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# Encapsulation of Plasmid DNA in Stabilized Plasmid–Lipid Particles Composed of Different Cationic Lipid Concentration for Optimal Transfection Activity

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In previous work (Wheeler *et al.* (1999) *Gene Therapy* **6**, 271–281) we have shown that plasmid DNA can be entrapped in "stabilized plasmid–lipid particles" (SPLP) using low levels (5–10 mol%) of cationic lipid, the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), and a polyethyleneglycol (PEG) coating for stabilization. The PEG moieties are attached to a ceramide anchor containing an arachidoyl acyl group (PEG-$CerC_{20}$). However, these SPLP exhibit low transfection potencies *in vitro* as compared to plasmid/cationic lipid complexes formed with liposomes composed of cationic and neutral lipid at a 1 : 1 lipid ratio. The objective of this study was to construct SPLPs with increased cationic lipid contents that result in maximum transfection levels. A phosphate buffer detergent dialysis technique is described resulting in formation of SPLP containing 7–42.5 mol% DODAC with reproducible encapsulation efficiency of up to 80%. An octanoyl acyl group was used as anchor for the PEG moiety (PEG-$CerC_8$) permitting a quick exchange out of the SPLP to further optimize the *in vitro* and *in vivo* transfection. We have demonstrated that this technique can be used to encapsulate either linearized DNA or supercoiled plasmids ranging from 3–20 kb. The SPLP formed could be isolated from empty vesicles by sucrose density gradient centrifugation, and exhibited a narrow size distribution of approximately 75 ± 6 nm as determined by cryo-electron microscopy. The high plasmid-to-lipid ratio observed corresponded to one plasmid per particle. The SPLP consist of a lipid bilayer surrounding the plasmid DNA as visualized by cryo-electron microscopy. SPLP containing a range of DODAC concentrations were tested for *in vitro* and *in vivo* transfection. *In vitro*, in COS-7 cells transfection reached a maximum after 48 h. The transfection efficiency increased when the DODAC concentration in the SPLP was decreased from 42.5 to 24 mol% DODAC. Decreasing the cationic lipid concentration improved transfection in part due to decreased toxicity. *In vivo* studies using an intraperitoneal B16 tumor model and intraperitoneal administration of SPLP showed

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maximum transfection activity for SPLP containing 24 mol% DODAC. Gene expression observed in tumor cells was increased by approximately one magnitude as compared to cationic lipid/DNA complexes. The SPLP were stable and upon storage at 4°C no significant change in the transfection activity was observed over a one-year period. Thus this phosphate buffer detergent dialysis technique can be used to generate SPLP formulations containing a wide range of cationic lipid concentrations to determine optimal SPLP composition for high transfection activity and low toxicity.

*Keywords:* Non-viral gene delivery, Cationic lipid, Cancer gene therapy, Plasmid encapsulation, Liposomes



## INTRODUCTION

Plasmid delivery has primarily relied upon two approaches; virus based gene delivery and non-viral gene delivery. Recombinant viral delivery systems while specific are rapidly cleared from the circulation limiting delivery to first pass organs such as the liver (Huard *et al.*, 1995; Worgall *et al.*, 1997). Non-viral delivery systems have included liposomes, cationic lipid–DNA complexes or lipoplexes, cationic polymers and cation coated nanoparticles (reviews see Ledley, 1995; Felgner, 1997; Lasic, 1997; Zabner, 1997; Sorgi and Huang, 1997; Chonn and Cullis, 1998; Maurer *et al.*, 1999; Pollard *et al.*, 1998). The highly charged lipoplexes and polymers are quite efficient in gene delivery to cells *in vitro* (Gao and Huang, 1991; Felgner *et al.*, 1994; Zabner *et al.*, 1995; Hofland *et al.*, 1996). However, these charged and often large systems are generally cleared rapidly following i.v. injection limiting the potential transfection sites to first-pass organs, such as lung liver and spleen (Thierry *et al.*, 1995; Hong *et al.*, 1997; Hofland *et al.*, 1997; Templeton *et al.*, 1997; Huang and Li, 1997; Liu *et al.*, 1997). The DNA is not completely sequestered by these carrier systems, and it is susceptible to degradation by nucleases (Wheeler *et al.*, 1999). High concentrations of cationic lipids are known to cause toxicity *in vivo* and *in vitro* (Harrison *et al.*, 1995, Li and Huang, 1997). Furthermore, these complexes often exhibit limited stability, have a tendency for aggregation and exhibit transfection potencies that can be sample and time dependent. Methods for passive encapsulation of plasmid into liposomes are highly inefficient and result in low DNA/lipid ratios (Fraley *et al.*, 1979; 1980; Lurquin, 1979; Wang and Huang, 1987).

The focus in our lab has been on the development of small (approximately 100 nm diameter), well-defined lipid-based plasmid carrier systems, where the plasmid is fully encapsulated within a lipid envelope. Previous studies have shown that plasmid DNA can be encapsulated with high efficiency (> 50%) by a detergent dialysis procedure using low cationic lipid concentration (Wheeler *et al.*, 1999). The encapsulation efficiency was a sensitive function of the cationic lipid concentration used. The particles formed, of approximately 70 nm diameter, contain one plasmid per particle and are stabilized with a polyethylene glycol (PEG) coating. The plasmid in these stabilized plasmid–lipid particles (SPLP) is fully protected from serum nucleases and the particles are stable during circulation in the blood stream *in vivo* (Monck *et al.*, 1999). It was shown that the transfection potency of SPLP is dependent on the type of ceramide used as anchor for the PEG polymer. The higher transfection potency observed with ceramide groups containing shorter acyl groups was attributed to the faster dissociation rate of PEG-Cer with shorter acyl groups, thereby destabilizing the particle and improving cell interaction and uptake into target cells. The inhibitory effect of PEG coating for association and fusion are well known (Holland *et al.*, 1996a,b). Initial studies have shown that the transfection activity of SPLP could be increased significantly by increasing the cationic lipid content in the SPLP (Zhang *et al.*, 1999). The formulation method described resulted in efficient encapsulation of DNA into well-defined SPLP containing up to

25 mol% cationic lipid. Citrate was used as counter-ion to efficiently shield the positive charge on the lipid intermediate structures, such as cylindrical micelles and lamellar sheets formed during dialysis to levels compatible with good entrapment. Citrate concentrations below the optimal range resulted in aggregate formation while at concentrations above the optimal range, interaction of the plasmid with lipid structures was inhibited and little or no encapsulation occurred. However this protocol was not successful for cationic lipid concentrations higher than 25–30 mol%.

