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Research Paper

A Scalable, Extrusion-Free Method for Efficient Liposomal Encapsulation of Plasmid DNA

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Purpose. A fully scalable and extrusion-free method was developed to prepare rapidly and reproducibly stabilized plasmid lipid particles (SPLP) for nonviral, systemic gene therapy.
Methods. Liposomes encapsulating plasmid DNA were formed instantaneously by mixing lipids dissolved in ethanol with an aqueous solution of DNA in a controlled, stepwise manner. Combining DNA-buffer and lipid-ethanol flow streams in a T-shaped mixing chamber resulted in instantaneous dilution of ethanol below the concentration required to support lipid solubility. The resulting DNA-containing liposomes were further stabilized by a second stepwise dilution.
Results. Using this method, monodisperse vesicles were prepared with particle sizes less than 200 nm and DNA encapsulation efficiencies greater than 80%. In mice possessing Neuro 2a tumors, SPLP demonstrated a 13 h circulation half-life *in vivo*, good tumor accumulation and gene expression profiles similar to SPLP previously prepared by detergent dialysis. Cryo transmission electron microscopy analysis showed that SPLP prepared by stepwise ethanol dilution were a mixed population of unilamellar, bilamellar, and oligolamellar vesicles. Vesicles of similar lipid composition, prepared without DNA, were also <200 nm but were predominantly bilamellar with unusual elongated morphologies, suggesting that the plasmid particle affects the morphology of the encapsulating liposome. A similar approach was used to prepare neutral egg phosphatidylcholine:cholesterol (EPC:Chol) liposomes possessing a pH gradient, which was confirmed by the uptake of the lipophilic cation safranin O.
Conclusions. This new method will enable the scale-up and manufacture of SPLP required for preclinical and clinical studies. Additionally, this method now allows for the acceleration of SPLP formulation development, enabling the rapid development and evaluation of novel carrier systems.

KEY WORDS: DNA; liposome; plasmid; systemic gene delivery.

INTRODUCTION

Systemic delivery of therapeutic genes to disease sites requires a vector with suitable properties. An ideal systemic vector should have the appropriate physical attributes to ensure favorable pharmacokinetics and delivery to disease sites. Following delivery to the target tissue, the vector must then be taken up by the tissue and express its DNA payload at sufficient levels to give a therapeutic effect. Importantly, a systemic vector must also be safe, well tolerated upon administration, and be non-immunogenic. The utility of viral vectors, cationic lipid–containing DNA complexes (lipoplexes), and polycationic polymer nucleic acid complexes (polyplexes) is limited in a systemic context (1).

Liposomes encapsulating small-molecule drugs such as doxorubicin (2) and vincristine (3) have been shown to be effective in humans. Liposomes can increase drug accumulation at the tumor site by 50 to 100 times compared to the administration of free drug (4–6). This is the result of the phenomenon referred to as "disease site targeting"—a passive targeting process that requires liposomes of around 100 nm in diameter and a circulation half-life >5 h. Although encapsulation of small-molecule drugs is relatively straightforward, encapsulating macromolecules, such as plasmid DNA, within a liposomal bilayer poses a greater challenge. Efficient encapsulation of plasmid DNA has been achieved using a detergent dialysis approach where lipid bilayers are formed around plasmid DNA as the detergent is removed (7). The resulting stabilized plasmid lipid particles (SPLP) contained DNA fully encapsulated within a lipid bilayer that incor-

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ABBREVIATIONS: Chol, cholesterol; DODAC, dioleyldimethylammonium chloride; DODAP, 1,2-dioleoyl-*N,N*-dimethyl-3-aminopropane; DODMA, 1,2-dioleyloxy-*N,N*-dimethylaminopropane; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; HBS, Hepes buffered saline; ^{3}H-CHE, tritium-labeled cholesteryl hexadecyl ether; OGP, octylglucopyranoside; PBS, phosphate buffered saline; PEG-CerC_{20}, 1-*O*-(2'-(ω-methoxypolyethyleneglycol)2000)-2-*N*-arachidoylsphingosine; PEG-S-DSG, 3-*O*-(2'(ω-methoxypolyethyleneglycol)2000)-1,2-distearoyl-*sn*-glycerol; QELS, quasi-elastic light scattering; SPLP, stabilized plasmid lipid particles; SVF, spontaneous vesicle formation; TE, Tris EDTA buffer; TEM, transmission electron microscopy; TNS, potassium 2-(*p*-toluidino)-6-naphthalenesulfonic acid.

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porated a cationic lipid (DODAC), a fusogenic lipid (DOPE), and a PEG-lipid (PEG-CerC_{20}). These particles possessed an average diameter of less than 100 nm, and unlike DNA-cationic lipid complexes formed by mixing preformed cationic lipid–containing vesicles with plasmid DNA, fully protect DNA following incubation with *Escherichia coli* DNase I. SPLP prepared using this approach have a circulation half-life of >6 h, accumulate in distal tumors following intravenous administration in tumor-bearing mice, and lead to reporter gene expression in murine tumors (8). SPLP have also been prepared by destabilizing preformed empty vesicles in ethanol at 40% v/v and incubating in the presence of plasmid DNA (9). This ethanol destabilization method yielded DNA encapsulation efficiencies of approximately 65%.

Although SPLP show considerable potential for systemic gene transfer, the detergent dialysis method of preparation suffers from a number of limitations. The method is exquisitely sensitive to minor changes in the ionic strength of the formulation buffer; changes as small as 10 mM result in a dramatic decrease in encapsulation efficiency (10). Even when SPLP are formed under ideal conditions, the detergent dialysis method results in the formation of large numbers of empty vesicles that are typically separated from SPLP by gradient ultracentrifugation (10). The detergent dialysis method is also difficult to scale to the size required to support preclinical and clinical development. For this reason, we sought to develop a more simple, robust, and fully scalable method for the encapsulation of plasmid DNA in SPLP. The method reported here, a type of spontaneous vesicle formation, produces SPLP with the same desirable properties as those prepared by detergent dialysis. We discuss the use of this method for the preparation of other types of drug delivery systems formerly prepared using extrusion methods.

MATERIALS AND METHODS

Lipids and Plasmid DNA

The cationic lipids dioleyldimethylammonium chloride (DODAC) and 1-*O*-[2′-(ω-methoxypolyethyleneglycol)2000]-2-*N*-arachidoylsphingosine (PEG-CerC_{20}) were synthesized as described previously (11,12). The cationic lipid 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODMA; Fig. 1A) was synthesized using methodology derived from that used previously for the synthesis of a DOTMA precursor (13). 3-(Dimethylamino)-1,2-propanediol (1.43 g, 12 mmol) and 95% sodium hydride (NaH, 2.52 g, 100 mmol) were stirred in benzene (60 ml) under argon for 30 min. Oleyl bromide (10.0 g, 30 mmol) was added and the reaction refluxed under argon for 18 h. The reaction mixture was quenched with ethanol, washed (3 × 250 ml distilled water), dried (magnesium sulfate), and evaporated. The organic phase was dried over magnesium sulfate and evaporated. The crude product was purified on a silica gel column (1% methanol in chloroform). It was subsequently decolorized by stirring for 30 min in a suspension of activated charcoal (1 g) in ethanol (75 ml) at 60°C. The charcoal was removed by filtration through Celite and the ethanol solution concentrated to yield 4.8 g (65%) of pure product.

