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Stabilized plasmid-lipid particles: construction and characterization

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A detergent dialysis procedure is described which allows encapsulation of plasmid DNA within a lipid envelope, where the resulting particle is stabilized in aqueous media by the presence of a poly(ethyleneglycol) (PEG) coating. These 'stabilized plasmid-lipid particles' (SPLP) exhibit an average size of 70 nm in diameter, contain one plasmid per particle and fully protect the encapsulated plasmid from digestion by serum nucleases and E. coli DNase I. Encapsulation is a sensitive function of cationic lipid content, with maximum entrapment observed at dioleoyldimethylammonium chloride (DODAC) contents of 5 to 10 mol%. The formulation process results in plasmid-trapping efficiencies of up to 70% and permits inclusion of 'fusigenic' lipids such as dioleoylphosphatidylethanolamine (DOPE). The in vitro transfection capabilities of SPLP are demonstrated to be strongly dependent on the length of the acyl chain contained in the ceramide group used to anchor the PEG polymer to the surface of the SPLP. Shorter acyl chain lengths result in a PEG coating which can dissociate from the SPLP surface, transforming the SPLP from a stable particle to a transfection-competent entity. It is suggested that SPLP may have utility as systemic gene delivery systems for gene therapy protocols.

Keywords: *plasmid encapsulation; nonviral gene delivery; intracellular delivery; gene therapy; liposomes*

Introduction

Currently available gene delivery systems for gene therapy protocols have limited utility for systemic applications. Viral systems, for example, are rapidly cleared from the circulation, limiting potential transfection sites to 'first-pass' organs such as the lungs, liver and spleen. In addition, these systems induce immune responses which 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.[1–4] Plasmid DNA–cationic lipid complexes can also result in toxic side-effects both *in vitro*[5] and *in vivo*.[6]

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).[7–9] The accumulation of these drug delivery systems at disease sites, which includes sites of infection and inflammation as well as tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue.[10]

A gene delivery system containing an encapsulated plasmid for systemic applications should therefore be small (<100 nm diameter) and must exhibit extended circulation life-times 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 interact readily 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 show that a straightforward detergent dialysis procedure can produce stabilized plasmid-lipid particles (SPLP) which satisfy the demands of plasmid encapsulation, small size and serum stability. Furthermore, we show that the transfection properties of these systems can be modulated by employing poly(ethyleneglycol) (PEG) coatings which can dissociate from the SPLP, transforming the particle from a stable particle to a transfection-competent entity.

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Results

Entrapment of plasmid DNA into lipid particles by employing detergent dialysis

Previous work has shown that incubation of plasmid DNA with cationic lipids can result in a hydrophobic particle which is soluble in organic solvent.[11] It is of interest to determine whether this hydrophobic particle can be surrounded by an outer monolayer of lipid, which would then result in small, plasmid-containing particles stabilized in an aqueous medium. Detergent dialysis is a logical technique for achieving this, as the detergent may be 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 which are stable in aqueous suspension.

Initial experiments employed the cationic lipid DODAC, the plasmid pCMVCAT, the non-ionic detergent octylglucopyranoside (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 mm) 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, during dialysis to substitute the detergent associated with the particles for POPC, extensive precipitation of the suspension was observed. A method to stabilize the plasmid-containing particles against aggregation and precipitation during the dialysis process was therefore required.

Previous studies have shown that a PEG coating can prevent aggregation of LUVs induced by covalent coupling of protein to the surface of the LUVs,[12] and can inhibit fusion between LUVs.[13] It was therefore of interest to determine whether the stabilizing properties of a PEG coating could prevent aggregation during dialysis. However, the use of the standard PEG-phosphatidylethanolamine (PEG-PE) was contraindicated because the PEG-PE molecule bears a net negative charge and could displace the cationic lipid from the plasmid, as has been noted for other negatively charged lipids.[14] As a result, PEG_{2000} was linked to ceramide as the hydrophobic anchor to produce a neutral molecule. Two ceramide anchors were synthesized which differed in the length of the ceramide acyl chain ($CerC_{14}$ and $CerC_{20}$). When 10 mol% PEG-$CerC_{20}$ was incorporated in the detergent mixture with POPC, DODAC and plasmid DNA, precipitation was not observed during detergent dialysis. 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. As shown in Figure 1a, the encapsulation achieved is a sensitive function of the DODAC content, with encapsulation levels of 30% or higher at about 9% to 12% DODAC. It should be noted that addition of plasmid to preformed vesicles with the same lipid composition, followed by DEAE chromatography, resulted in complete plasmid retention on the column.

