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Encapsulation of Nucleic Acid–Based Therapeutics

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INTRODUCTION

Liposomes represent one of the most clinically advanced drug-delivery systems with the range of medical applications extending from chemotherapy of cancer and infectious disease to vaccines and gene therapy (1,2). However, liposomal formulations of genetic drugs such as antisense oligonucleotides and plasmid DNA (pDNA) for systemic applications are difficult to achieve (3). The large size and highly-charged nature of these molecules mitigates against the formation of small, neutral, serum-stable carriers, which are required to achieve the long circulation times necessary for efficient accumulation at disease sites such as sites of tumor growth and inflammation. In particular, passive encapsulation of pDNA in liposomes is very inefficient due to the large size of these molecules. Efficient entrapment requires interaction between the lipid components of the carrier and the nucleotide-based drugs. However, this interaction is very difficult to control. For example, complexes formed through electrostatic interaction between negatively-charged polynucleotides and cationic liposomes exhibit broad size distributions. These complexes efficiently transfect cells in vitro; however, in vivo their large size and/or positive charge triggers rapid clearance from the circulation (4–8). They can also be highly toxic (8). Therefore, substantial effort has been focused on constructing lipid-based carriers with improved in vivo characteristics.

In this chapter, the recent advances in the development of small serum-stable carriers for nucleic acid therapeutics such as antisense and immune stimulatory oligonucleotides and pDNA are reviewed with the emphasis on our work. Two basic approaches for encapsulation will be described. The first of these employs incubation of large unilamellar vesicle (LUV) containing a cationic lipid and a polyethyleneglycol (PEG) coating with oligo- or polynucleic acids in the presence of ethanol (9–11). The use of membrane-destabilizing agents such as ethanol in conjunction with PEG-lipids offers a way to control the interaction between negatively-charged polyelectrolytes and cationic liposomes and results in the entrapment of the nucleic acids in small liposomes. The resulting particle will be referred to as "stabilized nucleic acid-lipid particle," or SNALP, and the approach of making it as preformed vesicle approach (PFV). The second method involves a detergent-dialysis procedure for the encapsulation of pDNA, resulting in the formation of "stabilized plasmid-lipid particles" (SPLP) (11–13). These SNALP and SPLP systems demonstrate long circulation lifetimes and preferentially accumulate at tumor sites and sites of inflammation following IV administration due the "enhanced permeability and retention" effect associated with the more permeable vasculature found in these disease sites (14). Highly specific transgene expression at distal tumor sites has been observed following IV injection of SPLP (15,16). It is also important to note that these systems naturally target antigen-presenting cells in vivo as they, like all other liposomal or particulate systems, are removed from the blood by the fixed and free macrophages of the mononuclear phagocyte system (reticuloendothelial system), resulting in accumulation in organs such as the liver and the spleen. Encapsulation of oligonucleotides containing immune stimulatory CpG motifs in liposomes (SNALP) results in an immune response that is enhanced compared to either lipid or oligonucleotide alone (17). This forms the basis for the application of liposome-encapsulated CpG oligonucleotides in immune therapy (18).

Both approaches are simple and allow efficient encapsulation of nucleic acid-based molecules such as oligonucleotides (9,10) and pDNA (8,10,12) in liposomes that are small in size (about 100 nm diameter) and stable in circulation, protecting the cargo from degradation. In the sections to follow, we will provide a brief overview of these methods.

METHODOLOGY

Entrapment of Polynucleotides Using the Preformed Vesicle Approach

The preformed vesicle (PFV) approach involves incubation of liposomes containing a cationic lipid and a PEG coating with polynucleotides in the presence of ethanol. Typically, LUV composed of distearoyl-phosphatidyl-choline (DSPC), cholesterol (Chol), 1-O-(2′-(ω-methoxy-polyethylene-glycol)

succinoyl)-2-*N*-myristoyl-sphingosine (PEG-CerC_{14}), and 1,2-dioleoyl-3-dimethylammoniumpropane (DODAP) are used. However, any zwitterionic lipid including dioleoylphosphatidyl-ethanolamine (DOPE) alone or in combination with Chol can be substituted for DSPC/Chol (10). DODAP was chosen as the cationic lipid because it has a protonable amino group. Its apparent pK_a is estimated to be between 6.6 and 7 (19). Entrapment can therefore be performed at low pH (pH 4) where DODAP is positively-charged and nonentrapped polynucleotides can be dissociated from the cationic lipid by neutralizing the pH and removed by subsequent anion exchange chromatography. Adjusting the pH to 7.5 also renders the surface charge of the liposomes neutral.