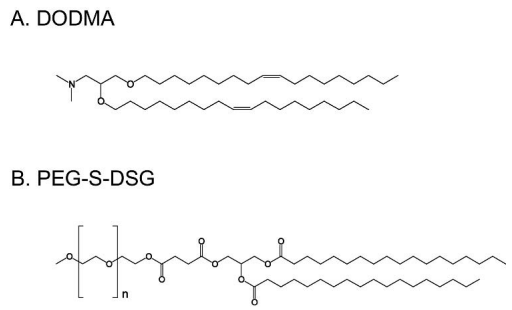

Fig. 1. Chemical structures of the lipids (A) DODMA and (B) PEG-S-DSG.

3-*O*-[2′(ω-methoxypolyethyleneglycol)2000]-1,2-distearoyl-*sn*-glycerol (PEG-S-DSG; Fig. 1B) was prepared by treating a solution of monomethoxypolyethylene glycol (average MW = 2000; PEG_{2000}) (30 g, 15 mmol) in pyridine (150 ml) with succinic anhydride (10 g, 100 mmol) overnight. The solvent was removed by rotovap and the solution diluted with water (200 ml). The solution was acidified with concentrated HCl and extracted with choloroform (3 × 150 ml). The organic fraction was dried over magnesium sulfate and concentrated to yield ~30 g of crude product as a white wax. Purification was carried out by flash column chromatography with chloroform containing 0–7% methanol. The pure product was taken up in water (300 ml) and lyophilized to give 20 g of PEG_{2000}-succinate (PEG_{2000}-S) as a white powder. PEG_{2000}-S (9 g, 4.3 mmol) was dissolved in benzene (100 ml) and treated with oxalyl chloride (4.44 g, 35 mmol). The solution was stirred for 2 h at room temperature and the solvent removed under vacuum. The residue was dissolved in ethanol-free chloroform (100 ml) and 1,2-distearoyl-*sn*-glycerol (3.0 g, 4.8 mmol) and triethylamine (3 ml, 20 mmol) added. The solution was stirred for 48 h, added to water (100 ml), acidified with HCl, and the organic phase collected. The aqueous phase was extracted with chloroform (2 × 100 ml). The organic phases were combined, dried over $MgSO_4$, filtered, and the solvent removed. The crude product was purified by column chromatography, eluting with chloroform containing 0–7% methanol. The product was decolorized by stirring for 30 min in a suspension of activated charcoal (1 g) in ethanol (100 ml) at 60°C. The suspension was filtered, the solvent removed, and the product taken up in water (300 ml) and lyophilized, yielding PEG_{2000}DSG (6.0 g) as a fluffy white powder.

Dioleoylphosphatidylethanolamine (DOPE) and egg phosphatidylcholine (EPC) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Distearoylphosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Synthetic cholesterol (Chol) and the detergent octylglucopyranoside (OGP) were obtained from Sigma-Aldrich Co. (Oakville, ON, Canada). Tritium-labeled cholesteryl hexadecyl ether (^{3}H-CHE) was obtained from Mandel NEN Products (Guelph, ON, Canada).

The pCMVluc plasmid, encoding the luciferase reporter gene under the control of the cytomegalovirus promoter, was propagated in *Escherichia coli* strain DH5α and purified by standard alkaline lysis/cesium chloride density gradient centrifugation (14,15).

SPLP Prepared by Detergent Dialysis

SPLP were prepared by detergent dialysis as described elsewhere (7,10). Briefly, DOPE, DODAC, and PEG-CerC_{20}

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at a molar ratio of 82.5:7.5:10 were dissolved in aqueous solutions of OGP. A pCMVluc plasmid solution was then added to give a solution of 0.4 mg/ml DNA, 10 mg/ml lipid, and 200 mM detergent. This solution was dialyzed for 48 h and unencapsulated DNA removed by anion exchange chromatography (DEAE Sepharose CL6B; Sigma). Empty vesicles were then removed by a one-step sucrose density ultracentrifugation. Fractions containing SPLP were consolidated and dialyzed with Hepes buffered saline (HBS) to remove sucrose. Isolated SPLP were concentrated by Amicon ultrafiltration to a final DNA concentration of 0.5 mg/ml. All samples were passed through sterile 0.2-μm filters and then stored at 5 ± 3°C until use.

Preparation of SPLP and Vesicles by Spontaneous Vesicle Formation

A flow diagram summarizing the process for preparing SPLP by spontaneous vesicle formation (SVF) is shown in Fig. 2. First, a plasmid solution was prepared by combining DNA in 10 mM Tris EDTA (TE) buffer with 100 mM citrate buffer (pH 5.0) and distilled deionized water to achieve a plasmid DNA concentration of 0.9 mg/ml in 20 mM citrate. A lipid solution in ethanol was prepared by dissolving Chol, DSPC, DODMA, and PEG-S-DSG at molar ratios of 55:20:15:10 in absolute, anhydrous ethanol and then adding distilled water to achieve an ethanol concentration of 90 vol%. This lipid composition was similar to the lipid composition of preformed vesicles used previously to encapsulate plasmid (9), except the more stable DODMA was selected instead of the titratable cationic lipid 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODAP), and PEG-S-DSG replaced PEG-CerC_{20}. The total concentration of lipid in solution was 20 mM. Equal volumes of both lipid and plasmid solutions were heated to 37°C prior to vesicle formation.

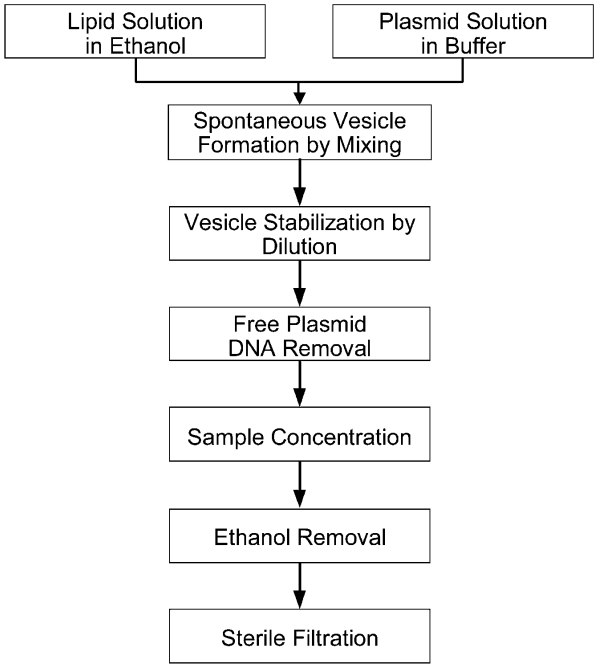

Fig. 2. Process flowchart for SPLP prepared by spontaneous vesicle formation. Please refer to Materials and Methods for a full description of the process.