The focus of this work was to develop a formulation protocol that permits efficient encapsulation of plasmid in SPLP containing 6–50 mol% cationic lipid and to determine the optimal cationic lipid concentration in SPLP for transfection *in vitro* and *in vivo*. Formation of SPLP with equimolar concentrations of cationic and neutral lipids (DODAC/DOPE) were of particular interest, permitting a direct comparison with plasmid/cationic lipid complexes of identical lipid composition. In this work it is shown that SPLP containing a range of 6–42.5 mol% cationic lipid can be formed by varying the phosphate concentration in the dialysis medium and that the plasmid DNA is encapsulated within a lipid bilayer. Optimal transfection potencies *in vitro* and *in vivo* are obtained with SPLP containing approximately 25 mol% cationic lipid. In a regional peritoneal tumor model, these optimized SPLP exhibit an increased tumor transfection activity of about one magnitude, as compared to the transfection observed with plasmid–cationic lipid complexes (lipoplexes). SPLP can be formed with plasmid ranging from 3000 to 50,000 bp in size.

## MATERIALS AND METHODS

### Materials

N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) was kindly provided by Dr. Steve Ansell (INEX Pharmaceuticals Corp., Burnaby, BC, Canada) and 1-O-[2′-(ω-methoxy-polyethyleneglycol) succinoyl]-2-N-octoylsphingosine (PEG-C8) was synthesized as described in Webb *et al.* (1998) and generously provided by Dr. Zhao Wang (INEX Pharmaceuticals Corp.). Dioleylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). Plasmids p BS KS$^+$ (2.964 kb), pINEX TK02 (4.262 kb) pGFPemd-c[R] (4.356 kb), pINEX TK05 (5.046 kb), pINEX L018 (pCMVLuc; 5.650 kb), pINEX D001 (5.963 kb), pINEX PL01 (8.151), pINEX P005 (11.125 kb), pINEX B001 (15.600 kb) were supplied by the Core Support Group (INEX Pharmaceuticals). λ-phage DNA (48.502 kb) was supplied by New England Biolabs (Mississauga, Ont., Canada). Monobasic potassium phosphate (ACS), bovine serum albumin, octyl-β-D-glucopyranoside (OGP), Triton X-100$^{TM}$ and DEAE-Sepharose CL-6B were obtained from Sigma (St. Louis, MO, USA). Dialysis membrane (Spetra/Por 2; Spectrum Medical Instruments), HEPES (BDH Analar), dibasic potassium phosphate (BDH Analar), sucrose (BDH Analar) and NaCl (BDH ACS) were obtained from VWR Scientific (Edmonton, AB, Canada). The B16/F10 mouse melanoma cell line was obtained from the Frederick Cancer Research Center (Frederick, MD, USA). C57/BL67 mice were obtained from Harlan Sprague-Dawley Inc (Chicago, IL, USA). The care and handling of the mice followed guidelines set out by the Canadian Council on Animal Care.

### Encapsulation of Plasmid DNA

Lipid dispersions were prepared from stock solutions of DODAC, DOPE and PEG-Cer$C_8$ in ethanol (typically 10 mg/ml each). Appropriate amounts of lipid were transferred into a glass test tube and the solvent removed under a stream of $N_2$ gas followed by storage under vacuum for 3–5 h. The dried lipid film (10 mg lipid per 1 ml formulation) was dissolved by gently mixing in a 1 ml solution containing 100 mM octylglucoside (stock solution 1 M OGP), 200–400 μg/ml plasmid (stock solution 1 mg DNA/ml in 10 mM Tris–HCl pH 8.0; 1 mM EDTA) in the appropriate dialysis buffer. The lipid/plasmid/detergent suspension was incubated

at RT for at least 30 min to dissolve the lipids completely. Following incubation the mixture was dialyzed for 2 days against 100–400 volumes of the appropriate ionic strength dialysis buffer (sodium phosphate pH 7.4 ± NaCl; filtered through 0.2 μm filter) with 2–3 changes of buffer. After dialysis the SPLP suspension was analyzed for particle size and encapsulation efficiency. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE Sepharose CL-6B column equilibrated in dialysis buffer. Where appropriate, empty liposomes were removed by sucrose density gradient centrifugation as described previously (Wheeler *et al.*, 1999; Zhang *et al.*, 1999) with the following modification. Formulations containing greater than 30 mol% DODAC were isolated on a 1% : 2.5% : 5% sucrose gradient and formulations containing 30 mol% and less DODAC were isolated on a 2.5% : 5% : 10% sucrose gradient. The gradients were centrifuged at 160,000× *g* for 12–18 h. The SPLP with > 30 mol% DODAC were buoyant on the 2.5–5% sucrose interface and SPLP with 30 mol% or less DODAC were buoyant on the 5–10% sucrose interface. The SPLP fraction was removed, dialyzed against HBS (20 mM HEPES in 150 mM NaCl, pH 7.4) and where required concentrated using Aquacide II (Calbiochem, San Diego, CA, USA) to 250–500 μg plasmid/ml.

### Evaluation of Plasmid Encapsulation Efficiency

The extent of the plasmid encapsulation in the SPLP was evaluated by measuring the accessibility of the dsDNA interchelating dye PicoGreen™ (Molecular Probes, Eugene, OR, USA) by the following method. Aliquots of the formulation taken directly from detergent dialysis were diluted 1 : 400 in dialysis buffer or HEPES buffered saline (pH 7.2) and 2 μl of Picogreen reagent was added to 1.0 ml of the diluted samples. The fluorescence was measured at an excitation wavelength of 495 nm and emission wavelength at 525 nm using an SLM-Aminco fluorometer (Rochester, NY, USA) both in the absence ($I$) and presence ($I_0$) of 10 μl 10% (v/v) Triton X-100. The percent plasmid encapsulation was calculated as $E\ (\%) = (I_0 - I)/I_0 \times 100$. It should be noted that this assay measures the extent that the lipid–plasmid association interferes with picogreen binding to the plasmid DNA. Association may be due to encapsulation within lipid vesicles as well as binding between lipid membrane surfaces due to aggregation (Zhang *et al.*, 1999). The presence of aggregation was determined separately for each preparation by QELS particle size analysis (below). To determine the total plasmid DNA concentration in the formulations PicoGreen fluorescence was measured in presence of 0.1% Triton X-100 and compared with plasmid DNA at standard concentrations.

### Particle Size Determination

Particle sizes were determined by quasi-elastic light scattering (QELS) using a Nicomp submicron particle seizer (Model 340, Nicomp particle sizing systems, Santa Barbara, CA, USA) operated in the volume-weighted vesicle mode.

### Lipid Analysis by HPLC

Lipids were quantified using a Beckman Gold series HPLC pump and autosampler system, fitted with a Beckman Ultrasphere Cyano 5 μm column (0.2 × 15 cm) (Beckman Instruments, Fullerton, CA, USA) and the samples were detected on an Alltech Model 500 ELSD – evaporative light scattering detector (Alltech Associates Inc., Deerfield, IL, USA). The lipid-containing samples (200 μl) were extracted with 1 ml each of HBS/methanol (2 : 1) and $CHCl_3$. The extract was vortexed for 1 min and the $CHCl_3$ layer was separated by centrifugation at 2500× *g* and loaded onto an autosampler for chromatography. The samples were eluted with a gradient from 100% solvent A to 90% solvent B over a time period of 8 min at 0.3 ml/min (Solvent A: 99.95% Chloroform, 0.05% Trifluroacetic acid; Solvent B: 90% isopropanol, 9.95 % water, 0.05% TFA).