Liposome preparation: Ethanolic suspensions of LUVs composed of DSPC/Chol/PEG-CerC_{14}/DODAP (20:45:10:25 mol%) were either prepared by addition of ethanol to extruded liposomes or by addition of lipids dissolved in ethanol to an aqueous buffer solution and subsequent extrusion. Both methods give the same entrapment results and will be described in greater detail in the following: (i) After hydration of a lipid film in 50 mM pH 4 citrate buffer and five freeze/thaw cycles LUVs were generated by extrusion through two stacked 100-nm filters (10 passes). Ethanol was subsequently slowly added under rapid mixing to a concentration of 40% (v/v). Slow addition of ethanol and rapid mixing are important as liposomes become unstable and coalesce into large lipid structures as soon as the ethanol concentration exceeds a certain upper limit. (ii) LUVs were prepared by slow addition of the lipids dissolved in ethanol (0.4 mL) to citrate buffer at pH 4 (0.6 mL) followed by extrusion through two stacked 100-nm filters (two passes) at room temperature. Dynamic light scattering measurements performed in ethanol and after removal of ethanol by dialysis show no significant differences in size, which is typically 75 ± 18 nm. The extrusion step can be omitted if ethanol is added very slowly under vigorous mixing to avoid high local concentrations of ethanol.

Entrapment of oligo- and polynucleotides: The oligo- or polynucleotide solution was slowly added under vortexing to the acidic ethanol-containing liposome dispersion, which typically contained 10 mg/mL of lipid. The resulting dispersion was incubated at 40°C for one hour, and then dialyzed for two hours against a 1000-fold volume excess of citrate buffer to remove most of the ethanol and twice against a 1000-fold volume excess of HBS [20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)/ 145 mM NaCl, pH 7.5]. At pH 7.5 DODAP becomes charge-neutral and polyelectrolyte bound to the external membrane surface is released from its association with the cationic lipid. Unencapsulated polyelectrolytes were subsequently removed by anion exchange chromatography on diethylaminoethyl (DEAE)-sepharose CL-6B columns equilibrated in HEPES- buffered saline (HBS) (pH 7.5). Finally, it should be noted that liposome formation and encapsulation could be combined in a single step by mixing the lipids dissolved in ethanol with an aqueous solution containing the polynucleotide.

Determination of trapping efficiencies: Trapping efficiencies were determined after removal of external polyelectrolytes by anion exchange chromatography. Oligonucleotide concentrations were determined by UV-spectroscopy. The absorbance at 260 nm was measured after solubilization of the samples in chloroform/methanol at a volume ratio of 1:2.1:1 chloroform/methanol/aqueous phase (sample/HBS). Alternatively, absorbance was read after solubilization of the samples in 100 mM octylglucoside. The antisense concentrations were calculated according to: c ($\mu g/\mu L$) = $A_{260} \times 1OD_{260}$ unit ($\mu g/mL$) × dilution factor ($mL/\mu L$), where the dilution factor is given by the total assay volume (mL) divided by the sample volume (μL). OD_{260} units were calculated from pairwise extinction coefficients for individual deoxynucleotides, which take into account nearest neighbor interactions. The Oligreen assay was used in some instances, obviating the need for prior removal of unencapsulated oligonucleotide. Fluorescence emission was read at 520 nm with the excitation set at 480 nm before and after addition of n-octyl β-D-glycopyranoside (OGP) to a final concentration of 50 mM. The addition of detergent results in slight (10%) quenching of the Oligreen fluorescence and this accounted for the calculation of trapping efficiencies. Oligonucleotide concentrations were determined relative to a standard curve. Plasmid DNA encapsulation efficiencies were determined by A^{260} as described above (1OD = 50 $\mu g/mL$) and/or with the PicoGreen assay (10). Adenosine triphosphate (ATP) encapsulation was determined by A^{260} with 1OD corresponding to 35.8 $\mu g/mL$ ATP. Lipid concentrations were determined by the inorganic phosphorus assay after separation of the lipids from the oligonucleotides by a Bligh and Dyer extraction.

Encapsulation of pDNA Using the Detergent Dialysis Procedure

The encapsulation of pDNA can also be accomplished with the use of a detergent dialysis procedure (12). In contrast to the PFV approach, the detergent dialysis procedure starts off with a micellar system and leads to encapsulation of pDNA in unilamellar liposomes called SPLP after detergent removal. Plasmid entrapment relies on a delicate balance between cationic lipid content and ionic strength of the solution.

Encapsulation of pDNA: Typically pDNA was encapsulated in SPLP composed of DOPE, dioleoyldimethylammonium chloride (DODAC), and 1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-dodecanoylsphingosine (PEG-$CerC_{20}$). Lipids (DOPE:DODAC:PEG-$CerC_{20}$, 84:6:10 mol%) were dissolved in ethanol or chloroform and dried to a lipid film. The lipid mixture was resuspended in HBS (5 mM HEPES, 150 mM NaCl, pH 7.5) containing 200 mM OGP and 0.4 mg/mL pDNA [e.g., Plasmid DNA Containing the cytomegalovirus promotor and coding for luciferase (pCMV)-luc]. The final lipid concentration was 10 mg/mL. The mixture of lipid, plasmid, and OGP was dialyzed for 16 to 18 hours against three changes

of a 1000-fold volume excess of HBS. Unentrapped plasmid was removed by DEAE-Sepharose CL-6B chromatography. Entrapment efficiencies were determined as described above for the PFV approach.

