[3] Stabilized Plasmid-Lipid Particles: A Systemic Gene Therapy Vector

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Introduction

Genetic drugs are a class of therapeutic agents with considerable potential for the treatment of human diseases such as cancer and genetic disorders. Although numerous methods exist for effective *in vitro* gene delivery, current systems have limited utility for systemic applications. Viral systems, for example, are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise transfection resulting from subsequent injections. In the case of nonviral systems such as plasmid DNA–cationic lipid complexes (lipoplexes), the large size and positively charged character of these aggregates also result in rapid clearance, and the highest expression levels are again observed in first-pass organs, particularly the lungs.[1,2–4] Plasmid DNA–cationic lipid complexes can also result in toxic side effects both *in vitro*[5] and *in vivo*.[6,7]

The need for a gene delivery system for treatment of systemic disease is obvious. For example, for cancer gene therapy there is a vital need to access metastatic disease sites as well as primary tumors. Similar considerations apply to other systemic disorders, such as inflammatory diseases. The design features for lipid-based delivery systems that preferentially access such disease sites are increasingly clear. It is now generally recognized that preferential delivery of anticancer drugs to tumor sites following intravenous injection can be achieved by encapsulation of these drugs in large unilamellar vesicles (LUVs) exhibiting a small size (<100 nm diameter) and extended circulation lifetimes (circulation half-life in mice >5 h).[8–10]

[1] L. Huang and S. Li, *Nat. Biotechnol.* **15,** 620 (1997).
[2] N. S. Templeton, D. D. Lasic, P. M. Frederik, H. H. Strey, D. D. Roberts, and G. N. Pavlakis, *Nat. Biotechnol.* **15,** 647 (1997).
[3] A. R. Thierry, Y. Lunardi-Iskandar, J. L. Bryant, P. Rabinovich, R. C. Gallo, and L. C. Mahan, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 9742 (1995).
[4] H. E. Hofland, D. Nagy, J. J. Liu, K. Spratt, Y. L. Lee, O. Danos, and S. M. Sullivan, *Pharm. Res.* **14,** 742 (1997).
[5] G. S. Harrison, Y. Wang, J. Tomczak, C. Hogan, E. J. Shpall, T. J. Curiel, and P. L. Felgner, *Biotechniques* **19,** 816 (1995).
[6] S. Li and L. Huang, *Gene Ther.* **4,** 891 (1997).
[7] P. Tam, M. Monck, D. Lee, O. Ludkovski, E. C. Leng, K. Clow, H. Stark, P. Scherrer, R. W. Graham, and P. R. Cullis, *Gene Ther.* **7,** 1867 (2000).
[8] R. T. Proffitt, L. E. Williams, C. A. Presant, G. W. Tin, J. A. Uliana, R. C. Gamble, and J. D. Baldeschwieler, *Science* **220,** 502 (1983).

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The accumulation of these drug delivery systems at disease sites, including sites of infection and inflammation as well as tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue.[11]

A gene delivery system containing an encapsulated plasmid for systemic applications should therefore be small (<100 nm diameter) and must exhibit extended circulation lifetimes to achieve enhanced delivery to disease sites. This requires a highly stable, serum-resistant plasmid-containing particle that does not interact with cells and other components of the vascular compartment. In order to maximize transfection after arrival at a disease site, however, the particle should readily interact with cells at the site and should have the ability to destabilize cell membranes to promote intracellular delivery of the plasmid. In this work we describe a straightforward detergent dialysis procedure used for the production of stabilized plasmid-lipid particles (SPLP) that satisfy the demands of plasmid encapsulation, small size, and serum stability. We will also indicate how the transfection properties of these systems can be modulated by changing the composition of the encapsulating lipid bilayer. The behavior of SPLP can be modified by employing poly(ethyleneglycol) (PEG) coatings which can dissociate from the SPLP,[12] by increasing the cationic lipid content,[13,14] by including other lipids such as cholesterol, or by incorporating cationic PEG-lipids (CPL) that enhance intracellular delivery.[15] Although most of this review will focus on technical aspects of SPLP production and characterization, we will also provide examples of their use in gene delivery and expression. A detailed protocol for producing SPLP is provided in the Appendix.

Construction and Characterization of Stabilized Plasmid-Lipid Particles

Encapsulation of Plasmid DNA within Liposomal System Using Detergent Dialysis Procedure

In recent years, a large number of liposomal systems have been designed for the systemic delivery of conventional drugs (including chemotherapeutic agents and antibiotics[16]), several of which have shown promise in the treatment of specific

[9] A. Gabizon and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6949 (1988).
[10] A. Chonn and P. R. Cullis, *Curr. Opin. Biotechnol.* **6,** 698 (1995).
[11] S. Kohn, J. A. Nagy, H. F. Dvorak, and A. M. Dvorak, *Lab Invest.* **67,** 596 (1992).
[12] J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).
[13] Y. P. Zhang, L. Sekirov, E. G. Saravolac, J. J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P. R. Cullis, and P. Scherrer, *Gene Ther.* **6,** 1438 (1999).
[14] E. G. Saravolac, O. Ludkovski, R. Skirrow, M. Ossanlou, Y. P. Zhang, C. Giesbrecht, J. Thompson, S. Thomas, H. Stark, P. R. Cullis, and P. Scherrer, *J. Drug Target.* **7,** 423 (2000).
[15] T. Chen, K. F. Wong, D. B. Fenske, L. R. Palmer, and P. R. Cullis, *Bioconjug. Chem.* **11,** 433 (2000).

cancers or other diseases and are now either in clinical trials or approved for use in humans.[10] Plasmid delivery systems such as cationic lipid-plasmid complexes have proved to be effective *in vitro* transfection agents,[17] but have limited ability for systemic applications. It is obviously desirable to develop liposomal systems for the *systemic* delivery of genetic drugs. This requires encapsulation of plasmid in small lipid vesicle systems. Unfortunately, techniques that had proved so useful for loading small, weakly basic drugs into LUVs[16] could not be adopted to the encapsulation of large molecules of plasmid DNA.

The solution came with the development of a detergent dialysis method for encapsulating plasmid in unilamellar lipid vesicles.[12] Previous work had shown that incubation of plasmid DNA with cationic lipids could result in a hydrophobic particle which was soluble in organic solvent.[18] This suggested the possibility that such a hydrophobic particle could be surrounded by an outer coating of lipid, which would then result in small, plasmid-containing particles stabilized in an aqueous medium. Detergent dialysis was recognized as a logical technique for achieving this, as the detergent was expected to solubilize the hydrophobic plasmid DNA–cationic lipid particles. The addition of phospholipid and subsequent removal of detergent by dialysis could then result in the exchange of the solubilizing detergent with phospholipid, leaving particles that were stable in aqueous suspension.

Initial experiments employed the cationic lipid DODAC, the plasmid pCMVCAT, the non-ionic detergent OGP, and the bilayer-forming lipid palmitoyloleoylphosphatidylcholine (POPC). When DODAC was added to plasmid in distilled water, the formation of large (>1000 nm diameter) precipitates was observed. However, the subsequent addition of OGP (200 m*M*) resulted in solubilization of the precipitate, forming an optically clear suspension consistent with entrapment of hydrophobic plasmid DNA–cationic lipid particles within detergent micelles. This optically clear quality was maintained when POPC solubilized in OGP was added. However, when dialysis was attempted to facilitate removal and substitution of the detergent associated with the particles for POPC, extensive precipitation of the suspension was observed. A method of stabilizing the plasmid-containing particles preventing aggregation and precipitation during the dialysis process was therefore required.

Previous studies had shown that a PEG-lipid coating could prevent aggregation and fusion of LUVs induced by covalent coupling of protein to the vesicle

[16] P. R. Cullis, M. J. Hope, M. B. Bally, T. D. Madden, L. D. Mayer, and D. B. Fenske, *Biochim. Biophys. Acta* **1331,** 187 (1997).

[17] P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7413 (1987).

[18] D. L. Reimer, Y. Zhang, S. Kong, J. J. Wheeler, R. W. Graham, and M. B. Bally, *Biochemistry* **34,** 12877 (1995).

surface.[19,20] This suggested that the stabilizing properties of a PEG coating could prevent aggregation during dialysis. However, the standard PEG-phosphatidylethanolamine (PEG-PE) could not be used because the PEG-PE molecule bears a net negative charge and would be expected to displace the cationic lipid from the plasmid as had been noted for other negatively charged lipids.[21] To address this issue, ceramide was used as a neutral hydrophobic anchor that was in turn linked to PEG_{2000} to produce a neutral molecule. Three ceramide anchors were synthesized, differing in the length of the ceramide acyl chain ($CerC_8$, $CerC_{14}$, and $CerC_{20}$). When 10 mol% PEG-$CerC_{20}$ was incorporated in the detergent mixture with POPC, DODAC, and plasmid DNA, precipitation was no longer observed during detergent dialysis (against 5 m*M* HEPES, 150 m*M* NaCl, pH 7.4). Further, a proportion of the plasmid was encapsulated, as measured by recovery of DNA after elution on a DEAE-Sepharose CL-6B anion exchange column, or by Picogreen associated fluorescence in the absence and presence of Triton X-100. The extent of plasmid encapsulation was found to be a sensitive function of the DODAC content, with encapsulation levels of 30% or higher at about 9 to 12% DODAC. Addition of plasmid to preformed vesicles with the same lipid composition, followed by DEAE chromatography, yielded no plasmid encapsulation as measured by complete plasmid retention on the column.

These results suggest that "stabilized plasmid-lipid particles" (SPLP) could be produced by detergent dialysis employing a POPC/DODAC/PEG-$CerC_{20}$ (79 : 11 : 10; mol : mol : mol) lipid mixture, as diagrammatically represented in Fig. 1. However, it has been shown that when POPC is employed as a "helper" lipid in plasmid DNA–cationic lipid complexes, very low transfection rates are observed, whereas when dioleoylphosphatidylethanolamine (DOPE) is present, much higher transfection rates are achieved.[22] The encapsulation properties of DOPE/DODAC/PEG-$CerC_{20}$ lipid mixtures were therefore investigated. As shown in Fig. 2, high levels of encapsulation were obtained only for a narrow range of DODAC content (6–7 mol%), as observed for the POPC-containing systems. Significant differences are that maximum encapsulation was greater (~70%) for the DOPE-containing system and that optimum encapsulation was observed at about 6 mol% DODAC, compared with approximately 9% DODAC for the POPC-containing particles. If PEG-$CerC_{14}$ was substituted for PEG-$CerC_{20}$ very similar plasmid encapsulation behavior was observed.

The lower encapsulation observed for POPC-containing SPLP may be partially attributed to the ionic strength of the dialysis medium. As will be discussed

[19] T. O. Harasym, P. Tardi, S. A. Longman, S. M. Ansell, M. B. Bally, P. R. Cullis, and L. S. Choi, *Bioconjug. Chem.* **6,** 187 (1995).
[20] J. W. Holland, C. Hui, P. R. Cullis, and T. D. Madden, *Biochemistry* **35,** 2618 (1996).
[21] Y. Xu and F. C. J. Szoka, *Biochemistry* **35,** 5616 (1996).
[22] H. Farhood, N. Serbina, and L. Huang, *Biochim. Biophys. Acta* **1235,** 289 (1995).