These results suggest that SPLP can be produced by detergent dialysis employing a POPC/DODAC/PEG-$CerC_{20}$ (79:11:10; mol:mol:mol) lipid mixture. 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.[15] The encapsulation properties of DOPE/DODAC/PEG-$CerC_{20}$ lipid mixtures were therefore investigated. As shown in Figure 1b, as the DODAC content was varied, an encapsulation profile for DOPE-containing systems similar to that obtained for the POPC-containing systems was

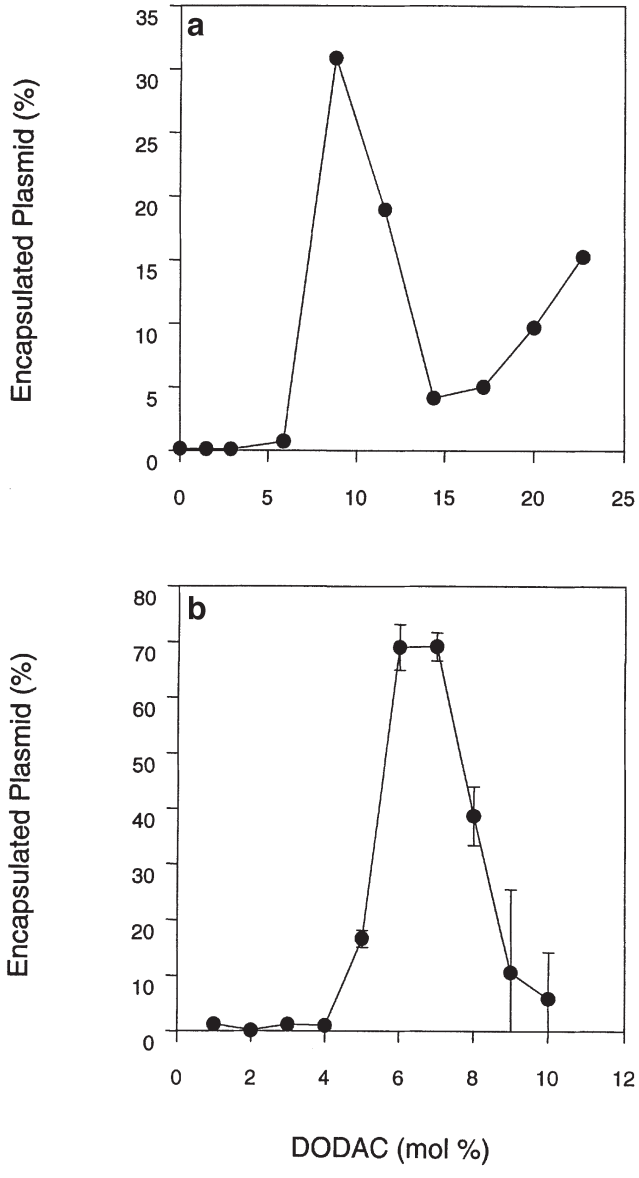

Figure 1 *Effect of DODAC concentration on the encapsulation efficiency of plasmid DNA (pCMVCAT) in SPLP. (a) Lipid composition POPC, DODAC and 10 mol% PEG-$CerC_{20}$. (b) Lipid composition DOPE, DODAC and 10 mol% PEG-$CerC_{20}$. Lipid (10 mg/ml total), dissolved in octylglucoside (0.2* m*), was mixed 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°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography, as outlined in Materials and methods.*

observed. Significant differences are that maximum encapsulation was greater (approximately 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.

In subsequent experiments DOPE / DODAC / PEG-Cer formulations were employed containing 6 mol% DODAC. For this fixed DODAC content, some batch-to-batch variability of encapsulation efficiency (typically over the range 50–70%) was observed when different batches of plasmid were employed. This variability resulted from small changes (up to ±1 mol%) in the DODAC concentration required for maximum encapsulation efficiency for different plasmid batches. Other factors which may influence encapsulation efficiency include the amount of the plasmid present and the size of the plasmid. The plasmid (pCMVCAT) concentration was varied over the range 25 to 400 μg / ml employing the DOPE / DODAC / PEG-CerC_{20} (84:6:10; mol:mol:mol) lipid mixture at a fixed total lipid concentration of 10 mg / ml. Encapsulation efficiencies of more than 50% were achieved over this range (data not shown). In addition, at a plasmid concentration of 400 μg / ml, similar levels of entrapment were observed for plasmids of 4.49 and 10 kbp in length (data not shown).

It is important to show that the detergent dialysis process does not inhibit the transfection potential of the encapsulated plasmid. In order to test this, the plasmid was extracted from SPLP as described in Materials and methods. Characterization of the extracted DNA by agarose gel electrophoresis indicated no DNA degradation or plasmid relaxation relative to the starting material. Furthermore, the luciferase activity measured in cells following transfection (mediated by calcium phosphate) with plasmid extracted from SPLP was equivalent to the activity observed for plasmid which had not undergone encapsulation, with activities of 0.44 ± 0.15 ng and 0.35 ± 0.2 ng, respectively for 0.5 μg plasmid per well.