The plasmid and lipid solutions were combined using a single peristaltic pump with dual pump heads (Watson Marlow), joined in opposition using a polypropylene T-connector (Cole-Parmer) and platinum-cured silicone tubing (Nalgene) both with an internal diameter of 1.6 mm. This configuration is conceptually similar to one that has been used to prepare lipoplex by the controlled mixing of preformed cationic liposomes with plasmid DNA (16). The two peristaltic pump heads were calibrated and adjusted to deliver 1 ml/s of mixed solution. The apparatus was then used to prepare SPLP by mixing the lipid and plasmid solutions, preheated to 37°C, resulting in the instantaneous formation of a liposomal suspension in 45% ethanol with approximately 60% DNA encapsulation. The volumes of lipid and plasmid solutions are not critical for the preparation of SPLP using this method. Solution volumes as low as 1 ml (0.9 mg DNA) and as large as 5000 ml (4.5 g DNA) have been used to prepare SPLP with similar results. Upon formation, the SPLP were then immediately diluted with 300 mM NaCl with 20 mM citrate pH 6.0 at 37°C using a similar apparatus. This second ethanol dilution step was found to stabilize further the metastable vesicles and resulted in a significant increase in the DNA encapsulation from 60% to 80–90%. The diluted vesicles were then incubated at 37°C for 30 min prior to removal of unencapsulated DNA by charged membrane filtration through a Mustang Q coin filter (Pall Corporation, Ann Arbor, MI, USA). After removal of unencapsulated DNA, encapsulated DNA was typically greater than 98% of that remaining. SPLP were concentrated using a tangential flow ultrafiltration system (Amersham Biosciences, Piscataway, NJ, USA) and diafiltered against 15 volumes of phosphate-buffered saline (PBS), pH 7.4, reducing the ethanol concentration to less than 0.5% [determined using a standard colorimetric alcohol dehydrogenase assay (Sigma)]. After ultrafiltration, samples were filtered through sterile 0.2-μm filters (Pall) and stored at 5 ± 3°C until use.

Empty vesicles of similar lipid composition, but lacking DNA, were prepared in a similar manner as SPLP, with the exception that an equivalent volume of 10 mM Tris-EDTA (TE) buffer was added to the initial citrate buffer solution instead of DNA.

Vesicles comprising EPC:Chol at a 55:45 molar lipid ratio were also prepared using this apparatus. Lipids were dissolved at 20 mM in 80% ethanol, and vesicles were formed by mixing these lipids with either PBS (pH 7.4) or 150 mM citrate (pH 4.0). The second dilution step was performed using the same buffer as the first mixing step. Following sample concentration by ultrafiltration, EPC:Chol vesicles were first diafiltered with 15 volumes of the original mixing buffer to ensure maintenance of the internal pH of the vesicles. Once the ethanol was removed, the vesicles were diafiltered against PBS, pH 7.4. These vesicles were then adjusted to the desired lipid concentration and filtered using sterile 0.2-μm filters.

Radiolabeled SPLP were prepared by adding ^{3}H-CHE at 0.5 μCi/mg lipid to the lipid-ethanol solution prior to mixing with the DNA solution.

Lipid Analysis

The total lipid content of SPLP and liposomes was calculated from the phospholipid content determined using the colorimetric method of Fiske and Subbarow (17). For SPLP,

lipid was first isolated from DNA using chloroform extraction (18). The apparent pK_a for the tertiary amino lipid DODMA in the lipid bilayer component of SPLP was determined as previously described (19) by measuring the fluorescence of potassium 2-(*p*-toluidino)-6-naphthalenesulfonic acid (TNS) over a pH range using a spectrofluorometer (SLM-Aminco) using excitation and emission wavelengths of 321 and 445 nm, respectively.

Particle Size Analysis

Particle size was determined using a Nicomp model 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). The instrument employs quasi-elastic light scattering (QELS) to determine the hydrodynamic equivalent spherical diameter of the particles. Volume-weighted, Gaussian distribution analysis was used to determine mean vesicle diameters and population standard deviations. Particle polydispersity was evaluated by the goodness-of-fit parameter χ^2, where a value of <3 indicates a monodisperse suspension (according to the instrument manufacturer).

DNA Content

Concentration and percent encapsulation of plasmid DNA in SPLP were determined using the membrane impermeable dye PicoGreen (Molecular Probes, Eugene, OR, USA) that binds specifically to double-stranded DNA and fluoresces only when bound. Picogreen fluorescence was measured with a spectrofluorometer (SLM-Aminco) using excitation and emission wavelengths of 495 and 525 nm, respectively. Plasmid encapsulation was determined by measuring fluorescence upon addition of PicoGreen to SPLP (Fi) and comparing this value to that obtained upon lysis of the SPLP lipid bilayers with 0.1% Triton X-100 (Ft):

$$\text{SPLP Plasmid Encapsulation (\%)} = \frac{(\text{Ft} - \text{Fi})}{\text{Ft}} \times 100\%$$

The total concentration of plasmid was then determined by comparing the fluorescence after addition of detergent (Ft) to a DNA standard curve prepared using a plasmid DNA standard:

$$\text{Total DNA Concentration} = \frac{(\text{Ft} - \text{b}) \times \text{Dilution}}{\text{m}}$$

where b is the y intercept of the DNA standard curve, and m is the slope of the standard curve.

The concentration of encapsulated plasmid DNA in SPLP was then determined by multiplying the total DNA concentration value by the value for percent plasmid encapsulation. *In vivo* experiments were performed using the concentration of encapsulated DNA to calculate DNA dose.

DNase Protection Assay

The ability of SPLP to protect encapsulated plasmid from exogenous DNase was assessed using a method modified slightly from that reported previously (7). The following samples were tested: naked plasmid DNA, SPLP prepared by SVF, and plasmid DNA added to empty vesicles with the same lipid composition as SPLP to achieve a similar DNA:lipid ratio as the SPLP. A 1-μg DNA aliquot of each sample was incubated with 500 U of DNase 1 (Life Technologies, Mississauga, ON, Canada) in a total volume of 100 μl React 4 buffer at pH 7.4 (Life Technologies) for 60 min at 37°C. Following incubation, DNA was isolated by sequentially adding 500 μl DNAzol (Life Technologies) and 1000 μl absolute ethanol. After centrifuging for 30 min at 13,000 × *g*, the supernatant was decanted and the DNA washed twice with 70% ethanol and dried. The resultant DNA pellets were dissolved in 30 μl Tris-EDTA buffer, pH 8.0, and analyzed by gel electrophoresis using a 0.8% agarose gel in Tris-acetate-EDTA, with a high DNA mass ladder (Invitrogen).