### Freeze-Fracture Electron Microscopy

Particle sizes were also determined by freeze-fracture electron microscopy provided by Dr. Kim Wong (Department of Biochemistry, University of British Columbia). Freeze-fracture as performed on a Balzers freeze-etching system, BAF 400D (Balzers, Balzers, Liechtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid Freon 22. The fractured surface was shadowed unidirectionally with platinum/carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a JEOL Model JEM 1200 EX microscope (Soquelec, Montreal, QC, Canada).

### Cryo-Electron Microscopy

A drop of buffer containing SPLP was applied to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed by blotting, leaving a thin layer of the suspension covering the holes of the carbon film. The grid was rapidly frozen in liquid ethane, resulting in vesicles embedded in a thin film of amorphous ice. Images of the vesicles in the ice were obtained under cryogenic conditions at a magnification of 66,000 and a defocus of −1.5 microns using a Gatan cryo-holder in a Philips CM200 FEG electron microscope.

### *In Vitro* Transfection Assay

COS-7 cells were seeded at a concentration of $4.0 \times 10^4$ cells per well in a 24 well dish in a total of 2.0 ml media one day prior to transfection. Cells in logarithmic growth phase were used to seed the dishes. At the time of transfection, cells were less than 20% confluent. Formulated plasmid DNA (both SPLP and complexed) was added to the cells at concentrations ranging from 0.1 to 1.0 μg DNA and remained in the culture medium throughout the experiment. At the completion of the incubation period, media was removed from each well and cells were washed twice with PBS prior to storage at −70°C. On the day of analysis, plates were brought to room temperature and 200 μl cell lysis buffer (0.1% Triton X-100, 250 mM $NaH_2PO_4$, pH 7.4) was added to each well. Plates were shaken on a rotary platform for 10 min at 100 rpm. The expression of the luciferase reporter gene was assayed as described below.

### Intraperitoneal Tumor Transfection

B16/F10 tumor cells ($1 \times 10^5$ cells in 200 μl PBS) were injected into the peritoneal cavity of female C57/BL6 mice. Seven days after tumor seeding, 30 μg of formulated plasmid DNA was injected intraperitoneally (i.p.) into the tumor bearing mice in a total volume of 500 μl PBS. Animals were sacrificed 6 or 24 h after transfection. Organs and tumors were collected, weighed, snap frozen in liquid nitrogen and stored at −70°C until analysis for luciferase gene expression. Frozen samples were brought to room temperature and transferred into individual Fast-Prep tubes pre-loaded with a small bead. A second bead was added to each tube prior to the addition of 0.5–1.0 ml of cell culture lysis reagent (Promega) supplemented with 1.0 mg/ml BSA. Samples were homogenized using the Fast-Prep FP120 (Savant Instruments, Farmingdale, NY, USA) for 10 s at a speed setting of 5. Homogenized samples were then incubated at room temperature for 15 min, subjected to centrifugation for 2 min at 14,000× *g* to remove debris and the clear lysate was assayed for luciferase activity.

### Luciferase Assay

Luciferase assays on both cultured cells or tissue homogenates were performed using Promega luciferase assay substrates (Promega E 1501) according to the procedure described by the manufacturer. A 20 μl aliquot of cell lysate (or tissue homogenate) was assayed for luciferase activity using a Dynex Technologies ML3000 microplate luminometer (Dynex Technologies, Chantilly, VA, USA). Luminescence readings were calibrated according to a standard curve obtained using a *Photinus pyralis* luciferase standard.

### Calcein Cell Viability Assay

The viability of *in vitro* cultured cells after incubation with SPLP was determined using a calcein assay technique. The media in the wells containing floating cells was collected into individual 6 ml tubes. The adherent cells remaining in the wells were trypsinized, harvested in PBS and added to the floating cells. The pooled cells were centrifuged at 1000× *g* for 10 min and the cell pellet was dissolved in 0.1 ml PBS. A 0.1 ml aliquot of the cell suspension was transferred to a 96 well plate to perform the viability assay. Calcein AM was added in 0.1 ml PBS at a final concentration of 2 μM per well. After 30 min fluorescence (Ex 485 nm; Em 520 nm) was measured on a BioLumin (Molecular Dynamics) fluorometer. The measured calcein fluorescence was proportional to the cell viability.

## RESULTS

### Encapsulation of Plasmid in SPLP Containing Equimolar Concentrations of Cationic Lipid and DOPE

Previous work showed that citrate can be used to modulate the ionic interaction between plasmid and lipid intermediate structures, that contain up to 25 mol% cationic lipid, during detergent dialysis resulting in efficient encapsulation of plasmid in lipid particles (Zhang *et al.*, 1999). However, at higher cationic lipid concentrations the ionic interaction was difficult to control and excess aggregation and precipitation was typically observed. Here we evaluated the shielding effect of phosphate during dialysis of a near equimolar cationic and neutral lipid mixture of DODAC and DOPE.

Plasmid encapsulation was studied as a function of DODAC concentration using 150 mM NaCl in 150 mM sodium phosphate buffer pH 7.4 as dialysis buffer (Fig. 1). The PEG concentration was kept constant at 15% and DOPE concentration varied as required to compensate for changes in DODAC concentration. The extent of plasmid encapsulation was estimated by determining the relative accessibility of the DNA interchelating fluorescent dye PicoGreen to the plasmid, in presence and absence of Triton X-100 as described in Materials and Methods. The particle size distribution was used as an additional criterion for SPLP formation and was required to be approximately 100 nm. Formation of large plasmid–lipid aggregates can lead to an overestimation of the encapsulation by the PicoGreen assay. The results in Fig. 1 demonstrate the sensitivity of the encapsulation efficiency to small changes in the cationic lipid concentration at a given ionic strength. The salt concentration of 150 mM NaCl in 150 mM sodium phosphate buffer pH 7.4 was optimal for SPLP formation with 42.5 mol% DODAC. A decrease in DODAC concentration of 2 mol% resulted in an up to 40% decrease in the encapsulation efficiency. At DODAC concentrations higher than 42.5 mol% formation of large polydisperse aggregates were detected under these conditions. In the absence of PEG-Cer, wholesale aggregation was observed (data not shown).