Separation of encapsulated plasmid from empty liposomes by sucrose density gradient centrifugation: The plasmid samples were applied to the top of a discontinuous sucrose gradient in 12.5-mL ultracentrifuge tubes. The gradient was formed with 3 mL each of 10% sucrose, 2.5% sucrose, and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at $160{,}000 \times g$ for two hours at 20°C. The lipid-encapsulated pDNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. The SPLP band was collected. Buffer-exchange and concentration was performed by diafiltration.

RESULTS

Preformed Vesicle Approach

The following paragraphs describe the factors that are important for encapsulation and summarize the physicochemical and in vivo characteristics of the resultant liposomal systems using oligonucleotides as an example. However, it should be noted that the PFV approach could be generally applied to the entrapment of negatively-charged polyelectrolytes including pDNA and nonnucleotide-based polyelectrolytes.

Encapsulation and Physicochemical Properties

Ethanol is required for entrapment to occur. Addition of increasing amounts of ethanol to 100 nm DSPC/Chol/DODAP liposomes leads to the formation of large lipid structures following oligonucleotide addition and a concomitant increase in oligonucleotide entrapment levels (Table 1). The

Table 1 Entrapment of Antisense Oligonucleotide in the Absence of Polyethyleneglycol-Ceramide[a]

% EtOH (v/v)	% Encapsulation
0	4.5 ± 0.5
20	20.5 ± 1.5
30	32.5 ± 2.5

[a]Encapsulation efficiencies are listed as a function of ethanol concentration for distearoyl-phosphatidyl-choline/cholesterol/1,2-dioleoyl-3-dimethylammoniumpropane large unilamellar vesicle (LUVs). The initial oligonucleotide-to-lipid ratio was 0.034 mol/mol (0.3 mg/mg). The LUVs used for these experiments were 99 ± 22 nm in size. The encapsulation values are given as mean $\pm$ SD.

increase in size and entrapment reflect a progressive reorganization of the LUVs into large multilamellar liposomes, which can be seen in the transmission electron microscope (TEM) micrograph presented in Figure 1C. At 40% (v/v) ethanol and higher, liposomes become unstable and fuse.

Incorporation of PEG-ceramide allows control of liposome size. The addition of ethanol was required for oligonucleotide encapsulation to occur. However, the size of the liposomes formed was large and could not be controlled. Therefore, regulatory components, which allow the control of fusion and aggregation processes responsible for the size increase, are required. It is known that incorporation of PEG-lipid conjugates into the liposomal membrane can inhibit liposome fusion and aggregation (20). PEG-lipids were therefore an obvious choice for regulating liposome size. In the presence of 2.5 to 10 mol% of PEG-Cer, oligonucleotides could be entrapped in liposomes that were not significantly larger than the parent liposomes from which they originated. Figure 2 depicts encapsulation efficiencies as a function of ethanol concentration for liposomes containing 10 mol% PEG-Cer. Maximum entrapment was reached at 40% ethanol and ethanol concentrations in excess of 25% (v/v) were required for entrapment to occur. No entrapment was found in the absence of ethanol. The amount of ethanol required for entrapment to occur was dependent on the PEG-Cer content of the liposomes, decreasing with decreasing amount of PEG. It should be noted that detergents such as octylglucoside could be substituted for ethanol.

Liposome size and entrapment efficiency. Both the size of the liposomes entrapping oligodeoxyonucleoliele (ODN) and the entrapment efficiency depend on the initial oligonucleotide-to-lipid ratio. Figure 3 shows that oligonucleotides can be efficiently entrapped at high oligonucleotide-to-lipid ratios. The entrapment efficiency is plotted as a function of the initial oligonucleotide-to-lipid ratio. The binding level at maximum entrapment is 0.16 mg oligonucleotide per mg of lipid (0.023 mol/mol, negative-to-positive charge ratio = 1.5). This corresponds to approximately 2200 oligonucleotide molecules per 100 nm liposome and demonstrates the high efficiency of this entrapment procedure. Entrapment efficiencies are about three orders of magnitude higher than obtained by passive encapsulation based on the trapped volume.

Upon increasing the oligonucleotide-to-lipid ratio, the size as well as the polydispersity of the samples increased slightly from 70 ± 10 nm for liposomes alone to 110 ± 30 nm for an initial oligonucleotide-to-lipid weight ratio of 0.2 mg/mg. Freeze-fracture electron microscopy showed an increase in the number of larger liposomes with increasing initial oligonucleotide-to-lipid ratios.