Low Cationic Lipid Content
Critical Cationic Lipid Content
High Cationic Lipid Content
Micelle
Detergent

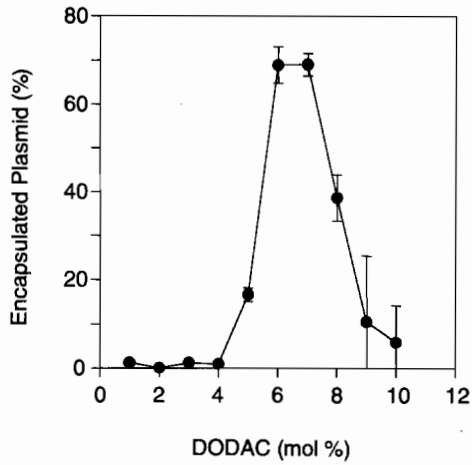


FIG. 2. Effect of DODAC concentration on the encapsulation efficiency of plasmid DNA (pCMV-CAT) in SPLP composed of DOPE, DODAC, and 10 mol% PEG-CerC$_{20}$. Lipid (10 mg/ml total), dissolved in octylglucoside (0.2 *M*), was mixed with plasmid DNA (50 μg/ml) in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube (12–14,000 molecular weight cutoff) and dialyzed against HBS for 36 h at 20°. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography (see text). Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).

in greater detail below, variation in lipid composition can alter the solute concentrations at which optimal encapsulation efficiency is achieved. Furthermore, the total lipid and plasmid concentrations have a significant effect on encapsulation efficiency. This was first shown for formulations of DOPE/DODAC/PEG-CerC$_8$

FIG. 1. Model of the formation and possible structure of SPLP. The first stage of dialysis is proposed to result in formation of macromolecular lipid intermediates, which may be in the form of lamellar sheets, cylindrical micelles, or leaky vesicles [M. Ollivon, O. Eidelman, R. Blumenthal, and A. Walter, *Biochemistry* **27,** 1695 (1988); P. K. Vinson, Y. Talmon, and A. Walter, *Biophys. J.* **56,** 669 (1989)]. If the cationic lipid content is too low (left panel), plasmid does not associate with these intermediates as dialysis proceeds, leading to formation of empty vesicles and free plasmid. At higher cationic lipid contents, plasmid associates with the lipid intermediates, drawn here as a bilayer sheet wrapped around the plasmid. If the cationic lipid content is at a critical level the presence of the plasmid reduces the net positive surface charge of the lipid intermediate to the extent that further association of plasmid is inhibited. As dialysis proceeds further, additional lipid would be expected to condense on this structure, leading to formation of a vesicle containing encapsulated plasmid, as indicated. In addition, empty vesicles and free plasmid would be expected. At high cationic lipid contents (right panel), the surface charge on the lipid intermediate structures is so high that two or more plasmids can associate with a given membrane sheet, leading to the formation of large aggregates.Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).

(42.5 : 42.5 : 15).[14] The percent plasmid encapsulation increased from roughly 20 to 70% as the total lipid concentration was increased from 1 to 10 mg/ml. Increasing the plasmid concentration from 100 to 1000 μg/ml resulted in a drop in encapsulation efficiency from 60 to 20%. Similar trends have been observed for other lipid compositions.

Isolation of Stabilized Plasmid-Lipid Particles by Density Centrifugation

The detergent dialysis process clearly results in plasmid-containing particles in which the plasmid is protected from the external environment. In addition, however, a population of empty vesicles is produced. To remove empty vesicles from plasmid containing SPLP a density gradient purification process was established taking advantage of the difference in density between SPLP and empty vesicles. The density gradient profile of a DOPE/DODAC/PEG-CerC_{20} (84 : 6 : 10; mol : mol : mol) SPLP preparation (initial plasmid-to-lipid ratio of 200 μg DNA to 10 mg lipid) was therefore examined employing sucrose density step gradient centrifugation. As shown in Fig. 3, after centrifugation at 160,000*g* for 2 h, the encapsulated DNA is present as a band localized at the 2.5% sucrose–10% sucrose interface in the step gradient. It is interesting to note that less than 10% of the total lipid (as assayed by the ^{3}H-CHE lipid marker) is associated with the encapsulated plasmid DNA, which in turn corresponds to 55% of the total input DNA. The plasmid-to-lipid ratio of these purified SPLP was determined to be 62.5 μg plasmid per μmol lipid. SPLP generated by detergent dialysis and purified by density gradient centrifugation may be concentrated by either ultrafiltration or dialysis against carboxymethyl cellulose to achieve plasmid concentrations of 1 mg/ml or higher.

Narrow Size Distribution of Stabilized Plasmid-Lipid Particles

The sizes of the empty lipid vesicles in the upper band and the isolated SPLP in the lower band of the sucrose density gradient were examined by quasi-elastic light scattering (QELS) and freeze-fracture electron microscopy techniques. As shown in Fig. 4, the QELS analysis indicated that the mean diameter of the empty vesicles was approximately 44 nm ($\chi^2 = 0.48$), whereas the isolated SPLP were larger, with a mean diameter of 75 nm ($\chi^2 = 0.14$). Freeze-fracture electron microscopy studies gave similar results. A size analysis of the particles in these micrographs indicated a size of 36 $\pm$ 15 nm for the empty vesicles and 64 $\pm$ 9 nm for the isolated SPLP.

Stabilized Plasmid-Lipid Particles: Plasmid Trapped inside Bilayer Lipid Vesicle

As mentioned above, purified SPLP composed of DOPE/DODAC/PEG-CerC_{20} (84 : 6 : 10) were determined to have a plasmid-to-lipid ratio of 62.5 μg plasmid per μmol lipid. When considering a 4.49 kb plasmid, such as pCMVCAT, this

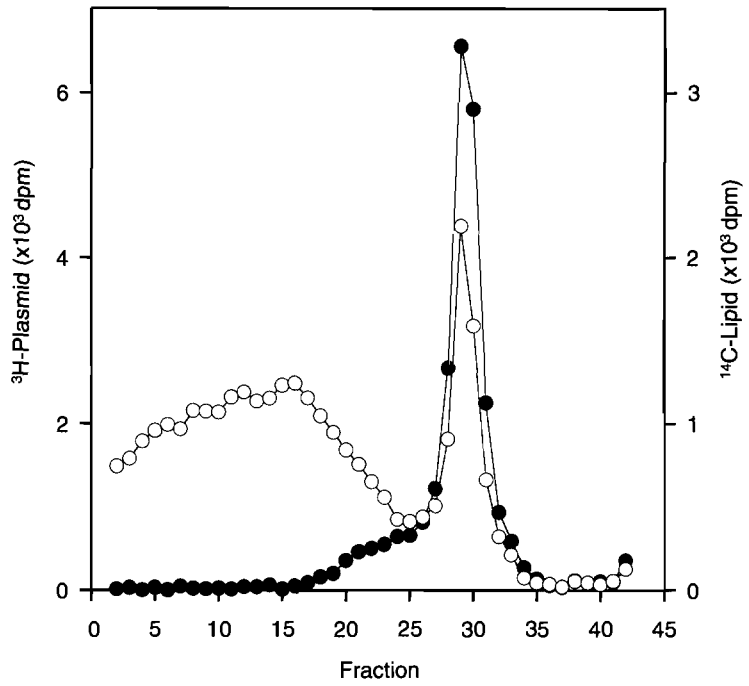


FIG. 3. Separation of SPLP from empty vesicles by discontinuous sucrose density gradient centrifugation. The solid circles indicate the behavior of the ^{3}H-labeled plasmid (pCMVLuc), whereas the open circles indicate the distribution of lipid as reported by the ^{14}C-labeled CHE lipid marker. SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84 : 6 : 10; mol : mol : mol) were prepared as indicated in the legend to Fig. 2, and an aliquot (1.5 ml containing approximately 50 μg of ^{3}H-plasmid DNA) was applied to a discontinuous sucrose density gradient (3 ml 10% sucrose, 3 ml 2.5% sucrose, 3 ml 1% sucrose; all in HBS). The gradient was then centrifuged at 160,000*g* for 2 h. [Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6**, 271 (1999).]

corresponds to a plasmid to particle ratio of 0.97 (for an SPLP with a diameter of 70 nm).[12] Thus each SPLP contains one plasmid molecule.

SPLP structure has been further characterized employing cryoelectron microscopy. Purified SPLP were prepared using the lipids DOPE : DODAC : PEG-CerC$_{20}$ (83 : 7 : 10) and pCMVluc plasmid DNA. Large unilamellar vesicles (LUV) with the same lipid composition were prepared by extrusion of the hydrated lipid mixture through 100 nm pore size filters. As shown in Fig. 5a, cryoelectron micrographs clearly reveal SPLP to consist of a lipid bilayer surrounding an internal structure consistent with entrapped plasmid DNA molecules. The minority of small (diameter ~30 nm), empty vesicles formed during the detergent dialysis

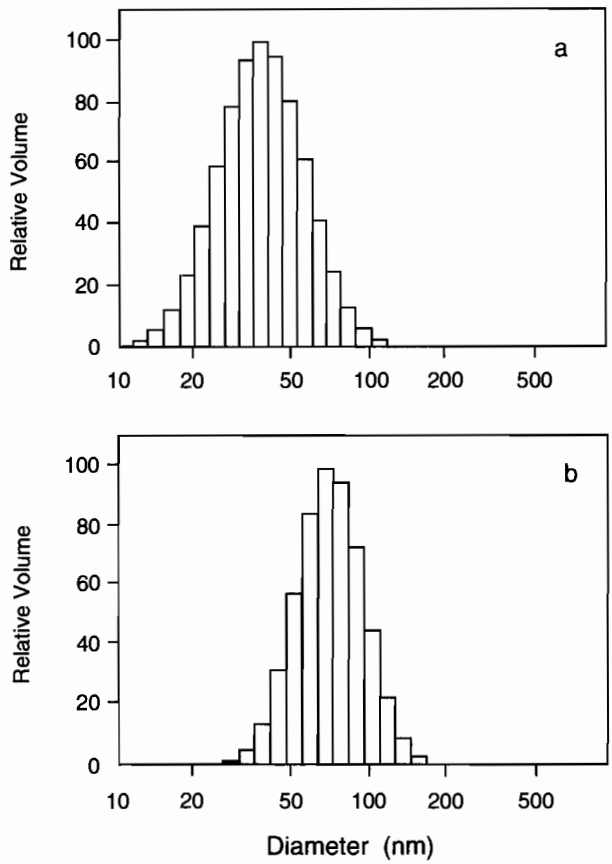


FIG. 4. Size distribution of SPLP and empty vesicles as determined by QELS. SPLP were prepared containing pCMVLuc as indicated in the legend to Fig. 2, and separated from empty vesicles by discontinuous sucrose density gradient centrifugation. (a) Size distribution for empty vesicles (upper band). (b) Size distribution for SPLP (lower band). The sizes were determined by quasi-elastic light scattering using a Nicomp Model 370 Sub-Micron particle sizer operating in the "solid particle" mode. [Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).]

process[23] that were not removed by density centrifugation do not exhibit the electron dense internal structure (see arrows in Fig. 5a). Nor is this internal structure observed in the LUV produced by extrusion (Fig. 5b). It may also be noted that SPLP visualized by cryoelectron microscopy have a remarkably homogeneous size (diameter 72 ± 5 nm), in close agreement with measurements of SPLP employing

[23] L. T. Mimms, G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds, *Biochemistry* **20,** 833 (1981).

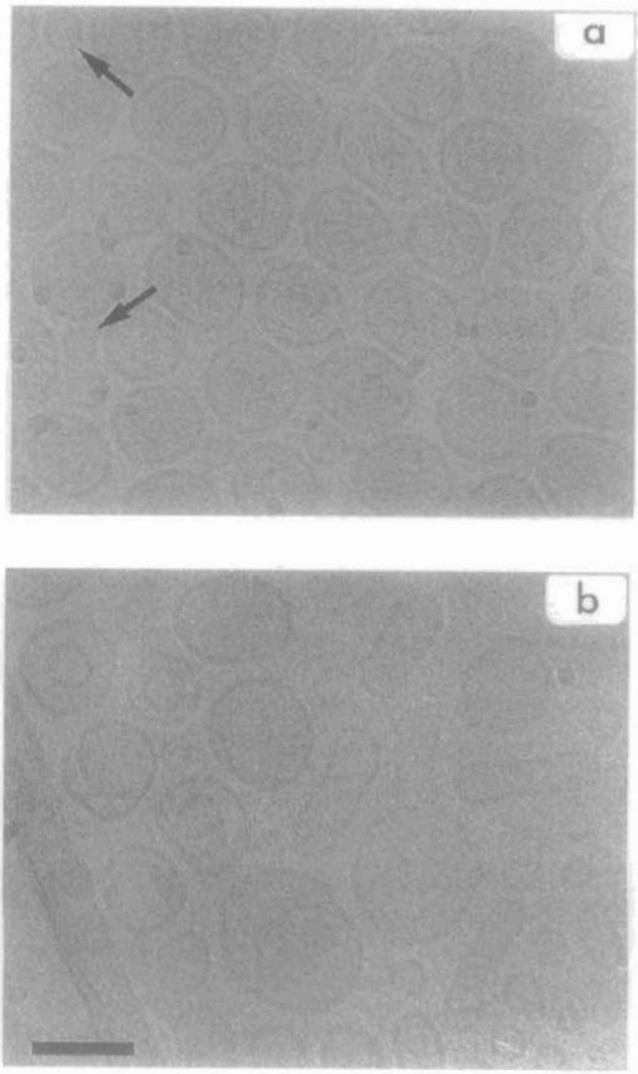


FIG. 5. Cryoelectron micrographs of (a) purified SPLP and (b) LUV prepared by extrusion. SPLP were prepared from DOPE : DODAC : PEG-CerC_{20} (83 : 7 : 10; mol : mol : mol) and pCMVluc and purified employing DEAE column chromatography and density gradient centrifugation. LUV were prepared from DOPE : DODAC : PEG-CerC_{20} (83 : 7 : 10; mol : mol : mol) by hydration and extrusion through filters with 100 nm diameter pore size. The arrows in panel (a) indicate the presence of residual "empty" vesicles formed during the detergent dialysis process that were not removed by the density centrifugation purification step. The bar in panel (b) indicates 100 nm. [Reprinted from P. Tam, M. Monck, D. Lee, O. Ludkovski, E. C. Leng, K. Clow, H. Stark, P. Scherrer, R. W. Graham, and P. R. Cullis, *Gene Ther.* **7,** 1867 (2000).]

freeze-fracture electron microscopy (diameter 64 ± 9 nm).[12] The homogeneous size and morphology of SPLP contrasts with the irregular morphology and large size distribution of the extruded vesicles. The narrow size distribution of SPLP was also reflected by QELS measurements (data not shown) indicating a mean diameter of 83 ± 4 nm. Plasmid DNA–cationic liposome complexes made from DOPE : DODAC (1 : 1; mol : mol) LUV exhibited a large, heterogeneous size distribution as determined by QELS (diameter 220 ± 85 nm, data not shown).