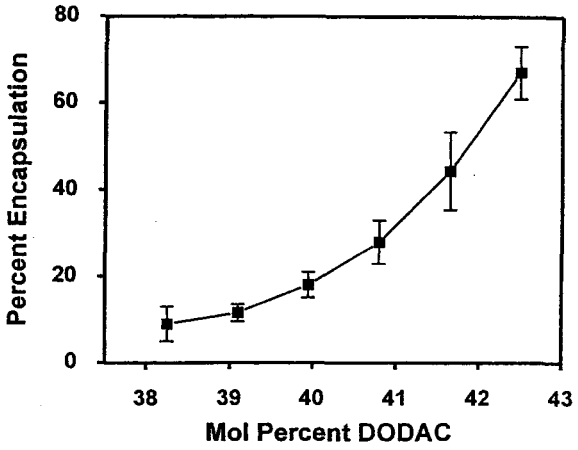


FIGURE 1 Encapsulation of plasmid in SPLP with different concentrations of DODAC. SPLP formulations composed of DODAC/DOPE/PEG-CerC$_8$ (42.5 − *x*:42.5 + *x*:15; mol:mol:mol) and pCMVLuc (pINEX L018) (200 μg/ml plasmid; 5 mg/ml lipid) were prepared with DODAC concentration varying between 38 and 42 mol% in detergent and dialyzed against 150 mM NaCl in 150 mM sodium phosphate buffer pH 7.4 as described in Materials and Methods. Encapsulation efficiency was determined by PicoGreen assay as outlined in Materials and Methods. Percent encapsulation is plotted as a function of DODAC concentration used for SPLP formation.

The salt concentration optimal for SPLP formation with equimolar concentration of DODAC and DOPE often required slight adjustments in the NaCl concentration for different plasmid preparations. Without adjustments the encapsulation efficiency can vary from 40–60% between plasmid batches.

### Effect of Lipid and Plasmid Concentration on Encapsulation Efficiency

The effect of the total lipid and plasmid concentration on the encapsulation efficiency was evaluated for SPLP with equimolar concentrations of DODAC and DOPE. A series of SPLP formulations were prepared where either the plasmid (0.1–1 mg/ml) or lipid (1–10 mg/ml) concentrations were varied in the preparations. Under identical ionic strength buffer conditions and 200 μg/ml plasmid, increasing lipid concentration resulted in an increase in the encapsulation efficiency (Fig. 2(a)). Note, that where encapsulation efficiency exceeded 70% a significant amount of aggregation (diameter > 150 nm) was often observed in these preparations both visually and as measured by particle size analysis, leading to an over-estimate of the encapsulation by the PicoGreen assay as pointed out earlier. At a constant lipid concentration of 5 mg/ml, increasing plasmid concentration resulted in a decrease of the plasmid encapsulation efficiency (Fig. 2(b)). These results suggest that the plasmids compete for the cationic lipid intermediate structures bearing a distinct charge density during dialysis and a larger proportion of plasmid was encapsulated when the total lipid concentration was increased.

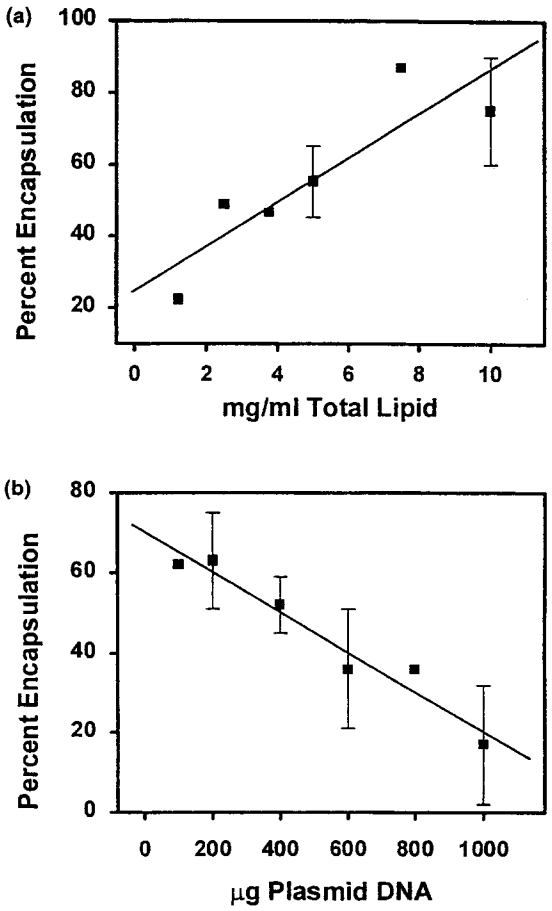


FIGURE 2 Effect of total lipid and plasmid concentration on encapsulation efficiency. Formulations composed of DODAC/DOPE/PEG-CerC$_8$ (42.5:42.5:15; mol:mol:mol) and pCMVLuc were dialyzed against 150 mM sodium phosphate buffer and 150 mM NaCl. In (a), the plasmid concentration was maintained at 200 μg/ml and the total lipid concentration was varied between 1 and 10 mg/ml. In (b), the total lipid concentration was maintained at 5 mg/ml while the plasmid concentration was varied between 100 and 1000 μg/ml. Encapsulation efficiencies of over 70% were typically accompanied with significant particle aggregation as measured by QELS. Percent plasmid encapsulation was plotted as a function of total lipid concentration (a) and plasmid concentration (b) used in the formulation.

### Conditions for SPLP Formation with Different Cationic Lipid Concentrations

A series of studies were performed to determine the optimal buffer ionic strength in the dialysis buffer for efficient encapsulation of plasmid (pINEXL018) in SPLP using a range of cationic lipid concentrations. Replicate SPLP formulations were prepared containing 30–42.5 mol% DODAC and these were dialyzed against a range of salt concentrations in the dialysis buffer (Fig. 3). The ionic strength in the dialysis buffer was titrated by varying the NaCl concentration from 0 to 200 mM in 150 mM sodium phosphate buffer pH 7.4.

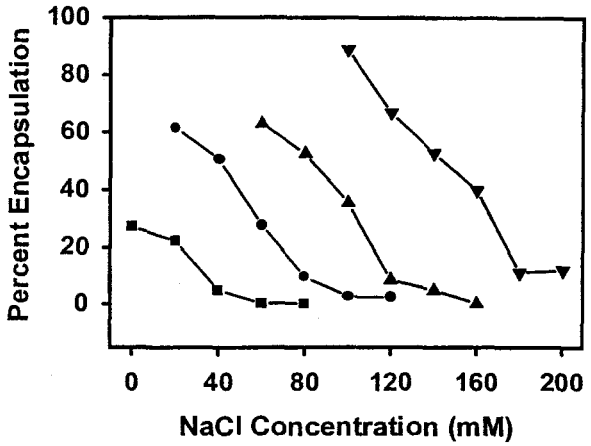


FIGURE 3 Encapsulation efficiency in SPLP containing different DODAC concentrations as a function of the salt concentration in the dialysis buffer. Formulations containing 30, 34, 38 and 42.5 mol% DODAC (DODAC: DOPE: PEG-cer$C_8$; 30:55:15; 34:51:15; 38:47:15 and 42.5:42.5:15; mol:mol:mol) were prepared. Replicate formulations were dialyzed against 150 mM sodium phosphate buffer containing 0–200 mM NaCl. Each of the SPLP formulations was prepared with 10 mg/ml total lipid and 400 μg/ml plasmid (pCMVLuc, pINEX L018). Percent encapsulation for SPLP with 30 (■), 34 (●), 38 (▲) and 42.5 mol% (▼). DODAC is plotted as a function of salt concentration in the dialysis buffer.