Morphology. Structural details were visualized by cryo-TEM. Figure 1A is a cryo-TEM image of a sample with an entrapped oligonucleotide-to-lipid ratio of 0.13 mg/mg. It confirms the coexistence of unilamellar liposomes with bi- and multilamellar liposomes. The membranes of the latter are in close contact. The inset of Figure 1A is an expanded view of a multilamellar

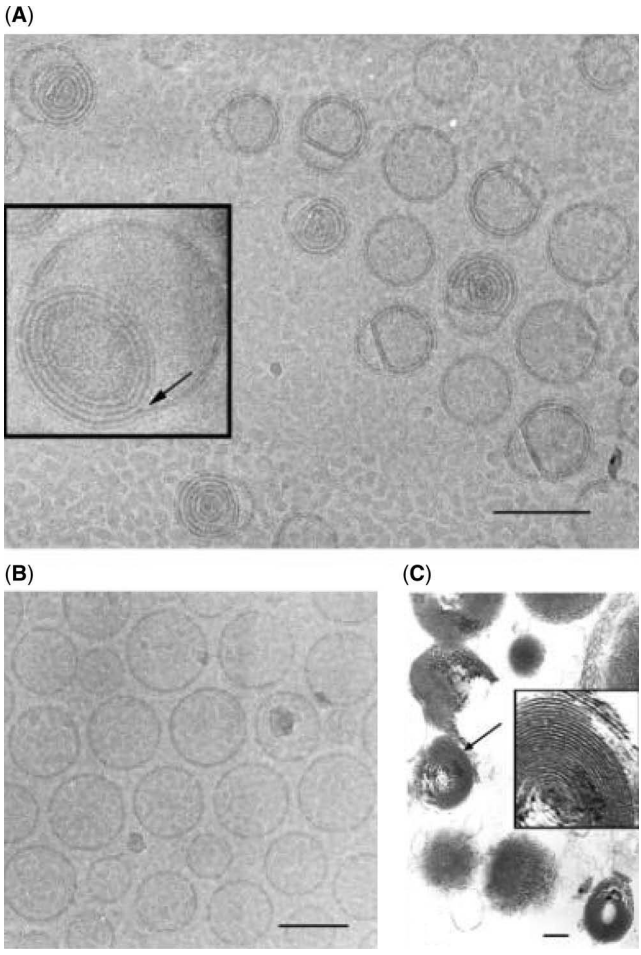

Figure 1 (**A**) Cryo-TEM picture of distearoyl-phosphatidyl-choline/cholesterol/1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-myristoyl-sphingosine/1,2-dioleoyl-3-dimethylammoniumpropane liposomes entrapping oligonucleotides. The inset is an expanded view of a multilamellar liposome showing two initially separate membranes forced into close apposition by bound oligonucleotides (*indicated by the arrow*). The entrapped antisense-to-lipid weight ratio was 0.125 mg/mg. Empty liposomes prepared the same way as the oligonucleotide-containing liposomes can be seen in (**B**). (**C**) TEM electron micrograph of a sample prepared in 30% ethanol in the absence of polyethyleneglycol-ceramide prior to anion exchange chromatography. The concentric bilayers of multilamellar liposomes can be clearly seen. The entrapment was 32% at an initial oligonucleotide-to-lipid weight ratio of 0.3 mg/mg. Throughout the figure, the bars represent 100 nm. *Abbreviation*: TEM, transmission electron microscope.

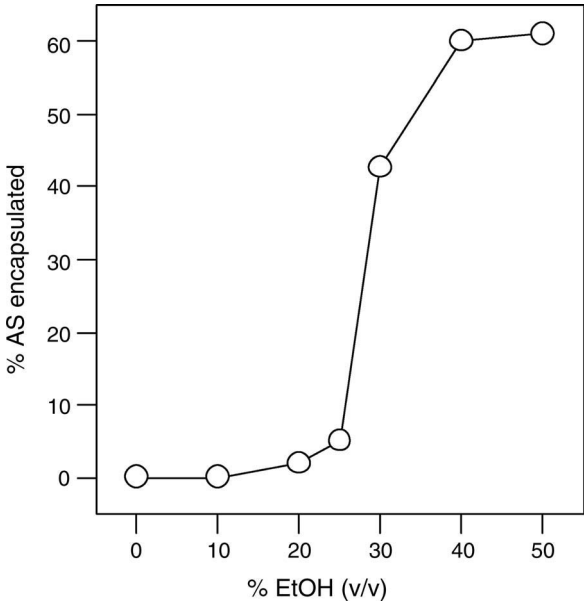

Figure 2 Encapsulation as a function of ethanol concentration. Oligonucleotides were added to distearoyl-phosphatidyl-choline/cholesterol/1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-myristoyl-sphingosine/1,2-dioleoyl-3-dimethylammonium propane liposomes in varying concentrations of ethanol at an initial oligonucleotide-to-lipid ratio of 0.24 mg/mg. *Abbreviations*: AS, antisense oligonucleotide; %EtOH(v/v), percentage of ethanol in volume/volume.

liposome and shows two initially separate membranes forced into close apposition by bound oligonucleotides. The number of multilamellar liposomes increases with increasing initial oligonucleotide-to-lipid ratio. The initial liposomes in the absence of antisense were unilamellar (Fig. 1B). The existence of multilamellar liposomes can only mean that more than one liposome participates in their formation and points to an adhesion-mediated mechanism of formation (2,10).