Stabilized Plasmid-Lipid Particle Protection of DNA from DNase and Serum Nucleases

It is important to demonstrate that the encapsulated plasmid in the particles obtained by the detergent dialysis process is, in fact, fully protected from the external environment. As a first measure of protection, the ability of recombinant DNase I to digest plasmid DNA in SPLP can be determined. Figure 6 illustrates the results of an experiment in which SPLP were prepared using DOPE/DODAC/PEG-CerC_{20} (84 : 6 : 10; mol : mol : mol) and pCMVLuc (200 μg/ml). Protection of SPLP plasmid is compared to protection of free plasmid and plasmid in plasmid cationic lipid complexes prepared with DODAC-DOPE (1 : 1) LUVs at a charge ratio of ±3.0. Samples containing 1 μg plasmid were exposed to 0, 100, and 1000 units of DNase I in a total volume of 1.0 ml HBS for 30 min at 37°. After incubation the plasmid was isolated and characterized by agarose gel electrophoresis. Figure 6 shows that

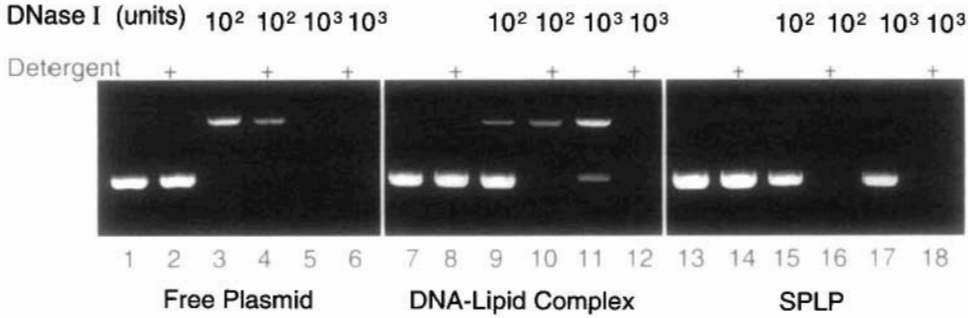


FIG. 6. Stability of free plasmid (lanes 1–6), plasmid encapsulated in SPLP (lanes 13–18), and plasmid in plasmid DNA–cationic lipid complexes (lanes 7–12) in the presence of DNase I. One μg of plasmid DNA was subjected to no treatment (lanes 1, 7, 13), exposure to detergent alone (1% Triton X-100) (lanes 2, 8, 14), exposure to 100 and 1000 units of DNase I alone (lanes 3, 9, 5 with 100 units and lanes 5, 11, 17 with 1000 units), and exposure to both detergent and DNase I (lanes 4, 10, 16 with 100 units and lanes 6, 12, 18 with 1000 units). The plasmid DNA–cationic lipid complexes consisted of DODAC : DOPE (50 : 50; mol : mol) LUVs (100 nm diameter) complexed to plasmid at a 3 : 1 charge ratio (positive-to-negative). [Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang , M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).]

free plasmid is completely digested by incubation with either 100 or 1000 units of DNase I. The plasmid in complexes (lipoplex) formed with cationic LUVs is marginally protected when compared to free DNA exposed to 100 units of DNase I, but is almost entirely digested by incubation with 1000 units. In contrast, plasmid DNA encapsulated in SPLP is completely protected from nuclease digestion unless detergent is added to disrupt the SPLP lipid bilayer prior to incubation with DNase.

A rigorous test of SPLP stability and protection of encapsulated plasmid involves incubation in serum. Serum contains a variety of nucleases, and serum proteins can rapidly associate with lipid systems,[24] resulting in enhanced leakage and rapid clearance of liposomal systems. The ability of serum nucleases to degrade plasmid is illustrated in Fig. 7. Intact pCMVCAT elutes in the void volume of the Sepharose CL-4B column, whereas after incubation with mouse serum (90%) at 37° for 30 min the plasmid is degraded into fragments that elute in the included volume (Fig. 7a). The behavior of the DOPE/DODAC/PEG-CerC_{20} (84 : 6 : 10) SPLP system where nonencapsulated plasmid has not been removed is shown in Fig. 7b. In this particular preparation, 53% of the plasmid DNA elutes with the lipid in the void volume, and 47% of the DNA, which represents degraded plasmid, elutes in the included volume. This indicates that 53% of the plasmid is encapsulated and protected from the external environment, in good agreement with a 55% trapping efficiency of this sample as determined by DEAE ion exchange chromatography.

A final test of the stability of the SPLP formulation is given in Fig. 7c, which illustrates the elution profile of the DOPE/DODAC/PEG-CerC_{20} (84 : 6 : 10; mol : mol : mol) SPLP system following removal of the external plasmid by DEAE chromatography and incubation in 90% mouse serum (30 min at 37°). In this case more than 95% of plasmid applied to the column eluted in the void volume, demonstrating the stability and the plasmid protection properties of the SPLP formulation. It should also be noted that SPLP containing PEG-CerC_{14} in place of PEG-CerC_{20} exhibit similar plasmid protection properties.

Variation of Cationic Lipid Content of Stabilized Plasmid-Lipid Particles over Wide Range

The SPLP systems described above fulfill many requirements of a systemic gene delivery system, but when used to transfect mammalian cells *in vitro* were found to have low transfection potency when compared to plasmid DNA–cationic lipid complexes. Initial attempts to increase the transfection potency of SPLP involved developing methods for increasing the DODAC content of the particles. Utilizing a formulation consisting of DOPE/DODAC/PEG-CerC_8, Zhang *et al.*[13]

[24] A. Chonn, S. C. Semple, and P. R. Cullis, *J. Biol. Chem.* **267,** 18759 (1992).

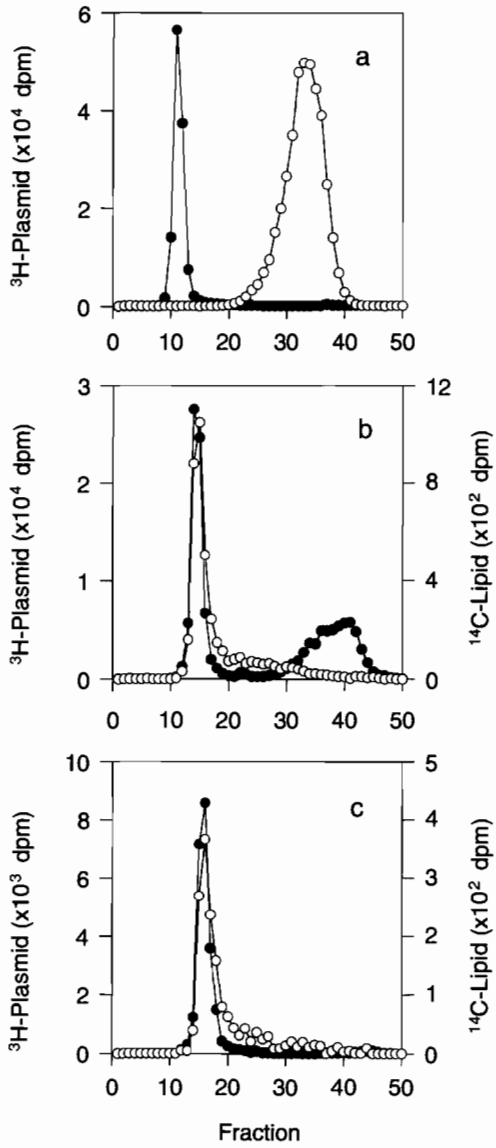


FIG. 7. Plasmid in SPLP is protected from serum nuclease cleavage. The stability of plasmid (pCMVCAT) in the free form or encapsulated in SPLP was determined in the presence of serum. The SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84 : 6 : 10; mol : mol : mol) were prepared as indicated in the legend to Fig. 2 and contained ^{14}C-labeled CHE as a lipid marker. Samples with 5 μg of ^{3}H-labeled plasmid DNA were incubated in the presence of HBS or 90% mouse serum for 30 min at 37° and eluted on a Sepharose CL-4B column equilibrated in HBS. (a) Elution profile of nucleic acid resulting from incubation of free plasmid in HBS (●) or 90% mouse serum (○). (b) Elution profile of nucleic

found that DODAC concentrations ranging from 7 to 30 mol% could be achieved by detergent dialysis against HEPES-buffered saline if appropriate concentrations of citrate were included in the dialysis medium. The encapsulation of DNA via cationic lipid requires ionic interactions between the positively charged lipid and the negatively charged DNA (Fig. 1). SPLP appear to form only when lipid structures possessing an appropriate surface charge are available for interactions with DNA.[12] If these interactions are too strong, as occurs at low ionic strength, encapsulation efficiency is high but is accompanied by the formation of large aggregates. If the ionic strength is too high, the DNA does not associate with the lipid, leading to the formation of small, empty vesicles and low encapsulation efficiencies. Therefore, it was reasoned that formation of SPLP with higher cationic lipid content might be possible if the ionic strength of the dialysis medium was raised to shield the higher net surface charge on the intermediate lipid structures. It was found that aggregation in samples containing higher DODAC concentrations could not be prevented simply by increasing the NaCl concentration. An effective solution to this problem was to use polyvalent anionic counterions such as citrate to produce stronger shielding effects. A study of plasmid encapsulation as a function of citrate concentration was performed for a lipid mixture composed of DODAC/DOPE/PEG-CerC$_8$ (20 : 65 : 15) and the pCMVLuc plasmid. At concentrations up to 60 m*M* citrate the dialyzed samples contained large (diameter > 150 nm) and polydisperse ($\chi^2 > 3.0$) particles. However, small, monodisperse particles (82 ± 40 nm) exhibiting high encapsulation efficiencies of 50–70% were formed when the dialysis medium contained 65–80 m*M* citrate. Increasing the citrate concentration further also resulted in formation of small particles, but the encapsulation efficiency decreased dramatically.

These results suggest two criteria for determining the optimum ionic strength for plasmid encapsulation: (1) formation of monodisperse ($\chi^2 < 3.0$) particles with diameter smaller than 100 nm and (2) an encapsulation efficiency greater than 50%. Studies to determine citrate concentrations that satisfy these criteria over a range of DODAC concentrations were performed, and the results are summarized in Fig. 8. The range of citrate concentrations giving rise to particles with diameter smaller than 100 nm and with encapsulation efficiencies of 50% or higher is represented by the solid circles. Higher citrate concentrations give rise to low plasmid encapsulation efficiencies of 30% or less, whereas citrate concentrations below the optimum levels resulted in large, polydisperse aggregates ($\chi^2 > 5$).

acid (●) and lipid (○) following incubation of SPLP in 90% mouse serum. (c) Elution profile of nucleic acid (●) and lipid (○) following incubation of SPLP with mouse serum where unencapsulated plasmid was removed by anion exchange chromatography prior to the serum treatment. [Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang, M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6**, 271 (1999).]