TABLE I Optimal phosphate buffer concentration for plasmid encapsulation

| SPLP DODAC concentration (mol%) | Optimal detergent dialysis buffer concentration |
|---|---|
| 7 | 150 mM NaCl |
| 20 | 90 mM $Na_2HPO_4$ |
| 24 | 110 mM $Na_2HPO_4$ |
| 30 | 140 mM $Na_2HPO_4$ |
| 34 | 150 mM $Na_2HPO_4$ + 50 mM NaCl |
| 38 | 150 mM $Na_2HPO_4$ + 90 mM NaCl |
| 42.5 | 150 mM $Na_2HPO_4$ + 140 mM NaCl |

TABLE II Diameter and lipid/DNA ratios of purified SPLP formulations

| DODAC concentration (mol%) | QELS diameter (nm)[a] | Lipid/DNA ratio (μg plasmid/μmol lipid) |
|---|---|---|
| 7 | 90.0 | 75.7 |
| 20 | 64.0 | 65.5 |
| 24 | 77.2 | 73.5 |
| 30 | 89.3 | 81.9 |
| 42.5 | 93.8 | 59.7 |

[a]QELS measured in particle size mode.

When the salt concentration in the dialysis buffer was too high, the association between the cationic lipid and the DNA was inhibited and little encapsulation was observed. Encapsulation efficiency increased at each DODAC concentration as the ionic strength of the dialysis buffer was decreased. Aggregation was often observed by QELS in preparations where the encapsulation exceeded 60–70%. To obtain efficient encapsulation (> 50%) with DODAC concentrations of 30 mol% and lower, the sodium phosphate concentration had to be further decreased from 150 mM to as low as 100 mM sodium phosphate (data not shown). By using encapsulation efficiency of > 50% and particle size of approximately 100 nm in diameter as criteria for SPLP formation, the optimal salt concentration required in the dialysis buffer was determined for DODAC concentrations from 7 to 42.5 mol% and summarized in Table I.

Following dialysis, the non-encapsulated plasmid was removed by DEAE Sepharose CL-6B chromatography and empty lipid vesicles were separated from loaded ones by sucrose density gradient centrifugation as previously described (Zhang *et al.*, 1999). Traces of aggregates formed during dialysis were also removed during gradient isolation, resulting in a very narrow particle size distribution. The lipid/DNA ratio was determined for each SPLP formulation by quantitative HPLC and PicoGreen assay and is presented in Table II. A constant lipid to DNA ratio ranging from 60 to 82 μg plasmid/μmol lipids was observed for isolated SPLP with pCMVLuc over the whole range of DODAC concentrations. This ratio is consistent with that previously reported for SPLP with lower DODAC concentrations (Wheeler *et al.*, 1999; Zhang *et al.*, 1999). The lipid composition of the isolated SPLP was determined by quantitative HPLC. It was found to be similar to the initial lipid composition used with may be a slight enrichment in the DODAC content of approximately 2% (data not shown).

### Effect of Plasmid Size on Encapsulation Efficiency and SPLP Size

The influence of plasmid size on SPLP formation was evaluated for formulations containing 7 and 24 mol% DODAC respectively. The plasmids used ranged from 2.9 to 15.6 kb and also included a λ-phage DNA of 48.5 kb. The formulations with different size plasmids (10 mg/ml lipid; 200 μg/ml plasmid) were dialyzed against identical salt conditions. An apparent direct relationship between the encapsulation efficiency and log of the plasmid size was observed for both 7 and 24 mol% DODAC containing SPLP formulation (Fig. 4(a)). Encapsulation efficiency decreased with increasing plasmid size. A similar effect was observed for the encapsulation efficiency of linearized and supercoiled plasmid (data not shown). In this case the encapsulation of the linearized plasmid (which has a larger diameter in solution) was at least 10% less efficient than for the supercoiled form ($p < 0.1$). SPLPs containing 7 mol% DODAC and different size plasmids were isolated by DEAE-chromatography and density gradient centrifugation. The particle size of the isolated SPLPs was analyzed by quasi-elastic light scattering operated in the volume weighting vesicle and particle mode and was plotted against plasmid size (Fig. 4(b)). The SPLP size determined in both vesicle and particle mode, increased with increasing plasmid size. This suggests that the decreased encapsulation efficiency observed with increasing plasmid size may reflect a limit to the size of the lipid sheet required to encapsulate the plasmid.

### Characterization of SPLP by Electron Microscopy

The SPLP structure was analyzed by cryo-electron microscopy and compared to large unilamellar vesicles (LUV) of the same lipid composition prepared by extrusion of a hydrated lipid mixture through 100 nm pore size filters (Hope *et al.*, 1985). The electron micrograph of SPLP shows a lipid bilayer surrounding an electron dense internal structure, which is consistent with plasmid DNA