Encapsulation is not dependent on a particular oligonucleotide or on lipid composition. Entrapment is a general feature of the interaction of negatively-charged polyelectrolytes with cationic liposomes. Figure 4 (black bars) shows that different oligonucleotides as well as pDNA can be efficiently entrapped in DSPC/Chol/DODAP/PEG-CerC_{14} liposomes. Encapsulation of pDNA in preformed vesicles was less efficient than encapsulation of short oligonucleotides. The maximum DNA-to-lipid ratio obtained with pDNA was about four times lower than that achieved for the oligonucleotides. Other nonnucleotide-based polyelectrolytes such as polyanetholsulfate and dextransulfate can also be encapsulated (unpublished results). In contrast to

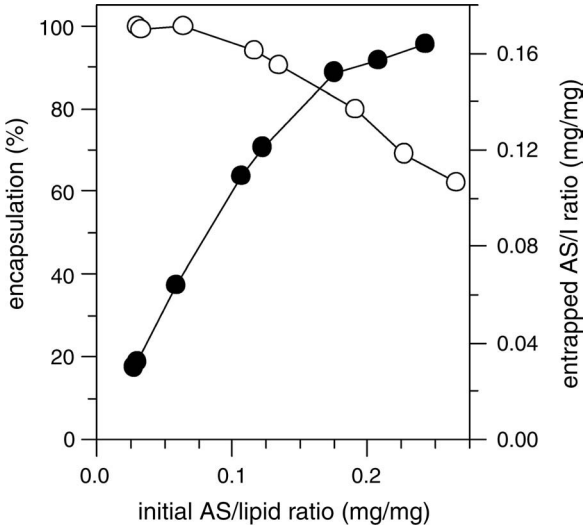

Figure 3 Plot of the entrapment efficiency expressed as the entrapped oligonucleotide-to-lipid ratio (*full circles*) and percent entrapment (*open circles*) as a function of the initial oligonucleotide-to-lipid ratio. The ratios are given in w/w. *Abbreviation*: AS, antisense oligonucleotide.

the efficient encapsulation of large molecules, entrapment of ATP, a small molecule with three negative charges, was less than predicted based on trapped volume calculations (10% at an initial ATP-to-lipid ratio of 0.2 mg/mg and 50 mM citrate buffer). This indicates that there is a critical size (length) and number of charges required for entrapment to occur. The entrapment procedure can be extended to other lipid compositions including DOPE systems (Fig. 4, white bars).

Pharmacokinetics

SNALP systems can exhibit plasma half-lives of up to 12 hours, significantly longer than the circulation half-life of free oligonucleotides and cationic liposome oligonucleotide complexes (Fig. 5). Increasing surface charge and PEG-coatings that dissociate from the liposome carrier can reduce the circulation half-life (9). For example, replacement of PEG-CerC_{20} with PEG-CerC_{14} results in a reduction of the half-life from 10 to 12 hours to 5 to 6 hours. This demonstrates a strong dependency of the circulation half-life on the length of the acyl chain contained in the hydrophobic ceramide group, which anchors the PEG coating to the membrane. The PEG-CerC_{14} lipid is able to rapidly exchange out of the lipid bilayer, with an in vitro half-life of approximately 1.1 hours. Under the same conditions, the exchange rate of the PEG-CerC_{20} is much slower (approximately 13 days), and this lipid is therefore able to provide steric protection against