Cryo Transmission Electron Microscopy

Cryo transmission electron microscopy (Cryo-TEM) analysis of SPLP and liposomes was performed at Uppsala University, Sweden, using a Zeiss EM 902A with cryo equipment.

Dye Loading of EPC-Chol Liposomes

The feasibility of preparing EPC:Chol vesicles with an acidic aqueous interior by stepwise ethanol dilution was investigated by formulating with either citrate buffer (pH 4.0) or PBS (pH 7.4). Vesicles at 5 mM lipid in PBS (pH 7.4) were incubated with 0.2 mM of the lipophilic dye safranin O (Sigma) at 37°C for 30 min. After 30 min, 500 μl aliquots were passed through a 2-ml Sepharose CL4B gel filtration column to separate free dye from the vesicles. Fractions were analyzed for lipid content and safranin O content, which was determined using a spectrofluorometer (SLM-Aminco) at an excitation wavelength of 516 nm and emission wavelength of 585 nm.

In Vivo Transfection, Plasma Clearance, and Tumor Accumulation Studies

All animal studies were performed in accordance with the guidelines of the Canadian Council on Animal Care. Ten to 14 days prior to SPLP treatment, 8-week-old male A/J mice (Harlan, IN, USA) were inoculated subcutaneously in the hind flank with 1.5×10^6 Neuro 2a cells (ATCC, VA, USA). For *in vivo* transfection studies, SPLP (200 μl total volume containing 2 mg total lipid and 100 μg total DNA) were administered by lateral tail vein injection. For plasma clearance and tumor accumulation studies, SPLP incorporating ^{3}H-CHE were used at the same lipid and DNA concentrations. At appropriate time points, mice were anesthetized and blood was collected by cardiac puncture into microtainer tubes. Plasma was separated from red blood cells via centrifugation and analyzed for ^{3}H-CHE by liquid scintillation counting using Picofluor 20 (Perkin Elmer) and a Beckman LS6500 (Beckman Instruments). For clearance and accumulation studies, tumors were harvested at the specified time points and homogenized in lysing matrix tubes containing 500 μl of distilled water. Liver lysate (100 μl) and 200 μl of all other tissue lysates were assayed for radioactivity by liquid scintillation counting with Picofluor 40 (Perkin Elmer). For gene expression studies, tumors were lysed with 750 μl of cell culture lysis reagent (CCLR, Promega, Madison, WI, USA), and 20 μl of lysate was assayed for luciferase activity in a 96-well microplate luminometer (Bernholdt Technologies, Oakridge, TN, USA) using the Promega Luciferase Assay reagent kit

(Promega) according to the manufacturer's instructions. Recombinant luciferase from the American firefly, *Photinus pyralis* (Roche, PQ, Canada) was used to calibrate luminescence readings.

RESULTS

Characterization of the Spontaneous Vesicle Formation Method

Lipid encapsulated plasmid DNA can be prepared by the dropwise addition of lipid dissolved in ethanol to a rapidly mixing aqueous buffer containing DNA. Small-scale SPLP samples were prepared using either the stepwise dilution method or the ethanol drop approach diluted in each case to a final ethanol concentration of 22.5% and incubated for 60 min before DNA encapsulation efficiency was determined (Fig. 3). SPLP prepared by the stepwise approach had a DNA encapsulation efficiency of 81% and were monodisperse with a mean vesicle diameter of 132 ± 55 nm ($\chi^2 = 0.6$). SPLP prepared by ethanol drop to an initial target ethanol concentration of 45% and then diluted dropwise to 22.5% ethanol had a DNA encapsulation efficiency of 74% and mean vesicle size of 116 ± 54 nm ($\chi^2 = 0.4$). SPLP prepared by a single-step ethanol drop procedure to 22.5% ethanol in pH 6.0 citrate buffer had a mean vesicle size of 107 ± 53 nm but was a polydisperse formulation ($\chi^2 = 14.1$), and DNA encapsulation was only 17%. These results suggest an ideal range of ethanol concentration, referred to here as the "DNA encapsulation zone" (Fig. 2). In this zone, lipid bilayers efficiently form around the DNA particles. This same SPLP formulation can be prepared with similar DNA encapsulation efficiencies and vesicle size at a starting concentration of 80–100% ethanol. When encapsulation was attempted in 75% ethanol, DNA encapsulation decreased dramatically and mean particle size increased (166 ± 101 nm, $\chi^2 = 4.5$), presumably as a result of reduced solubility of the lipid components prior to mixing (Fig. 4A). Clearly, vesicles formed by ethanol drop are exposed to variable ethanol concentrations with the first vesicles formed at lower ethanol concentrations, concentrations that preclude DNA encapsulation. This limitation of the ethanol drop method is more evident when attempting to prepare large-scale SPLP preparations. In this case, it also becomes difficult to achieve rapid mixing of ethanol with larger volumes of DNA solution. This limitation may contribute to low encapsulation efficiencies observed when we attempted to scale this method (data not shown).

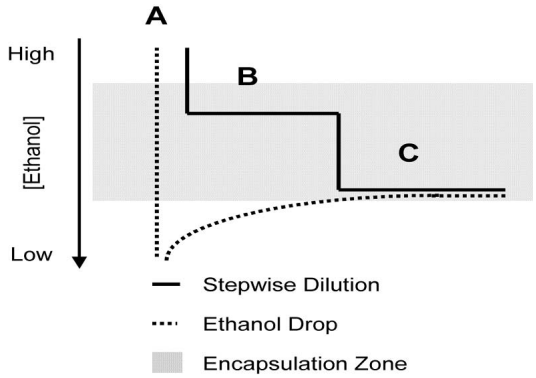

Fig. 3. Comparison of spontaneous vesicle formation by stepwise dilution to the ethanol drop method for encapsulating DNA. Lipids were dissolved in (A) 90% ethanol and diluted either stepwise to (B) 45% and (C) 22.5% ethanol, represented by the solid line, or (C) added dropwise into a stirred plasmid solution to a final ethanol concentration of 22.5%, represented by the dotted line.