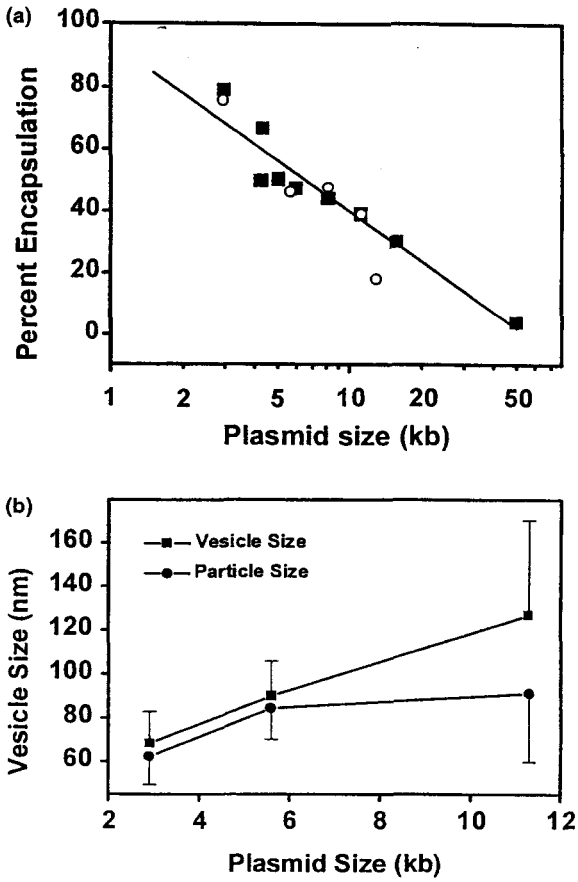


FIGURE 4 Effect of plasmid size on encapsulation efficiency and SPLP size. SPLP formulations were prepared with a series of plasmids ranging in size from 2.9 to 15.6 kb (see Methods) as well as 50 kb λ-phage DNA. The SPLP formulations were prepared at two DODAC concentrations, 7 and 24 mol% (DODAC : DOPE : PEG-cer$C_8$; 7 : 78 : 15 and 24 : 61 : 15; mol : mol : mol) and were dialyzed against 110 mM sodium phosphate buffer, pH 7.2 and 20 mM HEPES buffered saline pH 7.2 respectively. All formulations were prepared with 10 mg/ml total lipid and 200 μg/ml plasmid. No significant aggregation was observed in any preparation including those with encapsulation efficiency > 60%. Encapsulation efficiency was measured directly after dialysis and plotted as a function of plasmid size for SPLP containing 7 (■) and 24 mol% DODAC (○) in (a). Following removal of non-encapsulated plasmid by DEAE chromatography and separation of empty vesicles by sucrose gradient centrifugation, the size of SPLP containing 7 mol% DODAC and different size plasmid (2.9–15.6 kb) was analyzed by quasi-elastic light scattering using both "solid particle" (●) and "vesicle" (■) algorithms in a NICOMP particle size analyzer and plotted as a function of plasmid size (b). Error bars indicate the standard deviation calculated by the NICOMP program.

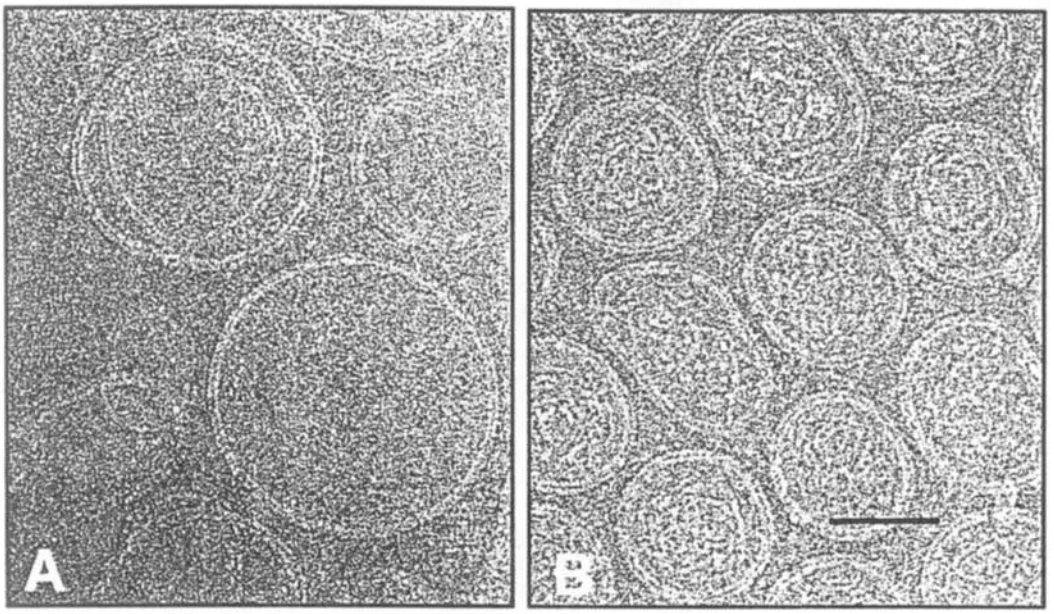


FIGURE 5 Cryo-electron micrographs of SPLP. LUV (A) were prepared by hydration and extrusion through polycarbonate filters with 100 nm pore size. The lipid concentration of the formulations was adjusted to about 2 mg/ml. Isolated SPLP (B) were prepared as described in Fig. 4(b) with pCMVLuc. The bar in panel (B) indicates 50 nm. Details of sample preparation and cryo-electron microscopy are given in Materials and Methods.

encapsulated in a lipid vesicle (Fig. 5(B)). This internal structure in SPLP is not seen in the LUV prepared by extrusion (Fig. 5(A)). The SPLPs exhibit a homogeneous morphology and size ($75 \pm 6$ nm diameter). This narrow size distribution of the SPLP was also seen by quasi-elastic light scattering ($90 \pm 16$ nm with a narrow Gaussian distribution ($\chi^2 = 0.2$) for a 5.65 kb plasmid) and by freeze-fracture electron microscopy (data not shown). SPLP formed with the same size plasmid exhibit an overall similar size distribution regardless of the DODAC concentration used (Table II).

### Effect of Cationic Lipid Concentration on the Transfection Potency of SPLP *In Vitro*

The transfection potency of isolated SPLP containing a range of different DODAC concentrations and luciferase as reporter gene, was investigated *in vitro* using COS-7 cells. Measurements of luciferase expression in COS-7 cells over a 72 h time course following transfection with 0.5 μg plasmid in SPLP containing 42.5, 38 and 34 mol% DODAC revealed that maximal expression occurred after 48 h of continuous incubation with the SPLP formulation (Fig. 6(a)). Decreasing the DODAC concentration in SPLP from 42.5 to 34 mol% increased the extent of luciferase expression approximately 5 fold after 48 h of transfection. A dose titration (0.05–1 μg plasmid/well) with SPLP containing 20–42.5 mol% DODAC revealed that the transfection potency for SPLP with 20–30 mol% DODAC is consistently higher than for SPLP with 42.5 mol% DODAC for each dose (Fig. 6(b)). The luciferase activities detected in cells treated with SPLP were comparable to those obtained for plasmid lipid complexes formed with pCMVLuc and DOPE/DOCAC (1 : 1; mol : mol) liposomes at a charge ratio of 1.5 : 1 (+ : − which was optimal for this cell line). Cell viability measured with a calcein hydrolysis assay, clearly increased with decreasing cationic lipid content in SPLP. Based on the transfection levels and cell viability SPLP containing 20–25 mol% DODAC were considered optimal for transfection *in vitro*.