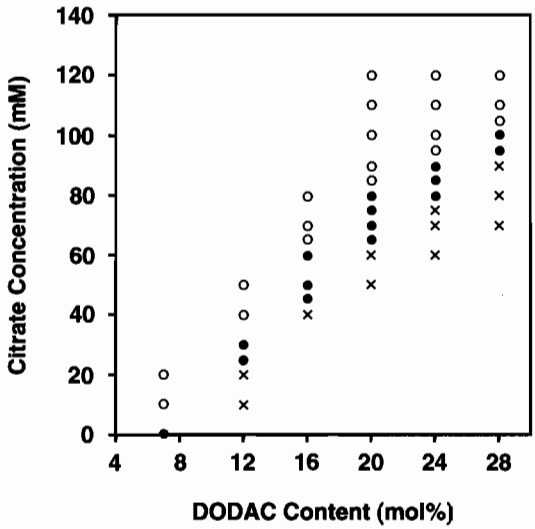


FIG. 8. Determination of the optimal citrate concentration range as a function of SPLP DODAC content to achieve maximum plasmid encapsulation in combination with minimum aggregation. Formulations were composed of DODAC/DOPE/PEG-CerC$_8$ (x : 85-x : 15; mol : mol : mol) and pCMVLuc (10 mg lipid and 100–200 μg plasmid per ml) and were prepared by detergent dialysis where the dialyzate contained the indicated sodium citrate concentrations as well as 150 m*M* NaCl, 10 m*M* HEPES (pH 7.2). The solid circles (●) indicate formulations that exhibited plasmid encapsulation efficiencies greater than 50% and a small, monodisperse size distribution as determined by QELS (diameter <100 nm, $\chi^2 < 3$). The open circles (○) indicate formulations that exhibited plasmid encapsulation efficiencies of less than 40% in combination with a small, monodisperse size distribution (diameter <100 nm, $\chi^2 < 3$). The crosses (×) indicate polydisperse formulations with large size distributions (diameter >100 nm, $\chi^2 > 3$). [Reprinted from Y. P. Zhang, L. Sekirov, E. G. Saravolac, J. J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P. R. Cullis, and P. Scherrer, *Gene Ther.* **6,** 1438 (1999).]

It was not possible to obtain satisfactory formulations for preparations containing 30 mol% DODAC simply by varying the citrate concentration. Aggregation persisted in formulations dialyzed in 70–90 m*M* citrate buffer, whereas at higher concentrations the plasmid encapsulation was less than 25%. Improved results were achieved, however, by leaving the citrate concentration constant at 100 m*M* and decreasing the NaCl concentration from 150 m*M* to 120–140 m*M*. This resulted in an increase in the plasmid encapsulation efficiency to 55–70% while the particle size remained small (<100 nm diameter).

Other multivalent salts can be used in the formulation of SPLP with high DODAC content. Utilizing a formulation consisting of DOPE/DODAC/PEG-CerC$_8$, Saravolac *et al.*[14] found that DODAC concentrations ranging from 7 to 42.5 mol% could be achieved by detergent dialysis against a sodium phosphate buffer.

Both of these latter examples highlight an important point in formulation of SPLP, especially when higher quantities of cationic lipid are required. Small variations in the cationic lipid to DNA charge ratio result in changes in the buffer concentrations required for optimal size and encapsulation. In practice, it is usually necessary to perform a citrate or salt titration in order to determine optimal conditions for SPLP formation. This is often necessary even for lower DODAC concentrations, where minor variations in lipid stock solution concentrations can lead to changes in final DODAC levels, and thus to buffer or salt concentrations required for optimal results. Recall that at a salt concentration of 150 m*M*, efficient encapsulation was only observed over a range of 6–7% DODAC. There are two general methods available for determining the appropriate conditions for preparing SPLP by detergent dialysis: one can select a buffer system and dialyze a range of lipid formulations that differ in cationic lipid content, or one can fix a formulation's lipid composition and perform citrate and/or salt titrations as necessary.

A second point to note in this context involves the challenges that can be encountered in the production of larger preparations of SPLP, such as would be required for a series of *in vivo* experiments. For example, when performing *in vitro* transfection experiments, one would typically begin with 10 m*M* lipid and 400 μg plasmid DNA in a 1 ml volume (solubilized at an OGP concentration of 200 m*M*). However, *in vivo* experiments require scale-up to starting quantities of 50–250 mg plasmid DNA in a volume of 125–625 ml. In order to confirm the optimal buffer concentrations for detergent dialysis, one would perform a series of test dialyses using 1 ml aliquots of the lipid-detergent mixture, and assay for size and encapsulation efficiency following overnight dialysis. Even so, it is possible that aggregation, or low encapsulation, may occur following dialysis of a large-scale preparation. If this occurs, one can resolubilize the plasmid-lipid mixture with OGP, adjust the buffer solute concentrations, and redialyze. The criteria for determining successful formulation is an average SPLP diameter less than 100 nm with a low χ^2 (<3).

All of the examples discussed above involve SPLP formed with the cationic lipid DODAC. It should be noted that SPLP have been formed from a number of other cationic lipids as well, including DOTMA, DODMA-AN, DSDAC, and DC-Chol,[25] giving rise to particles that exhibit a range of transfection potencies.

Formation of Stabilized Plasmid-Lipid Particles from Various Lipids

An effective systemic gene delivery system must possess traits that at first appear contradictory. Perhaps the clearest example of this is the need for particles that are stable while in the blood compartment yet are able to release their

[25] K. W. Mok, A. M. Lam, and P. R. Cullis, *Biochim. Biophys. Acta* **1419,** 137 (1999).

plasmid payload once they have accumulated at disease sites or are taken up by cells. This can potentially be accomplished through the use of systems capable of programmable fusion. Programmable fusion can be achieved by preparing SPLP with PEG-Cer molecules that exchange out of the SPLP at an optimized rate. The original SPLP particle is protected from serum nucleases and the proteins that lead to vesicle clearance by the PEG-Cer coating. Once sufficient PEG-Cer is lost from the particle, the remaining lipid bilayer becomes unstable and becomes more able to fuse with other lipid bilayers, enhancing the intracellular delivery of the plasmid to cells. To date we have focused on SPLP formed using PEG-CerC_{20}, PEG-CerC_{14}, or PEG-CerC_8. (The concentration of the last lipid must be adjusted to 15 mol% if stable particles are to be formed.) Particles made with PEG-CerC_{20} have long circulation lifetimes due to the slow rate of release of the PEG-lipid from SPLP, whereas those made with the shorter chain PEGs have circulation lifetimes and lipid exchange rates that are greatly reduced. Both *in vitro*[12] and *in vivo*[13,14] studies have demonstrated that transfection rates are increased for SPLP containing shorter-chain PEG-ceramides.

In addition to forming SPLP with different PEG-Cer molecules, we have made particles containing different phosphatidylcholines (POPC and DOPC), and DOPE-SPLP containing various concentrations of cholesterol. It is worth noting that the formation of SPLP from these or other different lipids may occur over a wide range of ionic strength conditions in the dialysis buffer. For example, SPLP composed of DOPC/DODAC/PEGCerC_{20}/Rho-PE (82.5 : 7.5 : 10 : 1) would only form at very low salt conditions ([NaCl] = 80–85 m*M*). In similar manner, SPLP composed of DOPE/DODAC/cholesterol/PEG-CerC_{20}/rhodamine-PE (30.5 : 14 : 45 : 10 : 0.5) were formed following dialysis against 20 m*M* HEPES, 30 m*M* citrate, 130 m*M* NaCl, pH 7.2. Both the citrate and NaCl concentrations were lower than obtained for the analogous system without cholesterol (DOPE/DODAC/PEG-CerC_{20}/rhodamine-PE (75.5 : 14 : 10 : 0.5), for which the optimal dialysis buffer was 20 m*M* HEPES, 40 m*M* citrate, 150 m*M* NaCl, pH 7.2. Thus, even the addition of a neutral molecule such as cholesterol may alter the optimized buffer solute concentrations for SPLP formation.

Post-Insertion of Cationic PEG Lipids into Stabilized Plasmid-Lipid Particles

A key finding of *in vitro* transfection studies involving cationic lipid-plasmid DNA complexes and SPLP relates to the significantly lower transfection levels achieved with the latter.[25] This stems from the lower levels of lipid and DNA delivered to cells by these particles, and highlights the need for increased transfection potency. Although some of the modifications listed above have significant effects on transfection potency, they may be insufficient.

We have described a new class of cationic PEG lipids (CPL) that were designed to enhance interactions of liposomes with cells by increasing nonspecific ionic

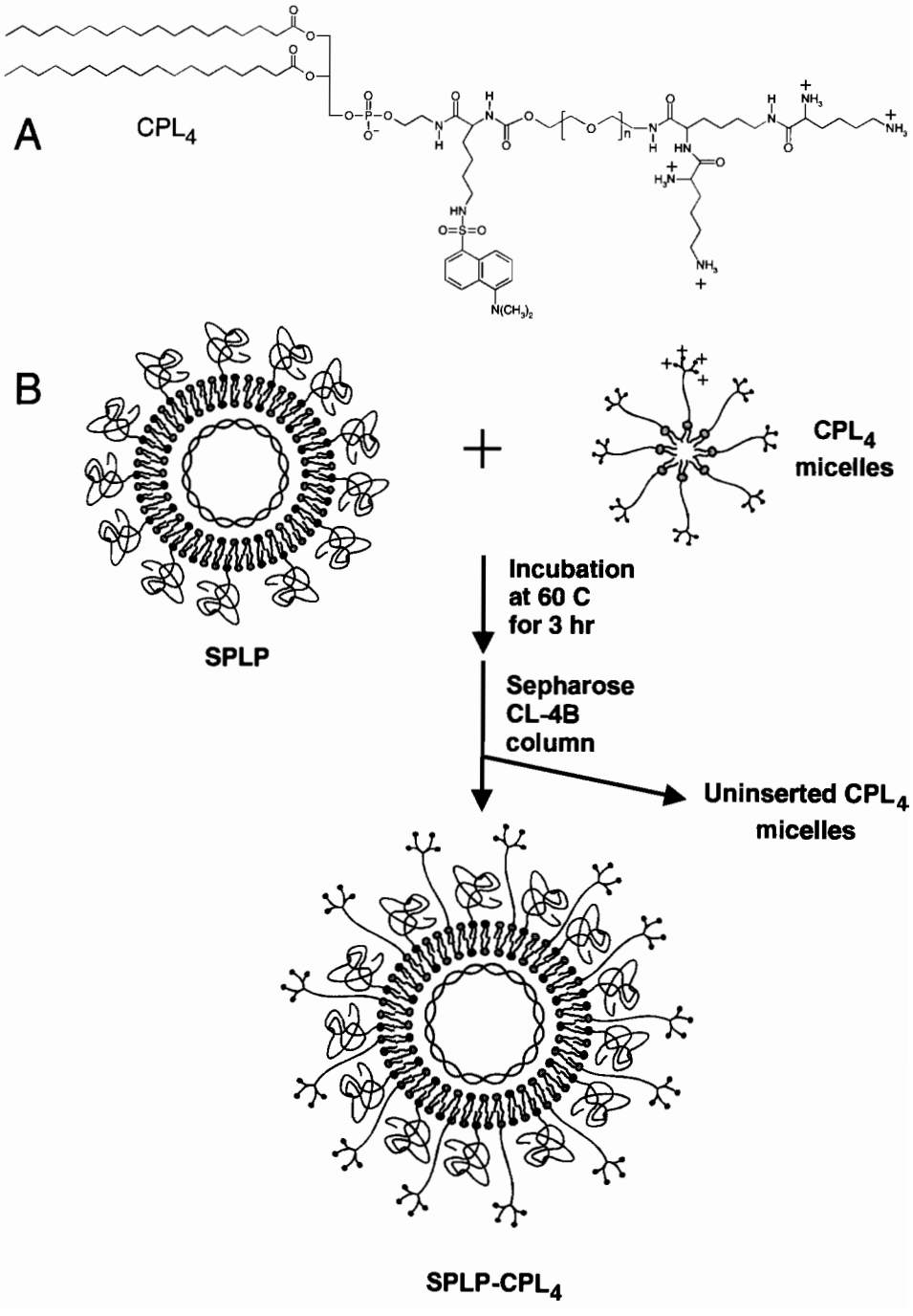
interactions.[15] These lipids consist of a hydrophobic distearoylphosphatidylethanolamine (DSPE) anchor coupled to a highly fluorescent N_ε-dansyl lysine moiety, which is in turn attached to a hydrophilic poly(ethylene glycol) (PEG) spacer linked to a cationic headgroup made of lysine residues. The most effective CPL, designated CPL_4 (Fig. 9A), has three lysine residues in the headgroup, giving a charge of +4 at the chain terminus. We have shown that these CPL_4 can be incorporated into pre-formed vesicles and into SPLP using a post-insertion technique (Figure 9B) leading to greatly enhanced uptake and gene expression in mammalian cells (L. Palmer, manuscript submitted).