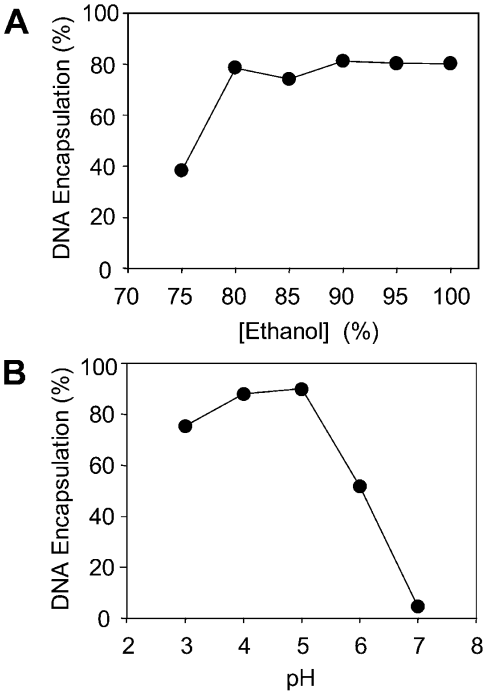

Fig. 4. Effects of (A) initial ethanol concentration and (B) pH on DNA encapsulation at the vesicle formation step. (A) The initial ethanol concentration in the lipid solution was varied, while the pH of the plasmid solution was kept constant at pH 5.0. (B) The pH of the plasmid solution was varied while the ethanol concentration was kept constant at 90%. DNA trapping efficiencies for the SPLP samples were determined after the dilution step with 300 mM NaCl, 20 mM citrate, pH 6.0. Data represent mean SD.

An important feature of the SPLP prepared here is the titratable cationic lipid DODMA. This lipid has an apparent pK_a of 6.8 in a lipid bilayer (as determined by TNS assay) and while predominantly uncharged at physiologic pH, possesses a positive charge under acidic conditions that is thought to promote the association of DODMA-containing bilayer fragments with negatively charged DNA. Because DNA hydrolyzes when incubated under acidic conditions (20), we varied the pH of the initial DNA buffer to determine the ideal pH for maximizing DNA encapsulation while minimizing DNA degradation. Predictably, DNA encapsulation efficiency dropped dramatically at mixing buffer pH values above 5 (Fig. 4B). The vesicles sizes changed little over the pH range 3 to 7 (110 ± 37 nm to 122 ± 55 nm), thus the pH of 5.0 was selected for the initial step of this process.

While developing the encapsulation approach described here, it was determined that the addition of a second dilution step increased the in-process stability of SPLP and at the same time increased the extent of DNA encapsulation from 60% to 70% seen after the initial mixing step to 80–90%. The pH and salt concentration of the dilution buffer were varied to determine the optimal conditions for promoting the increase in DNA encapsulation (Fig. 5). Dilution buffer containing 300 mM NaCl with pH adjusted from pH 4 to 10 was

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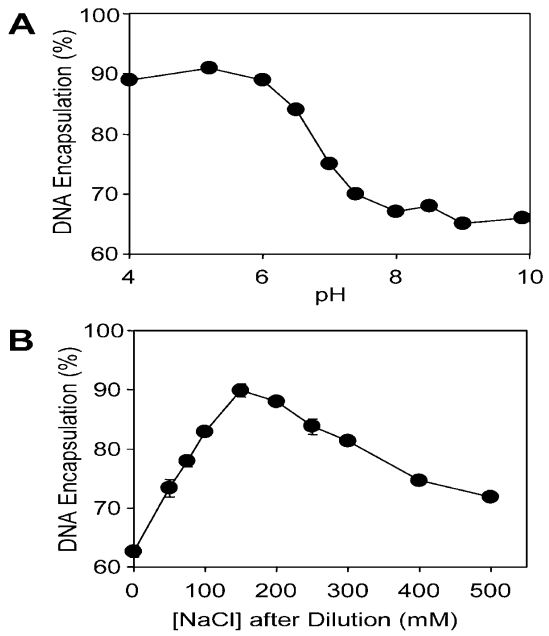

Fig. 5. Effect of varying (A) pH and (B) ionic strength on DNA encapsulation. Following the formation of SPLP by SVF. (A) The pH of the dilution buffer was varied while keeping the ionic strength of the diluted sample constant at 150 mM NaCl. (B) The ionic strength of the dilution buffed was varied while keeping the pH of the dilution buffer constant at pH 6.0. The dilution buffers possessed twice the concentration of the target NaCl concentration for the diluted sample (e.g., the dilution buffer was 300 mM NaCl, to achieve a salt concentration of 150 mM after dilution). Data represent mean SD.

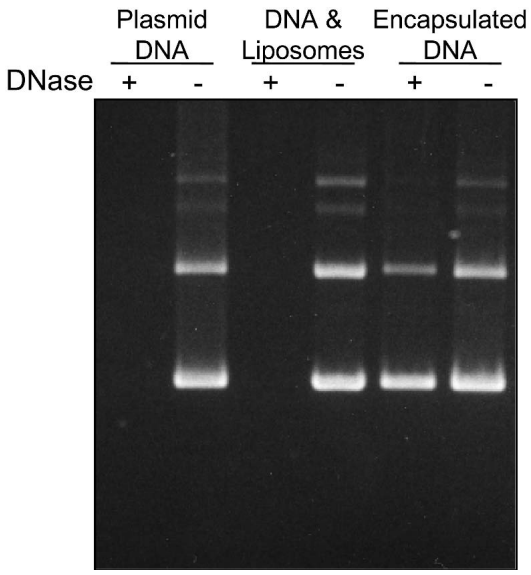

Fig. 6. SPLP prepared by spontaneous vesicle formation protect DNA from digestion by DNase 1. Lanes: Stability of free plasmid DNA, free plasmid in the presence of liposomes, and plasmid encapsulated in SPLP in the presence of DNase I. Each sample consisted of 1 μg of DNA and was incubated with either 500 U of DNase 1 (+) or without DNase 1 (–) at 37°C for 1 h prior to analysis by gel electrophoresis.

evaluated. Optimal DNA encapsulation was observed at buffer pH values of 4 to 6 (Fig. 5A). Above pH 6, DNA encapsulation diminished until there was no enhancement at pH 8 or above. This effect is thought to be a result of the net charge on the DODMA in the SPLP. The dilution buffer was set at pH 6.0 with citrate, and the optimal ionic strength of the dilution buffer was determined (Fig. 5B). Interestingly, dilution in 20 mM citrate buffer at pH 6.0 without NaCl yielded no additional DNA encapsulation compared to the mixed SPLP sample, suggesting that a simple reduction of ethanol concentration and shift in pH from 5.0 to 6.0 is insufficient to increase DNA encapsulation. Increasing the NaCl concentration in the dilution buffer to 300 mM resulted in an increase in DNA encapsulation to 90%. As the NaCl concentration in the dilution buffer was increased beyond 300 mM, the DNA encapsulation efficiency was reduced. In the presence of NaCl, supercoiled DNA is known to adopt a more compact structure (21). Therefore, the dilution step may be increasing DNA encapsulation by causing the DNA to adopt a more compact structure, increasing the charge density and providing more efficient association with lipid bilayer fragments that assemble to become liposomes containing DNA. Based on these observations, a dilution buffer of 300 mM NaCl with 20 mM citrate pH 6.0 was chosen for SPLP formation.