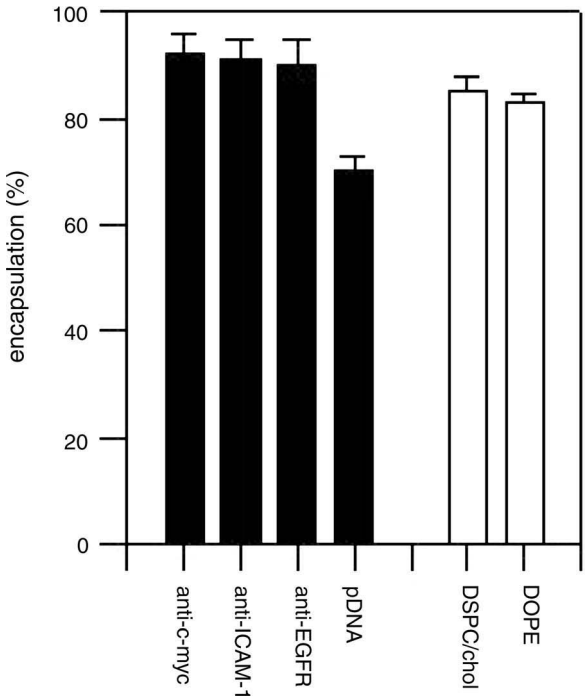

Figure 4 (*Black bars*) Entrapment of different antisense oligonucleotides as well as pDNA (pCMV-luc) in distearoyl-phosphatidyl-choline (DSPC)/cholesterol (Chol)/1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-myristoyl-sphingosine (PEG-CerC_{14})/1,2-dioleoyl-3-dimethylammoniumpropane (DODAP) liposomes. The initial oligonucleotide-to-lipid weight ratio was 0.1 mg/mg and 300 mM citrate buffer was used for oligonucleotide entrapment. The pDNA entrapment was performed in 50 mM citrate buffer at a pDNA-to-lipid weight ratio of 0.03 mg/mg. (*White bars*) Entrapment of anti-c-myc DSPC/Chol/PEG-CerC_{14}/DODAP (20/45/10/25 mol%) liposomes and dioleoylphosphatidyl-ethanolamine (DOPE)/PEG-CerC_{14}/DODAP (45/10/45 mol%) liposomes. The initial oligonucleotide-to-lipid weight ratio was 0.12 mg/mg for the DSPC/Chol system and 0.11 mg/mg for the DOPE system. The initial lipid concentration was 13 mM. The mRNA targets and sequences of the oligonucleotides are as follows: human c-myc (16-mr), 5′-AACGTTGAGGGGCAT-3′, human ICAM-1, 5′-GCCCAAGCTGGCATCCGTCA-3′ and human EGFR, 5′-CCGTGGTCATGCTCC-3′. *Abbreviations*: DOPE, dioleoylphosphatidyl-ethanolamine; DSPC, distearoyl-phosphatidyl-choline; Chol, cholesterol; pDNA, plasmid DNA.

interaction with plasma proteins and allows extended circulation times comparable to classical sterically stabilized liposomes containing PEG_{2000}-distearoylphosphatidylethanolamine (DSPE) (21,22). In summary, the circulation half-life of SNALP can be adjusted by selecting PEG-lipids with

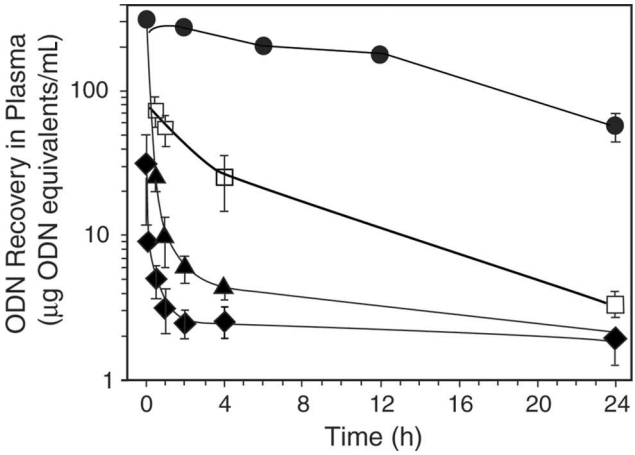

Figure 5 Circulation profiles in mice. The influence of surface charge and presence of a steric polymer surface coating on the circulation of SNALP was evaluated following IV administration in ICR mice. Formulations contained ^{3}H-labeled mICAM ODN and were administered at ODN doses of 15 mg/kg and approximate lipid doses of 100 mg/kg body weight. Formulations evaluated were: distearoyl-phosphatidylcholine (DSPC)/CH/1,2-dioleoyl-3-dimethylammoniumpropane/1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-dodecanoylsphingosine (●), DSPC/CH/dioleoyldimethylammonium chloride (DODAC)/1-O-(2′-(ω-methoxy-polyethylene-glycol)succinoyl)-2-*N*-myristoyl-sphingosine (□), DODAC/dioleoylphosphatidylethanolamine (1/1)/ODN complexes (♦), and free ODN (▲). The lipid ratios were 20/45/25/10 (mol/mol/mol/mol). The data points represent the mean ± SD from five animals.

different dissociation rates or by incorporation of cationic lipids with permanent charge (9).

Detergent Dialysis Approach

A different approach for the encapsulation of pDNA starts off with a micellar system (12). It involves solubilization of lipids and pDNA in octylglucoside-containing buffers and subsequent removal of the detergent by dialysis. This is in contrast to the preformed vesicle approach that employed subsolubilizing concentrations of ethanol or detergent rendering the liposomes morphologically intact. The physicochemical characteristics and in vivo properties of the resultant liposomal system, also called SPLP, will be described in the following.

