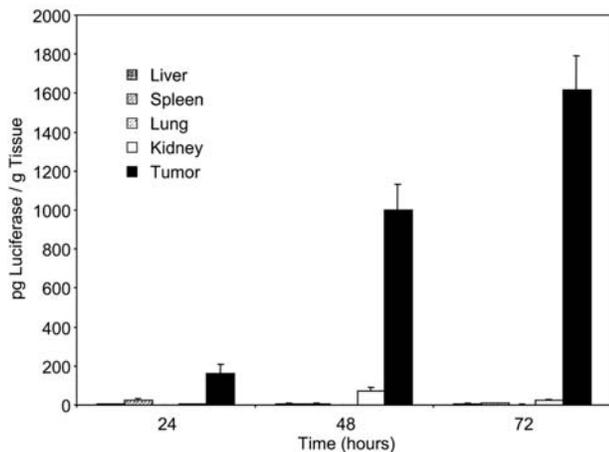


FIGURE 5.—SPLPs (encapsulating plasmid DNA with the gene for luciferase) give rise to tumor-specific gene expression following intravenous injection in neuro-2A tumor-bearing mice. Tumors were grown on the hind flank of [spo] mice following injection of 10^6 cells. Note that essentially all of the gene expression is in the tumor tissue, with minimal levels of expression in the liver, spleen, lung, and kidney. Reprinted with permission from Fenske et al. (2001).

Guided by our approach to develop SPLPs for delivery of DNA-based drugs, we developed a formulation process to produce LNs for delivery of antisense ODN drugs (Maurer, Wong, et al., 2001; Semple et al., 2001). Instead of a detergent, ethanol was used in this novel procedure, along with an ionizable aminolipid (positively charged in acidic buffer solutions), DODAP (1,2-dioleoyl-3-[dimethylamino]propane), to facilitate efficient encapsulation of antisense oligonucleotides in lipid vesicles. The advantage of this ionizable aminolipid is that it can subsequently be rendered neutral at physiological pH. This is essential because when delivery systems bearing a net cationic charge are administered intravenously, they exhibit rapid plasma clearance, distribute into the lung, liver, and spleen (references), and often exhibit liver and hemodynamic toxicities such as activation of complement and prolongation of clotting times (Schreier et al., 1997). We also used a PEG-lipid, PEG-ceramide, to sterically stabilize the liposomes and thereby prolong their circulation lifetimes. The end result is a mixture of multilamellar and unilamellar vesicles with small diameters (70 to 120 nm), capable of entrapping up to ~2,200 oligonucleotide molecules per 100 nm liposome (Maurer, Wong, et al., 2001; Figure 3). The stabilized antisense lipid particles (SALPs) exhibit extended circulation half-lives ranging from 5 to 6 hours for particles formed with PEGCerC₁₄ (PEG-ceramide containing 14 carbon fatty acid) to 10 to 12 hours for particles formed with PEGCerC₂₀ (PEG-ceramide containing 20 carbon fatty acid; Semple et al., 2001). As with SPLPs, they possess the combination of high entrapment efficiencies (up to 90%), small size, and extended circulation lifetimes necessary for effective *in vivo* delivery of antisense drugs.

When the pharmacokinetic and biodistribution properties of SALPs were first characterized in mice, a surprising discovery

was made. Repeated intravenous dosing of SALPs was found to lead to rapid clearance of the particle from the circulation, often with rapid death of the animal. Subsequent studies revealed that LNs encapsulating CpG ODNs led to a profound stimulation of the immune system, with release of cytokines and activation of natural killer (NK) cells (Mui et al., 2001). In retrospect, this should not have been surprising. The ability of bacterial and viral DNA to stimulate an innate immune response with release of inflammatory cytokines and interferons is well known (Heeg et al., 1998; Lipford et al., 1998; Lund et al., 2003). This has also been observed with dsRNA (double-strand RNA; Alexopoulou et al., 2001), ssRNA (single-strand RNA; Heil et al., 2004), and siRNA (Judge et al., 2005).

The strong response to bacterial DNA results from recognition of unmethylated CpG dinucleotides in a particular base context; mammalian DNA has a lower frequency of CpG sequences, and they are usually methylated (Hemmi et al., 2000). This recognition, and that of the other nucleic acids, is mediated by a class of innate pattern-recognition receptors, known as the Toll-like receptors (TLRs; Hemmi et al., 2000; Alexopoulou et al., 2001; Bauer et al., 2001; Kirschning and Bauer, 2001; Lund et al., 2003). Thus, whereas encapsulated antisense ODNs did not provide an effective means to silence a given gene, because of the immune response that overwhelmed any such effects, they did provide a potentially powerful way to stimulate the immune system for other applications, especially if the ODNs contained immunostimulatory CpG sequences.

In a sense, LNs containing CpG ODN can be viewed as an "artificial virus." These LNs resemble viruses in many aspects: they have a similar size, encapsulate nucleic acids, and have similar nucleic-acid-to-lipid ratios. Thus, like viruses, LN-CpG ODNs can be taken up by antigen-presenting cells (such as dendritic cells), where the interaction with TLR9 leads to stimulation and release of cytokines and interferons, which in turn activate NK cells to ANK cells, which then mediate antiviral, antibacterial, or anticancer activities. In addition to this innate response, increased antigen display leads to an adaptive response, with eventual production of antigen-specific antibodies and activation of T helper cells.