The ability of SPLP prepared by SVF to protect encapsulated DNA was tested by incubating with DNase I (Fig. 6). Naked DNA was completely degraded by DNase treatment. DNA mixed with empty vesicles with the same lipid composition as SPLP was also completely degraded, indicating that lipid alone did not interfere with the activity of the DNase. DNA in the SPLP sample showed no sign of degradation, indicating that the DNA is completely protected. A similar result was seen previously for SPLP prepared by detergent dialysis (7).

Liposome Formulations Prepared by Spontaneous Vesicle Formation

SPLP or empty vesicles similar in lipid composition to SPLP were prepared by SVF and analyzed for particle size, DNA and lipid content. Empty liposomes prepared by SVF had mean vesicle diameter of 120 ± 11 nm, $\chi^2 = 0.3$, and a narrower size distribution than SPLP (101 ± 37 nm, $\chi^2 = 0.7$). The efficiency of DNA encapsulation for SPLP prepared by SVF was 88% prior to free DNA removal and 96% in the final sterile filtered sample and following the free DNA removal step. For this formulation, the starting DNA:lipid ratio was 45 μg DNA/μmol lipid while the DNA:lipid ratio in the final filtered sample was 40 μg DNA/μmol, indicating that the ratio of encapsulated DNA to lipid remains constant throughout the formulation process. For small-scale SPLP formulation (<10 mg starting DNA), 50–60% recoveries of both encapsulated plasmid and lipid are typical in the final sterile filtered sample. Apart from the loss of unencapsulated DNA (8–15%), the major sources of product loss are holdup in the ultrafiltration cartridges and sterile filters. When encapsulating plasmid DNA at larger scale (>100 mg plasmid DNA), we routinely achieve DNA yields of 75%, as the in-process holdup losses represent a smaller proportion of the starting material. SPLP samples have been prepared with plasmid DNA concentrations as high as 9 mg/ml DNA. A variety of lipid compositions have been used to prepare SPLP using the SVF method, and plasmids varying in size from 4.4 to 15 kb have been encapsulated using a lipid com-

position containing a range of tertiary and quaternary cationic lipids, including DODAP and DODAC.

It was of considerable interest to determine whether SVF could be used to prepare liposomes suitable for the encapsulation of small-molecule drugs. Vesicles consisting of EPC:Chol (55:45 mol%) were also prepared by SVF. These vesicles possessed a mean particle diameter of 92 ± 41 nm, $\chi^2 = 1.7$, well within the typical size range of vesicles prepared by hydration from a lipid film followed by repeated extrusion through 100-nm polycarbonate membranes (22,23).

The morphologies of liposomes prepared by SVF were analyzed by Cryo-TEM (Fig. 7). Cryo-TEM has been used extensively to study liposome morphology, including DNA-lipid complexes (24). As reported previously, SPLP prepared by detergent dialysis are predominantly unilamellar (Fig. 7B) (7). SPLP and vesicles prepared by SVF are morphologically distinct from the unilamellar vesicles prepared by detergent dialysis or by extrusion (23). SPLP prepared using SVF appear to have three distinct particle morphologies: unilamellar vesicles similar in appearance to SPLP prepared by detergent dialysis; as well as bilamellar or oligolamellar vesicles that are more abundant and smaller than the unilamellar vesicles; and small dense particles (Fig. 7B). These dense particles have a narrow size range and appear to possess membrane structures. The 15% DODMA vesicles prepared by SVF have a very uniform bilamellar morphology (Fig. 7C). Many of these vesicles also exhibit protrusions of the outer bilayers that were not observed when the vesicles were prepared in the presence of DNA. DNA appears to play an important role in determining the morphology of SPLP. It may be that, in SPLP, the titratable cationic lipid DODMA in the inner bilayer is in contact with the DNA, effecting particle morphology in a manner that is not evident in the empty vesicles. Furthermore, higher concentrations of DODMA in the outer bilayer may make the empty vesicles more fusogenic, a property that has been described for analogous vesicles containing DODAP (19). Vesicles with tubular protrusions have previously been observed by cryo-TEM and attributed to an asymmetric distribution of the anionic lipid dioleoylphosphatidylglycerol (DOPG) between the outer and inner faces of the lipid bilayer in response to a transmembrane pH gradient (25), a mechanism that may be responsible for the structures observed in Fig. 7C.

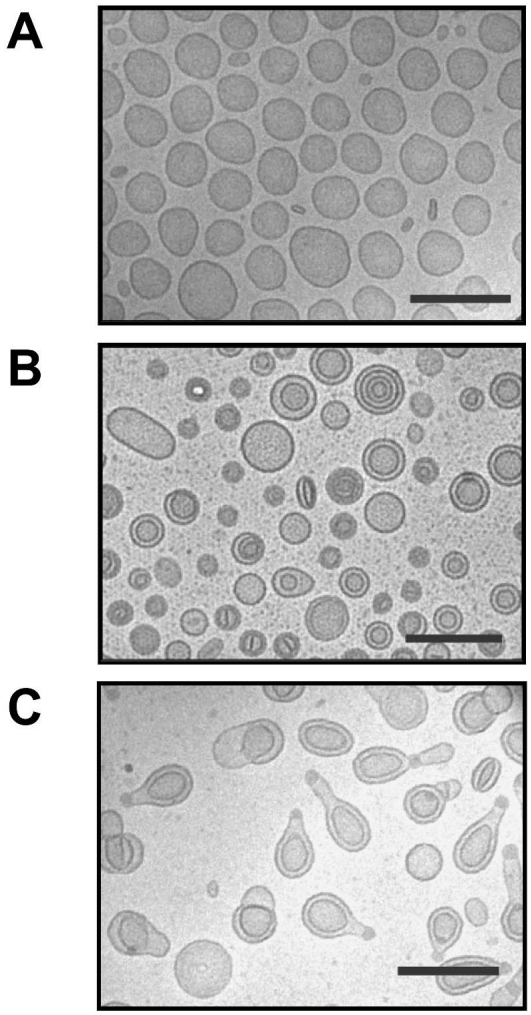

Fig. 7. Cryo transmission electron microscopy of SPLP. (A) SPLP prepared by detergent dialysis. (B) SPLP prepared by stepwise ethanol dilution. (C) Vesicles as in (B) but prepared without DNA. Scale bars = 200 nm.