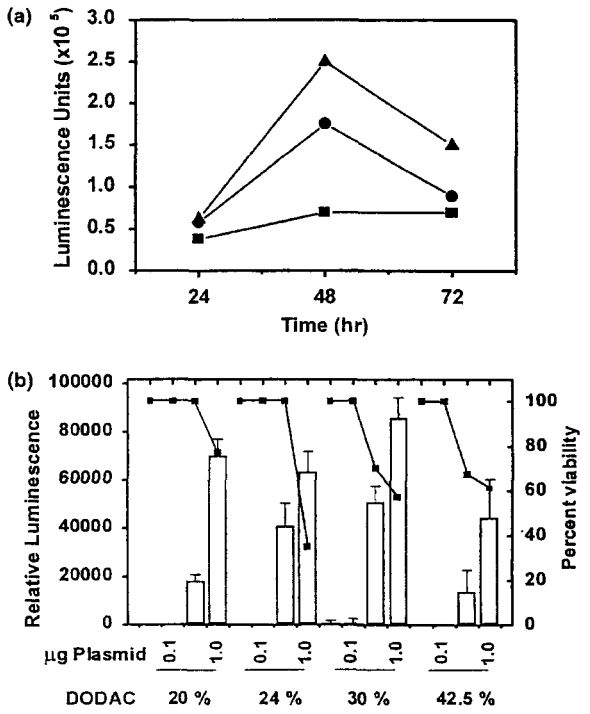


FIGURE 6 Effect of DODAC content in SPLP on the transfection activity *in vitro*. Plasmid (pCMVLuc) was encapsulated in SPLP containing different DODAC concentrations (DODAC : DOPE : PEG-cerC$_8$; $x$ : 85 − $x$ : 15; mol : mol : mol) using dialysis conditions as described in Fig. 3 and Table II. SPLP were isolated by sucrose gradient centrifugation. COS-7 cells were plated at 40.000 cells/well in a 24 well plate and incubated for 24 h. For the time course shown in (a) the medium was replaced after 24 h and SPLP containing 0.5 µg of pINEXL018 added and incubated for 24, 48 and 72 h. After incubation was complete the cells were assayed for luciferase expression as described in Methods and the luciferase activity plotted as a function of time for SPLP containing 42.5 (■), 38 (●) and 34 (▲) mol% DODAC. For the dose response shown in (b), COS-7 cells, were incubated as described above with 20, 24, 30 and 42.5 mol% DODAC SPLP at 0.05, 0.1, 0.5 and 1 µg pCMVLuc/well. The cells were incubated for 24 h and were assayed for luciferase to quantify transfection and calcein hydrolysis to estimate cell viability as described in Methods.

## SPLP are Stable During Storage

An isolated SPLP preparation containing 24 mol% DODAC was filter-sterilized (0.2 µm filter) and stored at 4°C for one year. At 3, 6 and 12 months aliquots of the formulation were removed from storage and assayed for luciferase transfection in a dose-response study and compared to the initial activity. In addition the particle size was characterized by quasi-elastic light scattering. Throughout the storage period the formulation retained both its transfection activity and small particle size (data not shown).

## Effect of Cationic Lipid Concentration on the Transfection Potency of SPLP *In Vivo*

The transfection activity of SPLP formulations containing DODAC ranging from 7 to 42.5 mol% were compared in an intraperitoneal B16 tumor model. B16 cells were seeded in the peritoneal cavity of C57BL/6 mice. After 7 days the tumors were on average approximately 200 mg in size and SPLP preparations containing pCMVLuc were administered intraperitoneally at a dose of 30 µg plasmid per mouse. The tumors were collected after 24 h and assayed for luciferase activity. Equal plasmid doses of both, sucrose gradient isolated (excess empty lipid vesicles removed) and non-isolated formulations were administered to determine if the presence of empty liposomes would affect the level of transfection *in vivo*. The luciferase expression detected in tumors treated with non-isolated SPLP containing 42.5 mol% DODAC was similar to the expression following transfection with complexes formed with DODAC/DOPE (1 : 1; mol : mol) liposomes and pCMVLuc (30 µg plasmid/mouse) of 100–200 pg/g tumor (data not shown). The complexes were formed at a 3 : 1 cationic lipid-to-DNA charge ratio, which was optimal for transfection in this tumor model. The highest luciferase expression was observed in tumors treated with isolated SPLP containing 24 and 30 mol% DODAC (Fig. 7). The luciferase activity detected was approximately 10 fold higher than that measured in tumors treated with cationic lipid/DNA complexes. Only very low levels of transfection were observed with SPLP containing 7 mol% DODAC. The presence of excess empty lipid vesicles reduced the transfection potency of SPLP approximately 4 fold, independent of DODAC concentration in the SPLP. Thus the highest transfection potency was obtained with isolated SPLP containing approximately 25 mol%

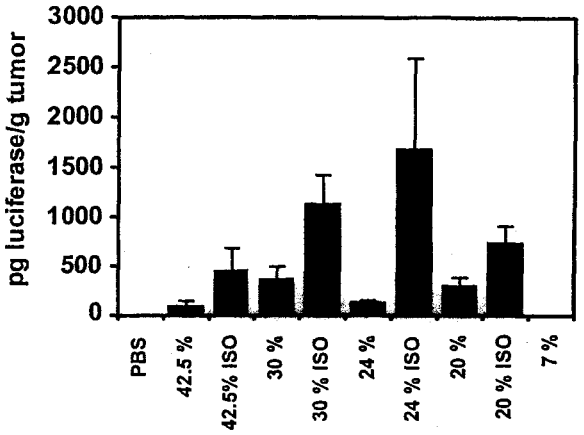


FIGURE 7 Transfection of intraperitoneal B16 tumors using isolated and non-isolated SPLP. Groups of female C57 mice were injected with 100,000 B16 tumor cells 7 days prior to administration of SPLP. Both isolated and non-isolated SPLP prepared with 7, 20, 24, 30 and 42.5 mol% DODAC (DODAC : DOPE : PEG-cer$C_8$; $x : 85 - x : 15$; mol : mol : mol) containing 30 μg pINEXL018 were injected into the peritoneal cavity of tumor-bearing mice. The tumors were removed 24 h post-injection and were assayed for luciferase transfection as described in Methods.

DODAC in both cells *in vitro* and in a regional tumor model *in vivo*.

## DISCUSSION

This study demonstrates that SPLP can be formed with a wide range of cationic lipid concentrations (6–42.5%) by adjusting the salt concentration in a phosphate buffer system used in the detergent dialysis process. The formulation process described even permits formation of SPLP with equimolar concentration of cationic lipid and neutral lipid which is similar to the lipid composition typically employed to form the plasmid/cationic lipid complexes (lipoplexes). The transfection potency of SPLP formed with equimolar concentrations of cationic and neutral lipids as determined in cells *in vitro* and in a regional tumor model *in vivo* was comparable to the one obtained with the plasmid–cationic lipid complexes formed with liposomes composed of DODAC : DOPE (50 : 50, mol : mol). But most important, reduction of the cationic lipid content in the SPLP to 24 mol% resulted in an approximate 10 fold increase in the transfection activity in tumors *in vivo*.

The SPLPs with the different DODAC concentrations all exhibit a homogeneous well-defined structure with plasmid DNA encapsulated within a lipid bilayer envelope. The cryo-electron microscopy data represent the first demonstration of a plasmid entrapped in a small well-defined lipid vesicle of approximately 75 nm diameter. This structure is clearly distinct from any structures described for plasmid/cationic lipid complexes. The complete encapsulation of the plasmid is consistent with the serum stability, protection from DNase and stability of SPLP in the blood stream (Wheeler *et al.*, 1999; Monck *et al.*, 1999). In contrast to the SPLP, plasmid/cationic lipid complexes are generally much larger than 200 nm in diameter and do not provide full protection of the associated plasmid.