Briefly, an aliquot of CPL_4 (in methanol), corresponding to approximately 5 mol% of the SPLP lipid, is incubated with SPLP (in HEPES-buffered saline) at 60° for 2–3 h. This results in the incorporation of about 80% of the CPL_4 into the SPLP, corresponding to a concentration of 4 mol% (relative to total lipid). Nonincorporated CPL is removed by gel filtration chromatography on a column of Sepharose CL-4B equilibrated in HBS. The SPLP-CPL_4 elute from the column near the void volume and can be easily identified by fluorescence of both the dansyl (CPL) and rhodamine (lipid) markers. Unincorporated CPL is retained by the column matrix and elutes later, effectively separated from SPLP.

When eluted in HBS, SPLP-CPL_4 form large aggregate structures, readily visualized by fluorescence microscopy using a rhodamine filter. Aggregation of SPLP-CPL_4 can be prevented by the addition of Ca^{2+}, which can be present during the entire insertion and isolation procedure, or can be added to the SPLP-CPL following removal of excess CPL. In the presence of 40 m*M* Ca^{2+}, the size of SPLP was determined by QELS prior to and following insertion of CPL_4, giving values of 80 ± 19 nm and 76 ± 15 nm, respectively. The values determined from freeze-fracture electron microscopy were 68 ± 11 nm and 64 ± 14 nm, respectively.

In Vitro Transfection Properties of Stabilized Plasmid-Lipid Particles

In previous sections we have established that SPLP encapsulate DNA in small uniform particles that confer significant protection from DNase. These properties, essential for a systemic delivery system, may actually hinder cell-surface binding, uptake, and concomitant intracellular delivery of plasmid. Several studies were undertaken to assess the transfection potency of SPLP. Initially, SPLP consisting of DOPE/DODAC/PEG-$CerC_{20}$ (84 : 6 : 10) and the plasmid pCMVLuc coding for the luciferase reporter gene were prepared. As shown in Fig. 10, incubation of these SPLP with COS-7 cells for 24 h resulted in little if any transfection activity. This was attributed to the presence of the PEG coating on the SPLP, which is expected to inhibit the association and fusion of the SPLP with cells in the same manner that PEG coatings inhibited fusion between lipid vesicles.[20] In this regard, previous studies on LUVs with PEG coatings attached to phosphatidylethanolamine (PE) anchors had demonstrated that, for PE anchors containing short acyl chains, the


A
CPL4
B
SPLP
CPL4 micelles
Incubation at 60 C for 3 hr
Sepharose CL-4B column
Uninserted CPL4 micelles
SPLP-CPL4

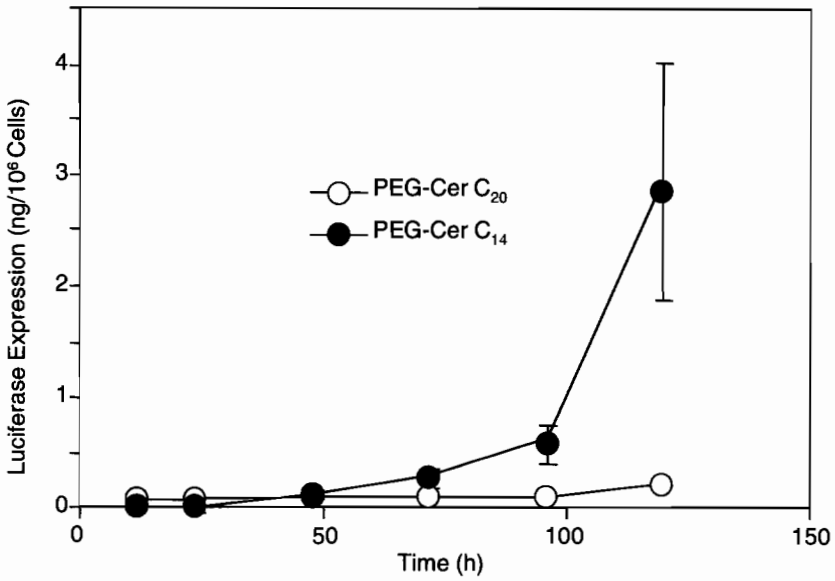


FIG. 10. Effect of PEG-Cer coating of SPLP on transfection activity *in vitro*. Plasmid (pCMVLuc) was encapsulated in SPLP (DOPE/DODAC/PEG-Cer; 84:6:10; mol/mol/mol) containing PEG-CerC$_{20}$ (○) or PEG-CerC$_{14}$ (●). Nonencapsulated plasmid was removed by anion exchange chromatography. The SPLP preparation (1 μg plasmid) was then added to COS-7 cells at a density of 2×10^4 per 24-well plate. The cells were incubated with the SPLP for the times indicated, following which the luciferase activity was measured. [Reprinted from J. J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. W. Graham, Y. P. Zhang , M. J. Hope, P. Scherrer, and P. R. Cullis, *Gene Ther.* **6,** 271 (1999).]

PEG-PE could rapidly exchange out of the LUV, rendering the LUVs increasingly able to interact and fuse with each other. To test this hypothesis we compared the transfection properties of SPLP containing PEG-CerC$_{20}$ or PEG-CerC$_{14}$. As shown in Fig. 10, after incubation with COS-7 cells for 24 h, the SPLP containing PEG-CerC$_{14}$ exhibited substantially higher levels of transfection compared with the system containing PEG-CerC$_{20}$. As indicated earlier, subsequent studies have revealed that SPLP containing PEG-CerC$_8$ possess even higher transfection

FIG. 9. Insertion protocol for the production of SPLP-CPL$_4$. (A) Structure of dansylated CPL$_4$. CPL$_4$ possesses four positive charges at the end of a PEG$_{3400}$ molecule attached to a lipid achor, DSPE. (B) Protocol for insertion of CPL$_4$ into preformed SPLP. The SPLP are composed of DOPE (light headgroups), DODAC (black headgroups), and PEG-CerC$_{20}$ (lipids with attached polymer). SPLP and CPL$_4$ are incubated together at 60° for 3 h, during which time CPL$_4$ monomers transfer from micelles and insert into the external monolayer of SPLP. Following insertion, unincorporated CPL$_4$ is removed using Sepharose CL-4B column chromatography. See text for further details.

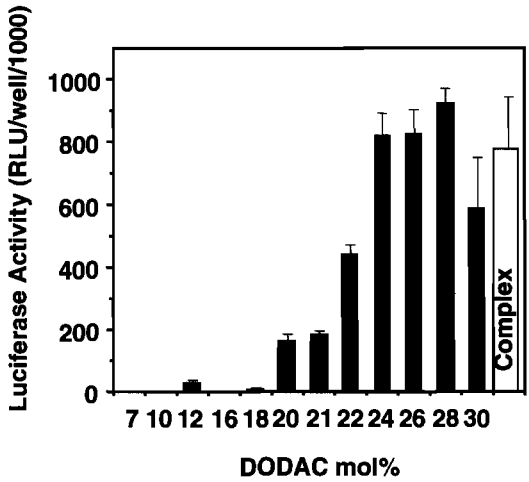


FIG. 11. Effect of DODAC content in SPLP on transfection activity *in vitro*. Plasmid (pCMVLuc) was encapsulated in SPLP containing 7-30 mol% DODAC, as described in the caption for Fig. 8. SPLP isolation by density gradient centrifugation was conducted as described in the caption for Fig. 3. SPLP were added to COS-7 cells (1 μg plasmid per well) and then incubated for 24 h, following which the luciferase activity was determined. The "complex" bar illustrates the transfection activity achieved with complexes of pCMVLuc and DODAC/DOPE (1 : 1; mol : mol) LUV at a charge ratio of 1.5 : 1 (+/−). [Reprinted from Y. P. Zhang, L. Sekirov, E. G. Saravolac, J. J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P. R. Cullis, and P. Scherrer, *Gene Ther.* **6,** 1438 (1999).]

potency. This was consistent with the ability of the shorter chain PEG-Cers to exchange off the SPLP surface at a greater rate than the PEG-CerC_{20}.

The effect of cationic lipid content in the SPLP on *in vitro* transfection was investigated in COS-7 and HepG2 cell lines.[13] The luciferase activities detected in COS-7 cells following transfection are shown in Fig. 11, where it is clear that the transfection activity was strongly dependent on the DODAC content in the SPLP. Luciferase activity was low for SPLP containing 7–8 mol% DODAC but increased substantially to reach a plateau value between 24 and 28 mol% DODAC. Importantly, the luciferase activities detected for SPLP containing high DODAC levels were comparable with those obtained for the plasmid-lipid complexes formed with pCMVLuc and DODAC/DOPE (1 : 1; mol : mol) liposomes.

The most dramatic enhancement in *in vitro* transfection has been observed for SPLP containing 4–5 mol% of CPL_4 in the presence of 8 m*M* Ca^{2+} (L. Palmer, manuscript submitted). Previously, we have shown that the incorporation of CPL_4 into LUVs can lead to a 50-fold increase in cell binding and uptake.[15] We have found that the presence of Ca^{2+} alone can increase tranfection potency of SPLP several hundredfold (A. Lam, manuscript submitted[26]). This suggests a possible

[26] A. M. Lam, Ph.D. Thesis (2000).

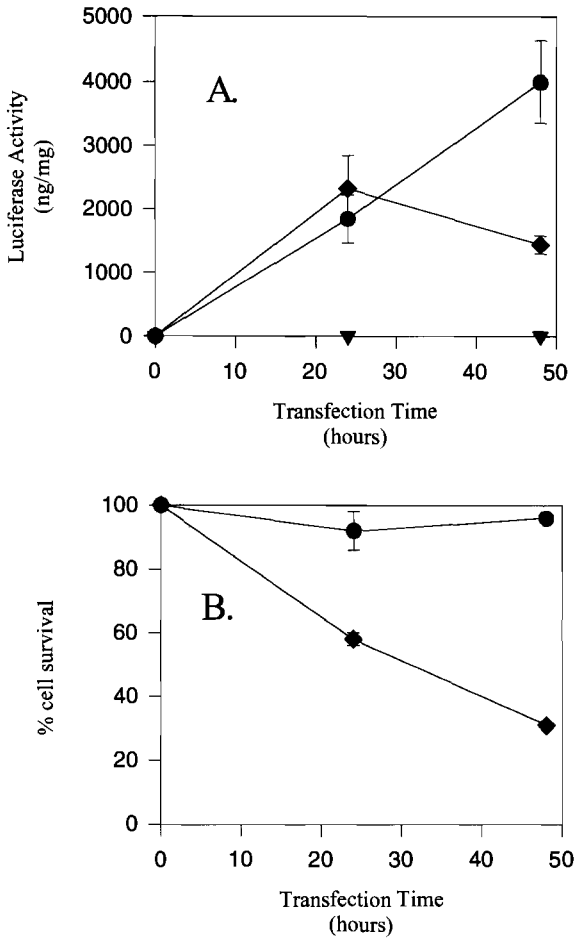


FIG. 12. (A) The transfection potency of SPLP-CPL_4 (●) containing 4 mol% CPL_4 and and lipofectin lipoplexes (◆) following extended transfection times with BHK cells. BHK cells were transfected in DMEM containing 10% FBS for 24 and 48 h with SPLP-CPL_4 and lipofectin lipoplexes (charge ratio of 1.5 : 1) containing 5.0 μg/ml pCMVLuc. Following transfection the luciferase expression levels and cell protein levels were determined in the cell lysate. The luciferase activity was normalized for protein content in the lysate and plotted as a function of transfection time. (B) The toxicity of SPLP-CPL_4 (●) containing 4 mol% CPL_4 and lipofectin lipoplexes (◆) as a function of transfection time, as assayed by cell survival based on the protein concentration in the cell lysate.

synergistic effect of Ca^{2+} and CPL_4, especially in light of the ability of Ca^{2+} to prevent aggregation of CPL_4-LUVs. Therefore, 4 mol% of CPL_4 was postinserted into SPLP composed of DOPE/DODAC/$PEGCerC_{20}$ (84/6/10), and transfection studies were performed on BHK cells in the presence of Ca^{2+}. As shown in Fig. 12A, a dramatic increase in transfection was observed for CPL_4-SPLP as compared with

SPLP alone, with a 10^6 enhancement observed following a 48 h incubation. While the transfection levels of CPL_4-SPLP were roughly equal to lipofectin lipoplexes at 24 h, by 48 h they were double that of lipoplex. Over this time period, the cytotoxic effects of lipoplex became apparent in lipoplex transfected cells. Such effects were not observed for the CPL_4-SPLPs.