Bilamellar structures were also very common for the EPC:Chol vesicles (data not shown). The lipophilic cation safranin O was next used to determine whether SVF could be used to prepare EPC:Chol vesicles possessing a transmembrane pH gradient. Liposomes possessing transmembrane pH gradients (acidic inside) have been used to encapsulate doxorubicin, vincristine, and other lipophilic amino-containing drugs within liposomes (26). Safranin O is taken up by model membrane systems in response to a membrane potential and is a useful tool to measure pH gradients across liposome membranes (27). When EPC:Chol vesicles were formulated with citrate buffer at pH 4, they demonstrated a significant increase in safranin O uptake of the cationic dye safranin (55% encapsulation efficiency) as compared to similar vesicles formulated with PBS pH 7.4 (9% encapsulation). Although the conditions for uptake were not optimized, this experiment clearly demonstrates that liposomal formulations possessing a pH gradient can be prepared by SVF.

Animal Studies with SPLP

Extended circulation lifetime is a prerequisite for disease site targeting and gene expression at distal tumor sites. To determine the clearance properties of SPLP, we prepared SPLP labeled with ^{3}H-CHE by SVF and evaluated their behavior in mice. At 24 h after intravenous administration, 40% of the injected dose was still present in the plasma, yielding a SPLP plasma half-life of 13 h (Fig. 8A). This plasma clearance profile was comparable to DOPE:DODAC:PEG-CerC_{20} SPLP that demonstrated a 10 h circulation half-life in mice (12). The accumulation of SPLP at distal tumor sites was significant, with 8% of the injected dose per gram of tumor accumulating 24 h after administration (Fig. 8B). Other tissues exhibited high levels of SPLP accumulation including the spleen, liver, adrenal glands, and small intestine each accumulating 44%, 26%, 14%, and 12% of the injected SPLP dose per gram of tissue, respectively (data not shown). Although, SPLP accumulation in these nontarget tissues was quite high, gene expression in these tissues was consistently two to three orders of magnitude lower than the tumor tissue, consistent with previous observations using SPLP prepared by detergent

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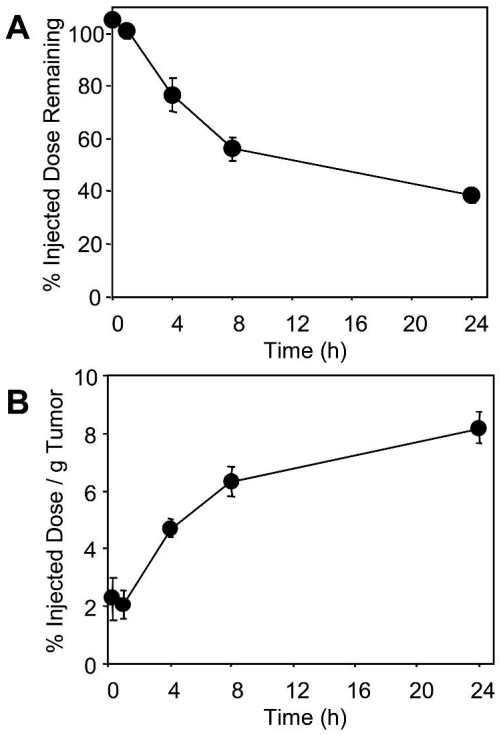

Fig. 8. *In vivo* (A) plasma clearance and (B) tumor accumulation of SPLP prepared by spontaneous vesicle formation. Each mouse received a single intravenous injection of 100 μg SPLP DNA. Error bars given as standard errors; n = 4 to 5.

dialysis. Figure 9 illustrates a direct comparison of tumor gene expression following intravenous administration of SPLP prepared by detergent dialysis and SVF, demonstrating that both methods yield particles with very similar patterns of luciferase gene expression over a 7-day period. Gene expression was highest 72 h after treatment with either preparation.

DISCUSSION

Controlled stepwise mixing has the advantage of maintaining an accurately and consistently mixed product throughout a process, facilitating scale-up of those processes that are sensitive to concentration effects. A significant benefit of the SVF method reported here is that it facilitates the rapid preparation of SPLP, allowing formulation development to proceed at a much faster pace than previous methods. Where it previously took 5 days to prepare a preclinical batch of SPLP by detergent dialysis (10), the same SPLP formulation can be prepared in a single day using this method. Spontaneous vesicle formation makes it possible to routinely prepare a large number of small-scale formulations (1 to 5 mg DNA) in a day, facilitating the accelerated optimization of formulation composition (e.g., lipid ratios, plasmid content) and process parameters (e.g., pH, ionic strength).

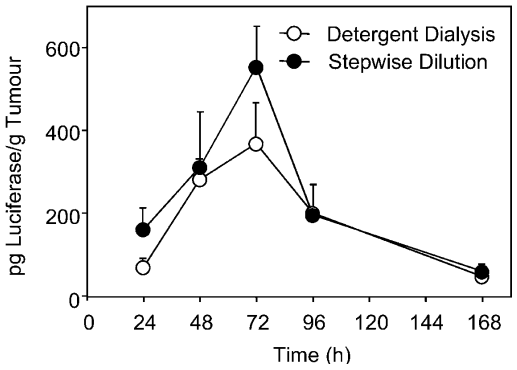

Fig. 9. *In vivo* luciferase gene expression following intravenous administration of SPLP prepared by spontaneous vesicle formation and detergent dialysis. Each mouse received a single intravenous injection of 100 μg SPLP DNA. Data for SPLP prepared with detergent dialysis and stepwise dilution are represented by open and solid circles, respectively. Error bars given as standard errors, n = 4.

Spontaneous vesicle formation represents an effective solution to the issues confounding SPLP preparation by detergent dialysis (7) or using ethanol-destabilized cationic liposomes (9). For the detergent dialysis approach, liposomes encapsulating DNA are formed using a 48-h dialysis step. Vesicle formation is very sensitive to the rate of detergent removal, and attempts to convert this dialysis step to a more rapid and scalable tangential flow process have failed (data not shown). The DNA trapping efficiency of the detergent dialysis process is quite low. Unencapsulated DNA, as well as empty liposomes, need to be removed from the formulation to give an acceptable DNA/lipid ratio. Although the removal of free DNA is straightforward, empty liposomes are removed using sucrose density ultracentrifugation (10), a step that is difficult to scale. Unlike the detergent dialysis approach, preparing SPLP using ethanol destabilized cationic liposomes does not require gradual detergent removal or ultracentrifugation steps (9). However, this method requires the formation of cationic vesicles prior to the encapsulation of DNA. The formation of vesicles by ethanol drop with an optional vesicle size reduction by extrusion through polycarbonate membrane filters is considerably more complicated and time consuming than the method described here. Once cationic liposomes of the desired size have been prepared, they need to be destabilized by ethanol addition to 40% v/v. Destabilization of vesicles with ethanol requires very slow addition of ethanol to a rapidly mixing aqueous suspension of cationic lipids to avoid localized areas of high ethanol concentration (>50% v/v) that would promote fusion and conversion of liposomes into large lipid structures (9). The addition of nucleic acid (oligonucleotide or DNA) must then be accomplished, again slowly, in a dropwise manner. The uncontrolled nature of both the vesicle destabilization and nucleic acid addition steps certainly poses challenges for reproducibly preparing SPLP at a scale suitable for preclinical evaluation.