Encapsulation and Physicochemical Properties

Plasmid DNA can be efficiently entrapped in liposomes. Encapsulation using the SPLP approach relies on the presence of a cationic lipid and a steric

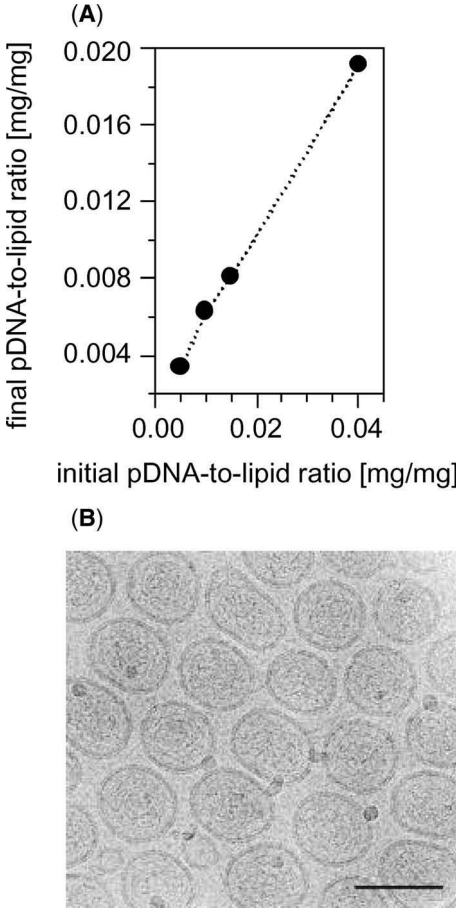

Figure 6 Encapsulation of plasmid DNA (pDNA) in small sterically stabilized liposomes [stabilized plasmid-lipid particles (SPLP)] using a detergent dialysis procedure. (**A**) Entrapped pDNA-to-lipid ratio as a function of the initial pDNA-to-lipid ratio (mg/mg). The initial lipid concentration was 10 mg/mL. (**B**) Cryo-electron micrograph showing the structure of SPLP. The location of the plasmid is indicated by the striated pattern superimposed on the liposomes. The bar represents 100 nm.

barrier lipid, as in the case of the PFV approach. Figure 6A demonstrates that pDNA can be efficiently entrapped in DOPE/DODAC/PEG-ceramide (84:6:10 mol%) liposomes (12,13,23). Encapsulation efficiencies are comparable to those obtained using the PFV approach for pDNA. The trapping efficiencies are a very sensitive function of the relative amounts of cationic lipid and PEG-ceramide and the ionic strength of the medium (3,12,13). With increasing size of the plasmid encapsulation, the efficiency decreased, coming down from

80% for a 2.9 kb plasmid to 35% for a 15.6 kb plasmid at an initial plasmid-to-lipid ratio of 0.02 mg/mg (13).

Size and morphology. The cryo-EM picture in Figure 6B shows that these plasmid-lipid systems have the morphological features of LUV. The encapsulated pDNA can be seen as a striated pattern superimposed on the liposomes. The average diameter from dynamic light scattering measurements is 70 nm. It should be noted that empty liposomes have been removed by ultracentrifugation as described in the "Methodology" section.

The mechanism of SPLP formation is not completely understood. Detergent dialysis involves a progression through different aggregate structures including spherical micelles, disk-like micelles and liposomes as more and more of the detergent is removed (24–26). In the presence of a cationic lipid, the surface charge density on these aggregates will increase in going from micelles to liposomes. For entrapment to occur, pDNA has to interact at a distinct point along this route. Both bilayer disks as well as liposomes can act as intermediate structure as both could form unilamellar liposomes internalizing the pDNA in response to a reduction of the surface area of one of their monolayers following DNA binding (27).

Pharmacokinetics, Tumor Accumulation, and Tumor Transfection of SPLP

The SPLP system is one of a few systems that have been directly compared to lipoplexes. The pharmacokinetics and biodistribution of the lipid as well as the pDNA was followed together with the levels of gene expression at a distal tumor site (8). Figure 7A shows the pharmacokinetics of SPLP in tumor-bearing mice in comparison to DODAC/DOPE lipoplexes. The clearance of SPLP from circulation can be described by a first-order process with a half time of 6.4 ± 1.1 hours. Relatively low levels of uptake by the lung and liver have been observed. Approximately 3% of the injected lipid dose accumulated at the tumor site. In contrast to SPLP, lipoplexes were rapidly cleared from circulation ($t_{1/2} \ll 15$ minutes) and accumulated predominantly in the lung and liver. Less than 0.5% of the injected dose was found at the tumor site after one hour and decreased at later timepoints.

The administration of SPLP results in reporter gene expression at the tumor site (Fig. 7B). Injection of free plasmid or lipoplexes resulted in no detectable gene expression at the tumor site. However, transfection was observed in the lung, liver, and spleen. SPLP, on the other hand, did not show detectable levels of gene expression in these organs.