It is likely that the increased positive surface charge of SPLP-CPL_4 will result in rapid serum clearance upon intravenous administration. However, this approach demonstrates the potential utility of postinsertion of a targeting ligand into SPLP. The pharmacology of native SPLP is discussed in the next section.

Circulation Lifetime of Stabilized Plasmid-Lipid Particles

The properties of small size, serum stability, and low levels of cationic lipid and the presence of the PEG coating suggest that SPLP should exhibit extended circulation lifetimes and disease site targeting properties following intravenous administration. A direct test of the pharmacokinetic properties of SPLP particles can be made by preparing SPLP containing trace amounts of ^{3}H-cholesteryl hexadecylether (^{3}H-CHE), a nonexchangeable lipid marker routinely used to label liposomes or vesicle preparations. The serum clearance of intravenously administered SPLP can then be determined by collecting blood at various time points and subjecting it to analysis for ^{3}H-CHE lipid by liquid scintillation analysis. This experiment reveals that PEG-$CerC_{20}$ containing SPLP are cleared from serum gradually with a measured serum half-life of 8.0 ± 1.1 h (Fig. 13). In contrast to the behavior of the SPLP system, ^{3}H-CHE labeled plasmid DNA-lipoplexes are rapidly cleared from the circulation ($t_{1/2} \ll 15$ min), appearing predominantly in the lung and liver (data not shown). The serum half-life of unprotected plasmid DNA is known to be less than 5 min.[27]

The levels of intact plasmid DNA in the circulation following intravenous administration of SPLP can also be determined directly by Southern blot hybridization and phosphorimaging analysis.[7] Quantitative analysis confirms that when naked plasmid is administered systemically, less than 0.01% of the injected dose remains intact in the circulation after 15 min. Only a small fraction of plasmid (<2%) is still intact in the circulation at 15 min following the administration of lipoplexes. In contrast, following intravenous injection of SPLP, approximately 85% of the injected plasmid DNA remains intact in the circulation at 15 min. The circulation half-life of intact plasmid DNA following injection of SPLP as measured by this method is calculated to be 7.2 ± 1.6 h, in good agreement with the circulation half-life of ^{3}H-CHE labeled SPLP, confirming the highly stable nature of SPLP in the circulation and validating the use of the ^{3}H-CHE lipid label as a surrogate marker for SPLP plasmid DNA in pharmacokinetic studies.

[27] A. R. Thierry, P. Rabinovich, B. Peng, L. C. Mahan, J. L. Bryant, and R. C. Gallo, *Gene Ther.* **4,** 226 (1997).

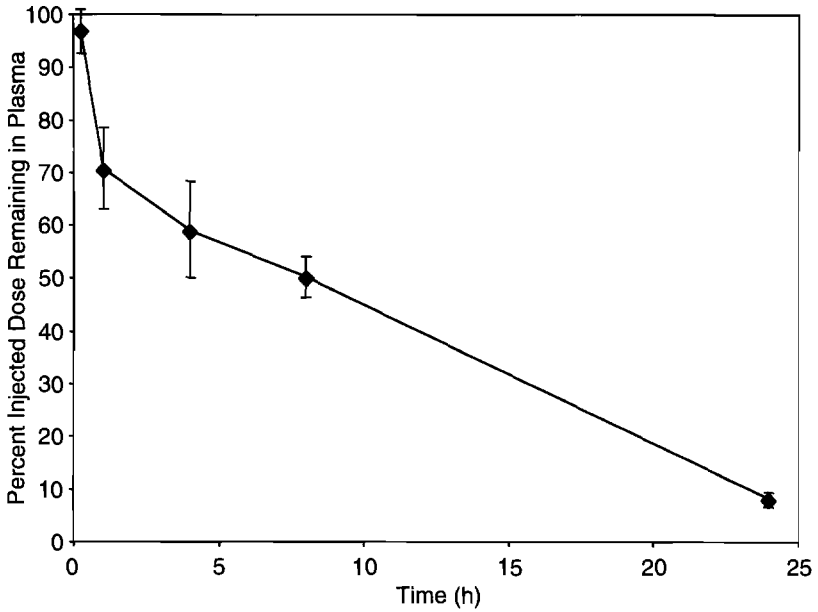


FIG. 13. SPLP serum clearance following a single intravenous administration in neuro-2a tumor-bearing A/J mice. On day 0, 1.5×10^6 cells were injected subcutaneously in the hind flank of each mouse (injection volume: 50 μl). When tumors were an appropriate size (about day 9), [^{3}H]CHE-SPLP (100 ug DNA) was administered i.v. in a total volume of 200 μl. The specific activity of the [^{3}H]CHE was 1 μCi/mg lipid. Each time point reflects the average results from 4 mice. [Reproduced from D. B. Fenske, I. MacLachlan, and P. R. Cullis, *Curr. Opin. Mol. Ther.* **3**, 153 (2001), with permission of PharmaPress Ltd.]

SPLP pharmacokinetics vary dramatically depending on the species of PEG-Cer employed for stabilization. Whereas SPLP containing PEG-CerC_{20} remain in the circulation for hours after injection, SPLP-CerC_{14} and SPLP-CerC_8 exhibit circulation half-lives of approximately 30 min and <5 min, respectively.[7] The pharmacokinetic behavior of SPLP containing different PEG-ceramides can be readily understood on the basis of the ability of these molecules to dissociate from the SPLP at different rates. In particular, it would be expected that SPLP-CerC_8 and SPLP-CerC_{14} would rapidly shed their PEG coating in the blood compartment, whereas the SPLP-CerC_{20} system should exhibit much longer PEG-Cer retention times. PEG-CerC_8 and PEG-CerC_{14} exhibit half-times for dissociation from liposomes of <1.2 min and ~1.1 h, respectively, under *in vitro* conditions, whereas PEG-CerC_{20} exhibits a dissociation half-time greater than 13 days.[12] The absence of a PEG coating would be expected to facilitate serum protein adsorption to the SPLP surface, leading to enhanced uptake by the mononuclear phagocytes of the reticuloendothelial system (RES). Thus the pharmacokinetic behavior of SPLP-CerC_8, SPLP-CerC_{14}, and SPLP-CerC_{20} is consistent with the ability of the

PEG coating to dissociate at rates dependent on the acyl chain component of the ceramide anchor. It should be noted that the rates of removal of the PEG coating *in vivo* are likely to be faster than under *in vitro* conditions. Thus a quantitative correlation between PEG dissociation rates *in vitro* and SPLP clearance rates would not be expected.

Tumor Accumulation of Stabilized Plasmid-Lipid Particles

^{3}H-CHE labeled SPLP may also be used to determine the biodistribution resulting from intravenous administration of SPLP. The biodistribution of SPLP can be determined by collecting tissue at various time points after administration and subjecting it to analysis for ^{3}H-CHE lipid. This approach reveals that SPLP containing PEG-CerC_{20} behave in a manner analogous to small, long circulating liposomes containing small molecule drugs such as doxorubicin. Whereas approximately 3–12% of the injected SPLP dose accumulates for every gram of tumor at the tumor site over 24 h, liposomes containing doxorubicin accumulate to the extent of 5–10% of the injected dose per gram of tumor in mouse models.[28]

The stability and long circulation lifetimes of the SPLP-CerC_{20} system would be expected to lead to maximum plasmid delivery to a distal tumor site when compared to other SPLP systems. The extent of DNA delivery to tumor tissue does vary greatly with the PEG-Cer in SPLP. Not surprisingly, relatively low levels of intact plasmid are delivered by SPLP-CerC_8 and SPLP-CerC_{14}. Preferential accumulation at the tumor site of the SPLP-CerC_{20} is consistent with the well-characterized behavior of liposomes that avoid immediate uptake by the RES and preferentially accumulate at disease sites such as tumors because of the permeable nature of the tumor vasculature. The benefits of this disease site targeting are clear in that the SPLP-CerC_{20} formulation delivers 43-fold and 1200-fold more plasmid to the tumor site than the more rapidly cleared SPLP-CerC_{14} and SPLP-CerC_8 formulations, respectively. The amounts of intact plasmid delivered to the tumor site by the SPLP-CerC_{20} system is substantial, corresponding to greater than 10% of the total injected dose per gram of tumor at the 24 h time point (Fig. 14).

The differential ability of the PEG-ceramides to dissociate from SPLP has also been shown to modulate the biodistribution of SPLP in other organs. The clearance of SPLP from the circulation and accumulation in the tumor is mirrored by accumulation in the liver. Whereas only 14% of the injected SPLP-CerC_{20} dose accumulates in the liver 3 h after SPLP-CerC_{20} administration, 85% and nearly 100% of SPLP-CerC_8 and SPLP-CerC_{14}, respectively, accumulate in the liver within 3 h of injection.[7] It is well known that rapid clearance of liposomes from the circulation is mediated by RES uptake, primarily the Kuppfer cells of the liver. Thus the accumulation of SPLP-CerC_8 and SPLP-CerC_{14} in the liver can be

[28] L. D. Mayer, M. B. Bally, P. R. Cullis, S. L. Wilson, and J. T. Emerman, *Cancer Lett.* **53,** 183 (1990).

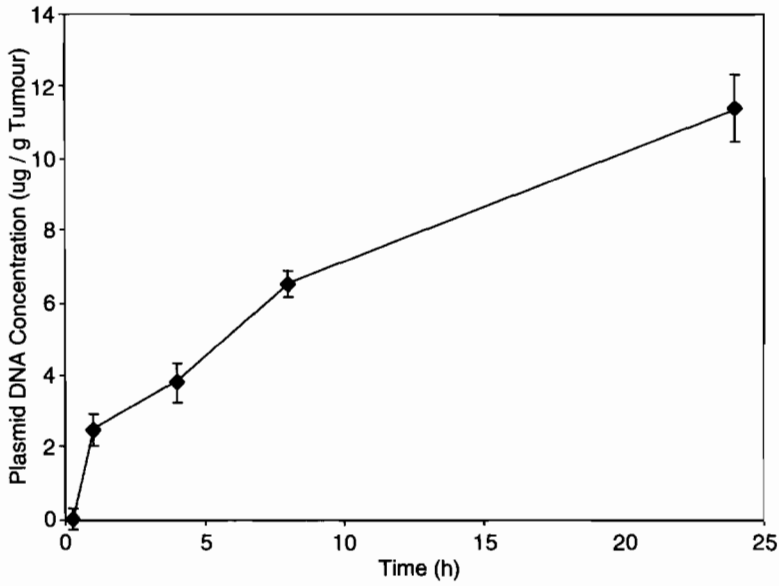


FIG. 14. SPLP accumulation in subcutaneous neuro-2a tumors following a single intravenous administration in A/J mice. For experimental details, see legend to Fig. 13. [Reproduced from D. B. Fenske, I. MacLachlan, and P. R. Cullis, *Curr. Opin. Mol. Ther.* **3,** 153 (2001), with permission of PharmaPress Ltd.]

attributed to dissociation of the PEG coating, opsonization by serum proteins, and uptake by the liver phagocytes.

A noteworthy feature of SPLP-CerC_{20} is the ability to bypass the lung, unique among cationic lipid containing gene delivery systems. This is likely due to the properties required for disease site targeting, namely small uniform size and low surface charge. A number of studies have characterized the transfection properties of plasmid DNA–cationic liposome complexes following intravenous administration.[2,29] High levels of transgene expression are usually observed in the lungs, with lower levels of expression in the spleen, liver, heart, and kidneys. Lipoplex-mediated gene expression in the lung appears to arise from deposition in lung microvasculature and reflects the rapid clearance of plasmid DNA–cationic lipid complexes from the circulation due to their large size (>200 nm diameter) and high cationic lipid content. This is consistent with the observation that murine B16 tumors seeded in the pulmonary vascular compartment can be transfected by intravenous administration of lipoplex.[30] The ability of SPLP to bypass the

[29] N. Zhu, D. Liggitt, Y. Liu, and R. Debs, *Science* **261,** 209 (1993).
[30] X. Zhou and L. Huang, *J. Contr. Release* **19,** 269 (1992).

lung may be considered a predictor for the transfection of distal tumors following systemic administration.