Neutral lipid complexes with 80% DNA encapsulation have previously been prepared by mixing DOPC/DOPE vesicles (1:1 molar ratio) with ethanol and calcium, followed by dialysis (28). Although these particles were under 200 nm and had longer *in vivo* circulation times than cationic lipid-DNA complexes, they accumulated predominantly in the liver and showed very little tumor accumulation. Numerous other approaches have been used to encapsulate plasmid in lipid particles, including reverse phase evaporation, ether injection, and the dehydration of DNA lipid complexes and subsequent rehydration (Table I). This dehydration and rehydration of plasmid lipid vesicles (DRV) produces particles greater than 500 nm that are not suitable for systemic delivery of genes but may show promise as DNA-based vaccines (29).

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Table I. Procedures for Encapsulating Plasmid in Lipid-Based Systems

Procedure	Lipid composition and molar proportions	Length of DNA	Trapping efficiency[a]	DNA-to-lipid ratio[a]	Diameter
Reverse-phase evaporation (30)	PS or PS:Chol (50:50)	SV40 DNA	30–50%	<4.2 μg/μmol	400 nm
Reverse-phase evaporation (31)	PC:PS:Chol (40:10:50)	11.9 kb plasmid	13–16%	0.23 μg/μmol	100 nm to 1 μm
Reverse-phase evaporation (32)	PC:PS:Chol (50:10:40)	8.3 kb, 14.2 kbp plasmid	10%	0.97 μg/μmol	ND
Reverse-phase evaporation (33)	EPC:PS:Chol (40:10:50)	3.9 kb plasmid	12%	0.38 μg/μmol	400 nm
Ether injection (34)	EPC:EPG (91:9)	3.9 kb plasmid	2–6%	<1 μg/μmol	0.1 to 1.5 μm
Ether injection (35)	PC:PS:Chol (40:10:50) PC:PG:Chol (40:10:50)	3.9 kb plasmid	15%	15 μg/μmol	ND
Detergent dialysis (36)	EPC:Chol:stearylamine (43.5:43.5:13)	Sonicated genomic DNA (approx. 250,000 MW)	11%	0.26 μg/μmol	50 nm
Detergent dialysis, extrusion (37)	DOPC:Chol:oleic acid or DOPE:Chol:oleic acid (40:40:20)	4.6 kb plasmid	14–17%	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)
Lipid hydration (38)	EPC:Chol (65:35) or EPC	3.9 kb, 13 kb plasmid	ND	ND	0.5 to 7.5 μm
Dehydration-rehydration, extrusion (400 or 200 nm filters) (39)	Chol:EPC:PS (50:40:10)	ND	ND	0.83 μg/μmol (200 nm) 1.97 μg/μmol (400 nm)	142.5 nm (200 nm filter) 54.6 nm (400 nm filter, ultracentrifugation)
Dehydration-rehydration (40)	EPC	2.96 kb, 7.25 kb plsmid	35–40%	2.65–3.0 μg/μmol	1–2 μm
Dehydration-rehydration (DRV) for DNA vaccines (29)	PC:DOPE:DOTAP various DOTAP molar ratios	pRc/CMV HBS 5.6 kb	94–97%	2.5–4.2 μg/μmol	580–700 μm
Sonication (in the presence of lysozyme) (41)	Asolectin (soybean phospholipids)	1.0 kb linear DNA	50%	0.08 μg/μmol	100–200 nm
Sonication (42)	EPC:Chol:lysine-DPPE (55:30:15)	6.3 kb ssDNA 1.0 kb dsRNA	60–95% ssDNA 80–90% dsRNA	13 μg/μmol ssDNA; 14 μg/μmol dsRNA	100–150 nm
Spermidine-condensed DNA, sonication, extrusion (43)	EPC:Chol:PS (40:50:10) EPC:Chol:EPA (40:50:10) or EPC:Chol:CL (50:40:10)	4.4 kb, 7.2 kb plasmid	46–52%	2.53–2.87 μg/μmol	400–500 nm
Ca^{2+}-EDTA entrapment of DNA-protein complexes (44)	PS:Chol (50:50)	42.1 kbp bacteriophage	52–59%	22 μg/μmol	ND
Freeze-thaw extrusion (45)	POPC:DDAB (99:1)	3.4 kb linear plasmid	17–50%	ND	80–120 nm
SPLP-detergent dialysis (7)	DOPE:PEG-Cer:DODAC (84:10:6)	4.4 to 10 kb plasmid	60–70%	62.5 μg/μmol (after removal of empty vesicles)	75 nm (QELS); 65 nm (freeze-fracture)
Ethanol-Ca^{2+} destabilization (28)	DOPC:DOPE (50:50) (20:45:10:25)	pCMV-CAT 4.4 kb	70–80%	5 μg/μmol	160 nm (QELS)
Ethanol destabilization (9)	DSPC:Chol:PEG-$CerC_{14}$:DODAP (20:45:10:25)	pCMV-Luc 5.7 kb	70%	22.6 μg/μmol	ND
SPLP detergent dialysis (46)	(PEG-DSG or POD):DOTAP:DOPE (20:11:69)	pCMV-Gal, pCMV-Luc, pCMV-GFP	Up to 46%	20 μg/μmol	53–60 nm (QELS)
SPLP-ethanol dilution (this work)	Various	4.4 to 15 kb plasmid	80–95%	40–45 μg/μmol	100–150 nm (QELS)

ND, not determined.
[a] Some values calculated based on presented data.

The simplicity of the stepwise dilution method to prepare rapidly liposomes of desirable size and encapsulate DNA with high efficiency is thought to result from the precise control of the conditions that lipids enter the aqueous environment and self arrange into lipid bilayer fragments and then liposomes. A common feature of liposomes formed by SVF is the abundance of bilamellar structures. Although we offer no explanation for why this occurs, it appears that this morphology has no effect on the stability or activity of SPLP *in vivo*. This method may also be an attractive alternative to the preparation of liposomes by extrusion (22). The fact that liposomes possessing transmembrane pH gradients can be prepared by SVF, as demonstrated by safranin O uptake, indicates that similarly prepared vesicles could be loaded with lipophilic cationic drugs such as doxorubicin and vincristine (26,27).

Using this SVF method, we have prepared a 225 mg DNA batch of SPLP from start (dissolution of lipids) to finish (sterile filtration) in one working day. The properties of SPLP manufactured at this scale were equivalent to those prepared at 1/10 or 1/100 of this scale using smaller process steps. To date, an appreciable hurdle preventing the development of systemic nonviral gene delivery has been achieving predictable and controlled manufacture of stable systems. This hurdle has been overcome: SPLP batches containing up to 5 g of DNA have been prepared using this approach and are now under clinical evaluation.

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