CONCLUSIONS

Polynucleotides have been encapsulated by a variety of methods (12). However, none of these procedures has yielded small, serum-stable particles in

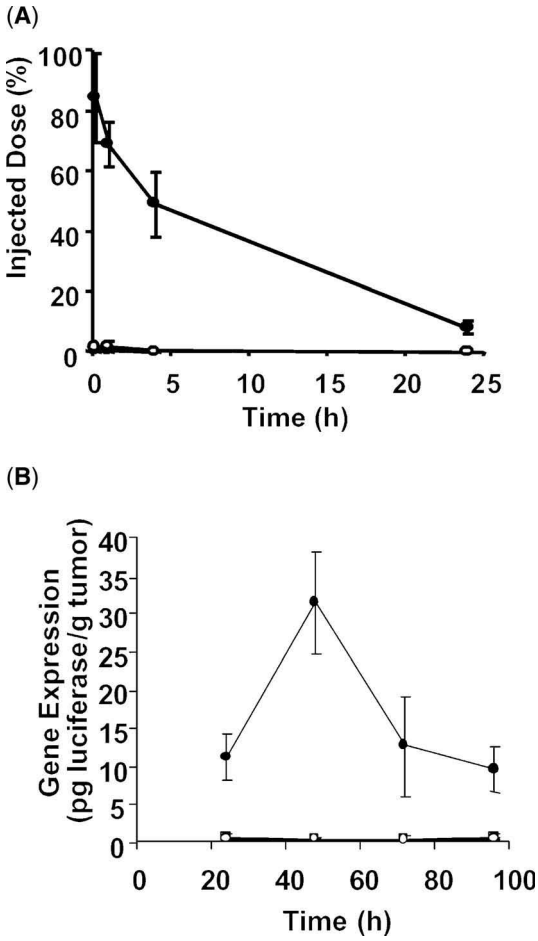

Figure 7 Pharmacokinetic properties and in vivo gene expression of stabilized plasmid-lipid particles (SPLP). (**A**) The levels of intact plasmid DNA (pDNA) in the circulation resulting from IV injection of naked plamid pDNA (□), lipoplexes (○), and SPLP (●) were determined by Southern blot analysis of plasma samples (100 μg pDNA/mouse). (**B**) Transgene expression at a distal tumor site resulting from IV injection of naked plamid pDNA (□), plamid pDNA-cationic liposome complexes (○), and SPLP (●).

combination with efficient encapsulation at high nucleic acid-to-lipid ratios that are required for clinical utility as a systemic drug carrier. We have developed two procedures, the preformed vesicle approach and the detergent dialysis procedure, that allow efficient encapsulation of nucleic acid-based molecules in liposomes that are small in size (about 100 nm diameter) and stable in circulation. The preformed vesicle approach can be generally

applied to the entrapment of negatively-charged polyelectrolytes including nonnucleotide-based molecules. Encapsulation by the detergent dialysis procedure is more difficult to control as it relies on a delicate balance of cationic and PEG-lipid content and the ionic strength of the solution. Plasmid DNA can be readily encapsulated using this approach; however, encapsulation of oligonucleotides in small liposomes is difficult to achieve. The entrapment of nucleic acids was found to be highly dependent on chain length, requiring a minimum length to occur and decreasing as the chain length became too long (10,13).

The development of procedures that allow efficient encapsulation of pDNA and oligonucleotides in small serum-stable liposomes has been a major advance toward systemic delivery of such drugs. Two of these systems have shown promising results and have progressed into formal preclinical and clinical testing, respectively (16,18): SNALP, consisting of immune stimulatory oligonucleotides encapsulated in a liposome also called Oligovax, and the SPLP system containing a therapeutic plasmid. Liposome-encapsulated immune stimulatory oligonucleotides promise great potential for the treatment of cancer and inflammatory and infectious diseases. Encapsulation protects these oligonucleotides from degradation, allowing the use of the natural, more specific phosphodiester sequences instead of synthetic backbone-modified oligonucleotides that exhibit a variety of nonspecific and toxic effects. Encapsulation can significantly enhance the immune stimulatory potency of these molecules, naturally targeting them to antigen, presenting cells such as the macrophages of the liver and spleen, which are responsible for removal of particulate systems from circulation (17). Intravenous administration of SNALP containing immune stimulatory CpG oligonucleotide resulted in significantly enhanced plasma cytokine levels and immune cell activation as compared to free oligonucleotide (17). The liposome-encapsulated oligonucleotides form a multimodal technology platform (18). For example, liposome-encapsulated oligonucleotides can be combined with a specific disease marker, for example, a tumor antigen, to direct a specific immune response against a particular disease, in this case against cancer. This technology can also be applied to the development of cancer vaccines, infectious disease vaccines or as an adjuvant to existing vaccines or alone to stimulate a protective immune response. In addition, liposome-encapsulated immune stimulatory oligonucleotides can enhance the potency of tumor antibodies such as Herceptin by enhancing antibody-dependent cellular cytotoxicity.

The plasmid-containing SPLP system can achieve highly selective protein expression at sites of disease after systemic administration, resulting in local therapeutic effects while minimizing systemic exposure. Different gene therapy approaches have been tested including delivery of a plasmid that encodes an enzyme that converts a prodrug into its active cytotoxic form and plasmids that express immune stimulatory proteins and toxins. The first of these approaches has progressed into clinical trials (16).

In summary, the preformed vesicle approach and detergent dialysis procedure have enabled development of nucleic acid-based therapeutics with clinical utility. Further applications of these liposomal systems with new nucleic acid-based therapeutics such as small interfering RNA for gene silencing are being developed and have demonstrated promising results (28).

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