Gene Expression Following Intravenous Injection of Stabilized Plasmid-Lipid Particles

Although PEG-containing SPLP are promising with respect to their ability to deliver intact plasmid DNA to disease sites, SPLP exhibit relatively low transfection efficiencies *in vitro* (Fig. 7) due to the ability of the PEG coating to inhibit cell association and uptake of PEG-containing liposomes.[20–22] However, the use of diffusible PEG-ceramides facilitates the formulation of stable particles containing a high percentage (79 to 84 mol%) of the fusogenic lipid DOPE. As the PEG-ceramide dissociates from the particle it is expected to become increasingly fusogenic. In particular, the SPLP lipid composition of DOPE, DODAC, and PEG-Cer is stabilized in the bilayer organization by the presence of the PEG-Cer component and will tend to assume the hexagonal (H_{II}) organization preferred by DOPE when the PEG-Cer dissociates. Liposomes having lipid compositions favoring H_{II} organization rapidly aggregate and fuse, releasing their entrapped contents. The inclusion of PEG-ceramides in SPLP may help to resolve the previously mentioned conflicting demands imposed upon carriers for systemic gene therapy. First, the carrier must be stable and circulate long enough to facilitate accumulation at disease sites. Second, the carrier must be capable of interacting with target cells in order to facilitate intracellular delivery. PEG coatings that dissociate from the carrier at the disease site are a potential solution to this problem.

A direct assessment of the *in vivo* transfection potential of SPLP reveals that SPLP-CerC_{20} are indeed transfection competent at disease sites following systemic administration (Fig. 15). A single intravenous administration of SPLP yields significant levels of gene expression within 24 h after administration. Marker gene expression increases considerably over the following 48 h, reaching maximal levels more than 72 h after administration. Remarkably, the greatest levels of gene expression observed upon systemic administration of SPLP-CerC_{20} are found in tumor tissue. Although moderate levels of marker gene expression are observed in all tissues assayed, at later time points the tumor gene expression is two orders of magnitude greater than that observed in other organs. (The maximal level of luciferase gene expression measured, 1.6 ± 0.18 ng/g corresponds to ~20 copies of active luciferase protein per tumor cell.) Most notable among the other organs assayed is the lung. While all other nonviral gene delivery systems are known to predominantly transfect lung upon intravenous administration, SPLP are a notable exception to this rule. The lack of marker gene expression in the lung correlates well with the ability of SPLP to bypass the lung on systemic administration. Unlike the lung, the liver does accumulate significant levels of SPLP. It is notable that although liver accumulation of SPLP-CerC_{20} may reach as much as 25% of the total

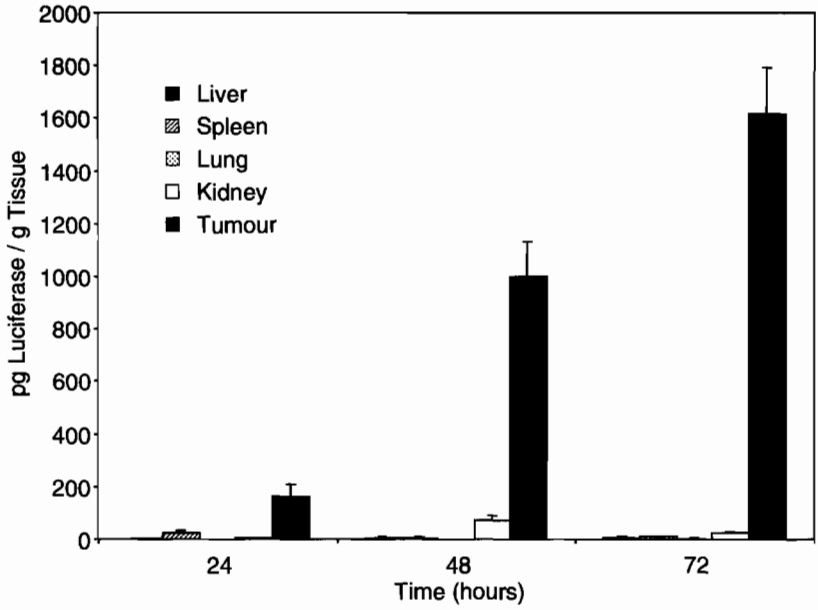


FIG. 15. Luciferase gene expression following a single intravenous administration of SPLP in neuro-2a tumor-bearing A/J mice. For experimental details, see legend to Fig. 13. [Reproduced from D. B. Fenske, I. MacLachlan, and P. R. Cullis, *Curr. Opin. Mol. Ther.* **3,** 153 (2001), with permission of PharmaPress Ltd.]

injected dose at later time points, the liver is relatively refractory to SPLP-mediated transfection.

The reasons for the marked preference for transfection of tumor tissue become apparent when one considers the various barriers that must be overcome in transfection process. The first requirement for transfection is delivery of the plasmid DNA to the disease site, readily facilitated by systems capable of disease site targeting. Once at the disease site, the transfection reagent must be interact with and be internalized by the target cell. On internalization the plasmid DNA needs to escape the endosomal compartment and translocate to the nucleus before gene expression can occur. It is probable that tumor tissue is more readily transfected than other tissues because tumor cells are more rapidly dividing than normal tissues. Tumor cells are highly endocytic, have a high mitotic index, and are highly active at the transcriptional and translational level. It has been shown that SPLP (in common with other nonviral gene delivery systems) require cell division for efficient transfection.[31]

[31] I. Mortimer, P. Tam, I. MacLachlan, R. W. Graham, E. G. Saravolac, and P. B. Joshi, *Gene Ther.* **6,** 403 (1999).

PEG-ceramide has an expected effect on the transfection potency of SPLP. Preliminary studies have determined that SPLP-CerC_{20} produce significantly higher levels of marker gene expression than SPLP-CerC_{14} and SPLP-CerC_8 when administered systemically in tumor-bearing mice. Neither SPLP-CerC_{14} nor SPLP-CerC_8 are able to mediate significant levels of luciferase gene expression at tumor sites, suggesting that the amount of intact plasmid DNA delivered to the tumor by SPLP-CerC_{14} and SPLP-CerC_8 is not sufficient to produce detectable levels of gene expression.[7] The SPLP-CerC_{20} mediated transfection levels are significant and offer a basis on which to optimize SPLP properties to reach higher levels. For example, the observation that the SPLP-CerC_{20} formulation is the only formulation that results in transfection at the tumor site emphasizes the obvious fact that little transfection can be achieved unless adequate levels of plasmid are delivered to the target tissue. SPLP-CerC_{20} are, however, the most stable preparations and the least intrinsically transfection potent of the formulations investigated when evaluated *in vitro*. In turn, this suggests methods for improving the tumor transfection potential of SPLP. For example, the utilization of PEG-ceramides containing acyl groups with intermediate chain lengths such as C_{16} or C_{18} may allow greater SPLP stability than SPLP-CerC_8 and SPLP-CerC_{14}, thus promoting greater delivery of intact plasmid to tumor sites. These compositions should also render the SPLP more transfection potent than SPLP-CerC_{20} after arrival at the tumor site because of an improved ability of the PEG coating to dissociate. Alternatively, methods for actively targeting SPLP to tumor cells after arrival at the tumor site may also facilitate target cell uptake and transfection. These and other methods for achieving high levels of SPLP-mediated gene expression at distal tumors following systemic administration are currently under investigation.

Summary

The ability of a systemically administered gene therapy vector to exhibit extended circulation lifetimes, accumulate at a distal tumor site, and enable transgene expression is unique to SPLP. The flexibility and low toxicity of SPLP as a platform technology for systemic gene therapy allows for further optimization of tumor transfection properties following systemic administration. For example, the PEG coating of SPLP is necessary to engender the long circulation lifetimes required to achieve tumor delivery. However, PEG coatings have also been shown to inhibit cell association and uptake required for transfection. The dissociation rate of the PEG coating from SPLP can be modulated by varying the acyl chain length of the ceramide anchor, suggesting the possibility of developing PEG-Cer molecules that remain associated with SPLP long enough to promote tumor delivery, but which dissociate quickly enough to allow transfection. Alternatively, improvements may be expected from inclusion of cell-specific targeting ligands in SPLP to promote cell association and uptake. Finally, the nontoxic properties of SPLP

allow the possibility of higher doses. A dose of 100 μg plasmid DNA per mouse corresponds to a dose of approximately 5 mg plasmid DNA per kg body weight. This compares well to small molecules used for cancer therapy, which typically are used at dose levels of 10 to 50 mg per kg body weight.

In summary, SPLP consist of plasmid encapsulated in a lipid vesicle that, in contrast to naked plasmid or complexes, exhibit extended circulation lifetimes following intravenous injection, resulting in accumulation and transgene expression at a distal tumor site in a murine model. The pharmacokinetics, biodistribution, and tumor transfection properties of SPLP are highly sensitive to the nature of the ceramide anchor employed to attach the PEG to the SPLP surface. The SPLP-$CerC_{20}$ system in which the PEG-Cer does not readily dissociate exhibits good serum stability, long circulation lifetimes, and high levels of tumor accumulation and mediates marker gene expression at the tumor site. The flexibility of the SPLP system offers the potential of further optimization to achieve therapeutically effective levels of gene transfer and clearly has considerable potential as a nontoxic systemic gene therapy vehicle with general applicability.

These features of SPLP contrast favorably with previous plasmid encapsulation procedures. Plasmid DNA has been encapsulated by a variety of methods, including reverse phase evaporation,[32-34] ether injection, [35,36] detergent dialysis in the absence of PEG stabilization,[34,35] lipid hydration and dehydration–rehydration techniques,[37-39]and sonication,[40-42] among others. The characteristics of these protocols are summarized in Table I. None of these procedures yields small, serum-stable particles at high plasmid concentrations and plasmid-to-lipid ratios in combination with high plasmid-encapsulation efficiencies. Trapping efficiencies comparable with the SPLP procedure can be achieved employing methods relying on sonication. However, sonication is a harsh technique that can shear nucleic acids. Size ranges of 100 nm diameter or less can be achieved by reverse-phase techniques; however, this requires an extrusion step through filters with 100 nm or smaller pore size which can often lead to significant loss of plasmid. Finally,

[32] R. Fraley, S. Subramani, P. Berg, and D. Papahadjopoulos, *J. Biol. Chem.* **255,** 10431 (1980).

[33] P. Soriano, J. Dijkstra, A. Legrand, H. Spanjer, D. Londos-Gagliardi, F. Roerdink, G. Scherphof, and C. Nicolau, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 7128 (1983).

[34] M. Nakanishi, T. Uchida, H. Sugawa, M. Ishiura, and Y. Okada, *Exp. Cell Res.* **159,** 399 (1985).

[35] R. T. Fraley, C. S. Fornari, and S. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3348 (1979).

[36] C. Nicolau and S. Rottem, *Biochem. Biophys. Res. Commun.* **108,** 982 (1982).

[37] P. F. Lurquin, *Nucleic Acids Res.* **6,** 3773 (1979).

[38] S. F. Alino, M. Bobadilla, M. Garcia-Sanz, M. Lejarreta, F. Unda, and E. Hilario, *Biochem. Biophys. Res. Commun.* **192,** 174 (1993).

[39] M. Baru, J. H. Axelrod, and I. Nur, *Gene* **161,** 143 (1995).

[40] D. G. Jay and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 1978 (1987).

[41] C. Puyal, P. Milhaud, A. Bienvenue, and J. R. Philippot, *Eur. J. Biochem.* **228,** 697 (1995).

[42] M. Ibanez, P. Gariglio, P. Chavez, R. Santiago, C. Wong, and I. Baeza, *Biochem. Cell Biol.* **74,** 633 (1996).