The high plasmid-to-lipid ratio of approximately 70 μg DNA/μmol lipid in the isolated SPLP containing pCMV Luc was independent of the DODAC concentration used and corresponds to one plasmid per SPLP. The plasmid-to-particle ratio was calculated assuming a lipid molecular area of 0.67 $nm^2$ (King *et al.*, 1985) and an average nucleotide molecular weight of 330. For a SPLP with a diameter of 75 nm (average size determined by cryo-electron microscopy) and a 5.650 kbp plasmid (pCMV Luc) the plasmid-to-lipid ratio of 70 μg DNA/μmol lipid corresponds to a plasmid-to-particle ratio of 0.99.

The SPLP can readily be sterilized by passing through a 0.22 micron filter and are highly stable during extended storage. No significant changes in their size, plasmid encapsulation and transfection potency was observed during storage at 4°C over a one-year period. Complexes are usually so unstable that they have to be formed immediately prior to administration and their size distribution makes filter-sterilization impossible.

Entrapment of plasmids of up to 20 kb in 80–100 nm diameter vesicles represents a difficult packing problem. For example, electron micrographs of

a supercoiled 4.4 kb plasmid revealed an extended length of about 520 nm and on average (in two dimensions) a diameter in the range of 350 nm (Lewis *et al.*, 1985). This would suggest an average diameter of > 1 μm for a 20 kb plasmid. The inverse relationship between encapsulation efficiency and plasmid size observed and the apparent limit for encapsulation of plasmids of 20 kb is therefore not surprising. The detergent dialysis process with cationic lipids described here must involve partial condensation of the plasmid to permit encapsulation in < 100 nm vesicles. The exact mechanism for plasmid encapsulation is not understood in detail. In an earlier study it was shown that the plasmid encapsulation efficiency was a sensitive function of the cationic lipid content and maximum entrapment was obtained with 6 mol% DODAC (Wheeler *et al.*, 1999). It was proposed that plasmid interacts with macromolecular lipid structures such as cylindrical micelles and lamellar sheets formed as intermediate structures during detergent dialysis. The results shown here indicate that the positive charge on these intermediate lipid structures can be shielded with phosphate. At phosphate concentrations above the optimum range the positive charge on the lipid structures formed during dialysis is shielded to the extent that interaction with the plasmid is inhibited, resulting in little or no plasmid encapsulation. At the critical optimal phosphate concentration the shielded charge on the lipid structures are sufficient to bind plasmid and encapsulation proceeds as outlined previously (Wheeler *et al.*, 1999). On the other hand, at phosphate concentrations below the optimum range the positive charge on the lipid structures is not shielded sufficiently resulting in strong plasmid lipid interaction with aggregate formation. Adjustments to the NaCl concentration can be used in combination with phosphate to fine-tune the system.

The SPLP are stabilized by a PEG coating. The PEG-ceramide lipids have a dual function by first regulating the degree of DNA interaction with cationic lipid during SPLP formation and secondly by providing programmable fusion (Holland *et al.*, 1996a,b) and circulation lifetimes *in vivo* (Lasic *et al.*, 1991; Monck *et al.*, 1999). The PEG-stabilized SPLP exhibit an extended shelve-life of at least one year without a detectable change in particle size and loss of transfection activity. However, the presence of a PEG coating can inhibit interaction and fusion between lipid vesicles (Holland *et al.*, 1996a,b) and the poor transfection observed with SPLP containing PEG-Cer$C_{20}$ *in vitro* was attributed to the inability of PEG to dissociate. SPLP containing PEG-Cer$C_{14}$ with a faster dissociation rate ($t_{1/2}$ = 1.2 h) compared to PEG-Cer$C_{20}$ ($t_{1/2} \geq 13$ days) were shown to have significantly improved transfection properties. The SPLP used in the present study contained PEG-Cer$C_8$ as the stabilizing agent with a very fast dissociation rate ($t_{1/2} \leq$ 1.2 min) to maximize the transfection and to permit evaluation of the effect of the cationic lipid content in the SPLP on the transfection potency *in vitro* and in the regional *in vivo* model, independent of PEG exchange. It should be noted that these systems would not be suitable for intravenous delivery for targeting disease sites such as distal tumors, since they will be highly unstable following interaction with biological fluids.

The most important finding in this study is the importance of the cationic lipid concentration in SPLP for efficient transfection *in vitro* and *in vivo*. The highest transfection activity was observed with SPLP containing approximately 25 mol% DODAC and the expression levels observed in tumors were about one magnitude higher than obtained with corresponding plasmid DNA–cationic lipid complexes. The SPLPs containing 20–30 mol% DODAC prepared by the citrate dialysis method (Zhang *et al.*, 1999) exhibit similar transfection potencies as SPLP with equivalent DODAC concentrations made by the phosphate dialysis method ($1.2 \pm 0.6$ and $1.7 \pm 0.8$ pg luciferase/g tumor for SPLP with 24 mol% DODAC formed by citrate and phosphate dialysis, respectively). There was no apparent toxicity associated with SPLP containing 24 mol% DODAC as determined by aspartate aminotransferase levels in the serum 24 h following i.p. injection. The higher concentration of cationic lipid in SPLP may result in enhanced association

with and uptake into cells. In this regard cellular delivery of plasmid with SPLP containing 6 mol% DODAC and stabilized with PEG-Cer$C_8$ was less than 3% of that observed with DODAC/DOPE (1 : 1) complexes (Mok *et al.*, 1999). In addition it is possible that it enhances interaction with the endosomal membrane facilitating intracellular delivery of the encapsulated plasmid. Excess lipid did not increase the transfection activity of SPLP *in vivo* in contrast to plasmid/lipid complexes (Song and Liu, 1998). The increase in transfection activity observed for SPLP containing higher cationic lipid concentrations and the reduced activity by excess empty vesicles suggest that cellular uptake of SPLP does represent a limiting step for efficient plasmid delivery. It is important to note that in this study only three lipid components DODAC, DOPE and PEG-Cer$C_8$ have been used. Using a broader range of lipids together with other components, such as conjugated targeting ligands, that increase cellular interaction and uptake, it may be possible to increase the transfection potential of SPLP even further. The formulation protocol described here provides a flexible system to assess different components for the further development of SPLP type gene delivery systems.

In summary, the results presented in this study show that SPLP can be constructed with a wide range of cationic lipid concentrations by including phosphate in the dialysis medium. The detergent dialysis protocol permits construction of highly flexible SPLP systems for non-viral gene delivery. SPLP with 24 mol% DODAC gives rise to tumor transfection that is superior to plasmid/cationic lipid complexes. Furthermore, these SPLP can be stored over extended period of time without any loss of activity. The well-defined structure sets SPLP clearly apart from lipoplexes and other cationic lipid or cationic polymer complexes.



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