The fact that LN-CpG ODNs are capable of acting as potent immunostimulatory agents suggests a variety of exciting applications for these agents. If this were to hold true in humans, LNs could have significant potential for vaccine development, expansion and activation of the NK cell population, and enhanced homing of NK cells to tumor sites. To explore this further, a liposome formulation, INX-0167, was developed and optimized to generate the strongest possible sequence-specific innate immune response. This was used to examine the immunopotency of INX-0167 and its ability to act as a vaccine adjuvant when administered subcutaneously with tumor-associated adjuvants. It was observed that LN-CpG ODNs were able to adjuvinate adaptive immune responses against coadministered tumor-associated antigens, inducing effective antitumor activity in a number of murine tumor models (de Jong et al., 2007). 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 tumors is a very significant result and highlights again the benefit of encapsulating therapeutic agents within an LNM vector.

LN for siRNA Drugs: Gene Silencing via RNA Interference

The techniques described above for the formation of SPLPs and SALPs were designed for small-scale preparations (Fenske et al., 2003), although they can be scaled up, with some technical challenges, for larger scale preparations suitable for animal studies (Fenske et al., 2002). Even so, they remain inadequate for preclinical and clinical studies. An important step forward was thus realized with the development of a simple and fully scalable method for the formation of SPLPs by spontaneous vesicle formation (Jeffs et al., 2005). This method is related to that used in the formation of SALPs as it involves the formation of particles in the presence of ethanol. The differences lie in the selection of lipids (which include DSPC, Chol, DODMA [1,2-dioleoyloxy-*N,N*-dimethyl-3-aminopropane, a more stable titratable cationic lipid], and PEG-S-DSG [PEG covalently bonded to distearoylglycerol, which replaces the PEG-Cer]) and the use of a peristaltic pump with dual pump heads to achieve mixing of lipids and RNA. This technology can be used to prepare SPLPs with similar properties to those formed by detergent-dialysis, but its strength 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 short interfering RNAs (siRNA). When the LNs encapsulate siRNA, the particles are commonly known as stabilized nucleic acid lipid particles, or SNALPs. siRNA are short, double-stranded RNA effector molecules involved in the mechanism of posttranslational gene silencing known as RNA interference (Aagaard and Rossi, 2007; Kong et al., 2007; Masiero et al., 2007; Sioud, 2007). When dsRNA molecules are introduced into a cell, they are recognized and cleaved by the enzyme Dicer (a member of the RNaseIII family of dsRNA-specific ribonucleases), resulting in 19- to 23-bp dsRNA duplexes. These are incorporated into the multiprotein complex RNA-induced silencing complex (RISC), where the antisense strand is used to guide RISC to recognize and cleave target mRNA. The end result is inhibition of gene expression, an endpoint for which siRNA appear to be much more potent than antisense ODNs (Kong et al., 2007).

Several recent studies highlight the therapeutic potential of SNALPs and RNA interference (RNAi) in the treatment of a variety of diseases. A particularly elegant example comes from a recent article describing a new approach for the treatment of high serum-cholesterol levels, based on the silencing of the liver apoB gene in nonhuman primates (Zimmermann et al., 2006). Initial 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

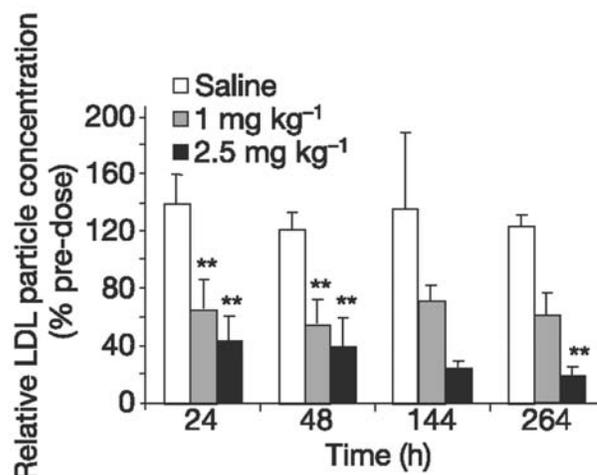


FIGURE 6.—Effect of RNAi-mediated silencing of apoB mRNA in non-human primates on the levels of circulating LDL. Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg/kg⁻¹ SNALP-siApoB-2, and measured for LDL. The results are expressed as a percentage of predose values and are expressed as mean \pm standard deviation. Data points marked with asterisks are statistically significant compared with saline-treated animals (* p < .05, ** p < .005). SNALP = stabilized nucleic acid lipid particles. Reprinted with permission from Zimmermann et al. (2006).

studies, and did not possess immunostimulatory activity. The siRNA was formulated in SNALP and evaluated for pharmacokinetics, efficacy, and safety in cynomolgus monkeys. Although the circulation half-life was shorter (72 minutes) than other liposomal DDS, the key result was a striking dose-dependent reduction of liver apoB mRNA levels following systemic administration of siRNA-SNALP. A single dose of siRNA in SNALP resulted in a reduction of liver apoB mRNA of 90% by 48 hours, an effect that persisted up to 11 days (see Figure 6). This led to maximum reductions in blood apoB-100, cholesterol, and LDL levels of approximately 78%, 62%, and 82%, respectively, which were also observed during the same time period. No reductions in HDL levels were observed. These results were found to exceed the results obtained with currently 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 RNAi-mediated effects, the positive results were buoyed further by the absence of any observed toxicities.

CONCLUSIONS

The examples discussed above demonstrate the major advances that have been made in the formulation of LNs and in their application to a variety of diseases. We anticipate that LNMs will soon begin to reach their full potential as an important class of therapeutic agents and will contribute to significant advances in the treatment of many classes of disease.

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