TABLE I

PROCEDURES FOR ENCAPSULATING PLASMID IN LIPID-BASED SYSTEMS

Procedure[a]	Lipid composition	Length of DNA	Trapping efficiency	DNA-to-lipid ratio	Diameter
Reverse phase evaporation[1]	PS or PS : Chol (50 : 50)	SV40 DNA	30–50%	<4.2 μg/μmol	400 nm
Reverse phase evaporation[2]	PC : PS : Chol (40 : 10 : 50)	11.9 kbp plasmid	13–16%	0.23 μg/μmol	100 nm to 1 μm
Reverse phase evaporation[3]	PC : PS : Chol (50 : 10 : 40)	8.3 kbp, 14.2 kbp plasmid	10%	0.97 μg/μmol	ND[b]
Reverse phase evaporation[4]	EPC : PS : Chol (40 : 10 : 50)	3.9 kbp plasmid	12%	0.38 μg/μmol	400 nm
Ether injection[5]	EPC : EPG (91 : 9)	3.9 kbp plasmid	2–6%	<1 μg/μmol	0.1–1.5 μm; Aug = 230 nm
Ether injection[6]	PC : PS : Chol (40 : 10 : 50) PC : PG : Chol (40 : 10 : 50)	3.9 kbp plasmid	15%	15 μg/μmol	ND
Detergent dialysis[7]	EPC : Chol : stearylamine (43.5 : 43.5 : 13)	sonicated genomic DNA (~250,000 MW)	11%	0.26 μg/μmol	50 nm
Detergent dialysis, extrusion[8]	DOPC : Chol : oleic acid or DOPE : Chol : oleic acid (40 : 40 : 20)	4.6 kbp plasmid	14–17%	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)
Lipid hydration[9]	EPC : Chol (65 : 35) or EPC	3.9 kbp, 13 kbp plasmid	ND	ND	0.5–7.5 μm
Dehydration–rehydration, extrusion (400 or 200 nm filters)[10]	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[11]	EPC	2.96 kbp, 7.25 kbp plasmid	35–40%	2.65–3.0μg/μmol	1–2 μm
Sonication (in the presence of lysozyme)[12]	asolectin (soybean phospholipids)	1.0 kbp linear DNA	50%	0.08μg/μmol	100–200 nm
Sonication[13]	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[14]	EPC : Chol : PS (40 : 50 : 10) EPC : Chol : EPA (40 : 50 : 10) or EPC : Chol : CL (50 : 40 : 10)	4.4 kbp, 7.2 kbp plasmid	46–52%	2.53–2.87 μg/μmol	400–500 nm
Ca^{2+}-EDTA entrapment of DNA–protein complexes[15]	PS : Chol (50 : 50)	42.1 kbp bacteriophage	52–59%	22 μg/μmol	ND
Freeze–thaw, extrusion[16]	POPC : DDAB (99 : 1)	3.4 kbp linear plasmid	17–50%	ND	80–120 nm
SPLP (this work)	DOPE : PEG-Cer : DODAC (84 : 10 : 6)	4.4–10 kbp plasmid	60–70%	62.5 μg/μmol	75 nm (QELS); 65 nm (freeze-fracture)

[a] Key to References: [1] R. Fraley, S. Subramani, P. Berg, and D. Papahadjopoulos, *J. Biol. Chem.* **255,** 10431 (1980);
[2] P. Soriano, J. Dijkstra, A. Legrand, H. Spanjer, D. Londos-Gagliardi, F. Roerdink, G. Scherphof, and C. Nicolau, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 7128 (1983);
[3] M. Nakanishi, T. Uchida, H. Sugawa, M. Ishiura, and Y. Okada, *Exp. Cell. Res.* **159,** 399 (1985);
[4] A. Cudd and C. Nicolau, *Biochim. Biophys. Acta* **845,** 477 (1985);
[5] R. T. Fraley, C. S. Fornari, and S. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3348 (1979);
[6] C. Nicolau and S. Rottem, *Biochem. Biophys. Res. Commun.* **108,** 982 (1982);
[7] J. C. Stavridis, G. Deliconstantinos, M. C. Psallidopoulos, N. A. Armenakas, D. J. Hadjiminas, and J. Hadjiminas, *Exp. Cell Res.* **164,** 568 (1986);
[8] C. Y. Wang and L. Huang, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7851 (1987);
[9] P. F. Lurquin, *Nucleic Acids Res.* **6,** 3773 (1979);
[10] S. F. Alino, M. Bobadilla, M. Garcia-Sanz, M. Lejarreta, F. Unda, and E. Hilario, *Biochem. Biophys. Res. Commun.* **192,** 174 (1993);
[11] M. Baru, J. H. Axelrod, and I. Nur, *Gene* **161,** 143 (1995);
[12] D. G. Jay and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 1978 (1987);
[13] C. Puyal, P. Milhaud , A. Bienvenue, and J. R. Philippot, *Eur. J. Biochem.* **228,** 697 (1995);
[14] M. Ibanez, P. Gariglio, P. Chavez, R. Santiago, C. Wong, and I. Baeza, *Biochem. Cell. Biol.* **74,** 633 (1996);
[15] J. Szelei and E. Duda, *Biochem. J.* **259,** 549 (1989);
[16] P. A. Monnard, T. Oberholzer, and P. Luisi, *Biochim. Biophys. Acta* **1329,** 39 (1997).

it may be noted that the plasmid DNA-to-lipid ratios that can be achieved for SPLP are significantly higher than those achievable by any other encapsulation procedure.

Appendix: SPLP Formulation Protocol

Scope

This sample protocol describes the preparation of SPLP for systemic gene delivery and expression. The batch described initially contains 40 mg of plasmid DNA in a total volume of 100 ml. This protocol provides detailed steps for

Preparation of lipids
Salt curve analysis
Removal of unencapsulated DNA by DEAE-Sepharose chromatography
Concentration by Amicon ultrafiltration
Sucrose density isolation procedure
Sterilization and storage

Materials

1. Sterile bottles or round bottom flasks
2. Sterile graduated cylinders
3. Sterile crimp-top glass vials
4. Spectrum Spectra/Por Molecular porous 25 mm and 45 mm membrane tubing 12,000–14,000 MWCO
5. Large chromatography column (2 cm radius)
6. Amicon ultrafiltration apparatus (350 ml and 180 ml)
7. Diaflo 76 mm and 62 mm ultrafiltration membranes
8. Seton 1 inch × 3.5 inches open top polyclear ultracentrifuge tube
9. Assorted sterile syringes and 18G 1/2 inch needles
10. Millipore 0.22 μm syringe filtration unit
11. Sterile Pasteur pipettes
12. Picogreen DNA quantitation reagent

Preparation of Lipids

1. Prepare 20 l of 10× HBS (1× = 10 m*M* HEPES and 150 m*M* NaCl pH 7.4).
2. Weigh out the required lipids and OGP on an analytical balance, or alternatively use lipid stock solutions previously prepared in methanol. For a 100 ml preparation with an initial lipid concentration of 10 mg/ml, 200 m*M* OGP, and the composition DOPE : DODAC : $PEGCerC_{20}$ (82.5 : 7.5 : 10), the following would be required: 664 mg DOPE, 47 mg DODAC, 289 mg $PEGCerC_{20}$, 5848 mg OGP, and 40 mg plasmid DNA.

3. In a sterile bottle or round bottom flask, add 1/3 total formulation volume of filter sterilized 1× HBS and add the DOPE, $PEGC_{20}$, OGP, and DODAC. Dissolve the lipids and OGP by magnetic stirring.

4. While waiting for the lipids to dissolve, cut six 8 cm strips of dialysis tubing so that there are a few extra centimeters of bag on both sides. Presoak the dialysis bags in filter-sterilized water for at least 30 min in preparation for the salt curve.

5. Once the lipids have dissolved add the DNA at 400 μg DNA/10 mg lipid.

6. Using a sterile graduated cylinder, add 1× HBS to bring the total volume to 100 ml and mix.

Test Formulate: Preparing for Salt Curve

1. Dialyze 1 ml aliquots of the above preparation overnight in 3 liters of the following buffers prepared using the 10× HBS stock:

[NaCl] (m*M*)
120
130
140
150
160
170

2. The next day, set aside 500 μl of each sample for QELS analysis and a PicoGreen assay.

PicoGreen Assay

(i) Prepare 1/40 dilutions of all samples (i.e., 25 μl stock sample +975 μl 1× HBS).

(ii) Prepare duplicates of 1/400 dilutions of all samples (i.e., 100 μl of above dilution +900 μl of 1× HBS).

(iii) Prepare plasmid DNA standard curve. Add 200 μl of a 10 μg/ml DNA standard to 1.8 ml HBS, and use this solution to prepare a serial dilution from 1 to 0.0625 μg/ml. Each standard and sample will have a volume of 1 ml.

(iv) Carry out PicoGreen analysis according to manufacturer's instructions in the absence and presence of 0.1% Triton X-100. The excitation and emission wavelengths of PicoGreen are 495 and 525 nm, respectively. Slit width = 4 nm. For each standard and sample, the steps are

- Add 2 μl PicoGreen to standard or sample.
- Transfer to cuvette, and measure fluorescence for 10–20 s (−Triton value).

- Add 10 μl 10% Triton X-100 to cuvette and mix thoroughly.
- Measure fluorescence for 10–20 s (+Triton value).

The % encapsulation = (([+Triton] − [−Triton]) × 100)/[+Triton]

3. The optimal NaCl concentration is determined by the percent of plasmid encapsulation and particle size. The optimal NaCl provides for 50–80% encapsulation and a single population with a particle size of $\leq$100 nm. Particle sizes greater than 120 nm indicate a "crashed" preparation where the vesicles have aggregated. Determine the optimal NaCl concentration and prepare 4 × 4 liters of dialysis buffer for a 48-h dialysis.

4. Dialyze the initial lipid solution for 48 h using the optimal buffer conditions. Transfer the dialysis bags to fresh dialysis buffer every 12 h.

5. Perform a PicoGreen assay and QELS on the resulting material. Expect a percent encapsulation of approximately 50–80%. If this is not achieved, perform another salt curve before proceeding to the next step.

Removal of Unencapsulated DNA by DEAE-Sepharose Chromatography

1. For every 0.64 mg of DNA loaded on the DEAE-Sepharose CL-6B (Sigma) column, 1 mL of stationary volume will be required. Pour the column and wash with 10 column volumes of filter sterilized 1× HBS.

2. Once the column has settled, slowly load the formulation suspension against the column wall in a circular motion and allow it to flow through the resin. Elute with HBS.

3. Use small glass test tubes to determine the point at which the cloudy formulation begins to elute from the column. Once the formulation appears, collect into a sterile flask or bottle. Collect a final volume equal to 1.5 times the sample volume to completely elute all of the formulation from the column.

Concentration by Amicon

1. Assemble the Amicon apparatus.
   (a) Place the membrane shiny side up into the bottom plate and the rubber seal on top of the membrane.
   (b) Screw on the bottom plate tightly.
   (c) Hydrate the filter with a steady stream of filter sterilized water for 10–20 min.
   (d) Add the sample and adjust the pressure so as not to exceed 50 psi, with a flow rate of 1 drop/sec.
   (e) Concentrate the sample down to the desired volume (typically 4× concentration).

Sucrose Density Isolation Procedure

1. Prepare desired volumes of 2.5%, 5.0%, and 10.0% w/v sucrose in 1× HBS and filter sterilize into a sterile container. Store at 4°.
2. Pull a long glass pipette to a small point using forceps and a Bunsen burner. Place the elongated pipette into the ultracentrifuge tube (Seton 1 inch × 3.5 inches open top polyclear) and pour 14 ml 2.5% sucrose solution into the pipette using a second sterile pipette.
3. Pour the sucrose layers in order of increasing density: 14 ml of 2.5%, 10 ml of 5.0%, and 7 ml of 10.0%. Load 5 to 7 ml of SPLP on top of the gradients.
4. Balance all the tubes with 2.5% sucrose or 1× HBS to within 0.01 g and place the tubes in the SW-28 buckets. Run for 18 h at 28,000 rpm at 20°.
5. The following day, slowly aspirate the lower band using an 18-gauge needle with a 10 ml syringe. Pool all of the lower bands and place the formulation into a dialysis bag overnight in 1× HBS to remove the sucrose.
6. Perform a PicoGreen assay to determine the percent encapsulation and DNA concentration.

Final Concentration by Amicon

1. Concentrate the sample until the desired concentration of DNA is achieved (typically >0.5 mg DNA/ml).

Sterilization and Storage

1. Filter sterilize the final volume through a sterile Millipore 0.22 μm filter unit in a biological safety cabinet and adjust the final volume so that the DNA concentration is exactly 0.5 mg/ml. Store SPLP in sterile vials at 4° for up to 2 years. Analyze the particle size, percent encapsulation, and lipid concentration via QELS analysis, PicoGreen assay, and HPLC analysis, respectively. Agarose gel electrophoresis can also be used to verify the integrity of the plasmid.