Plasmid DNA in stabilized plasmid-lipid particles is protected from DNase I 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 DNase I to digest plasmid DNA in DOPE-containing particles was examined. SPLP were prepared for the DOPE / DODAC / PEG-CerC_{20} (84:6:10; mol:mol:mol) lipid mixture and pCMVLuc (200 μg / ml). Protection of plasmid in the SPLP was compared to protection of plasmid in complexes with DODAC-DOPE (1:1; mol:mol) LUVs and to free plasmid. Samples containing 1 μg plasmid were exposed to 0, 100 and 1000 units of DNase I for 30 min at 37°C. After incubation the plasmid was isolated as described in Materials and methods and characterized by agarose gel electrophoresis. As shown in Figure 2, free plasmid is completely digested by incubation with both 100 and 1000 units of DNase I. The plasmid complexed with cationic LUVs is somewhat protected compared with free DNA when exposed to 100 units of DNase I, but is almost entirely digested by incubation with 1000 units. In contrast, plasmid DNA in the SPLP is digested only when detergent is added to disrupt the SPLP before incubation with DNase I.

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,[16] resulting in enhanced leakage and rapid clearance of liposomal systems. The ability of serum nucleases to degrade plasmid is illustrated in Figure 3a. Intact pCMVCAT elutes in the void volume of the Sepharose CL-4B column, whereas after incubation with mouse serum (90%) at 37°C for 30 min the plasmid is degraded into fragments which elute in the included volume. The behavior of the DOPE / DODAC / PEG-CerC_{20} (84:6:10; mol:mol:mol) SPLP system where non-encapsulated plasmid has not been removed is shown in Figure 3b. In this particular preparation, 53% of the plasmid DNA elutes with the lipid in the void volume and 47% of the DNA, which

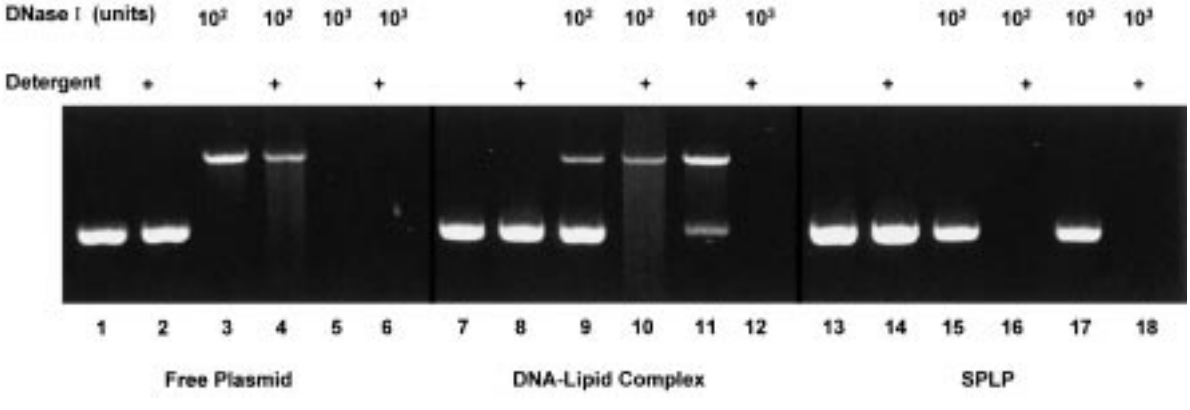

Figure 2 *Stability of free plasmid, plasmid encapsulated in SPLP and plasmid in plasmid DNA–cationic lipid complexes in the presence of DNase I. Each of the sample types was subjected to six different protocols, giving rise to six lanes for each sample. These protocols consisted of no exposure to DNase I or detergent (lanes 1, 7, 13), exposure to detergent alone (lanes 2, 8, 14), exposure to 100 and 1000 units of DNase I alone (lanes 3, 9, 15 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). These experiments utilized 1 μg of plasmid DNA (pCMVLuc), 1% Triton X-100 and 100 or 1000 units of DNase I. These components were combined in a total volume of 100 μl of 5 mm HBS and 10 mm $MgCl_2$, and incubated for 30 min at 37°C before preparation for gel electrophoresis as outlined in Materials and methods. The plasmid DNA–cationic lipid complexes were prepared as indicated in Materials and methods and consisted of DODAC:DOPE (50:50; mol:mol) LUVs (100 nm diameter) complexed to plasmid at a 3:1 charge ratio (positive-to-negative).*

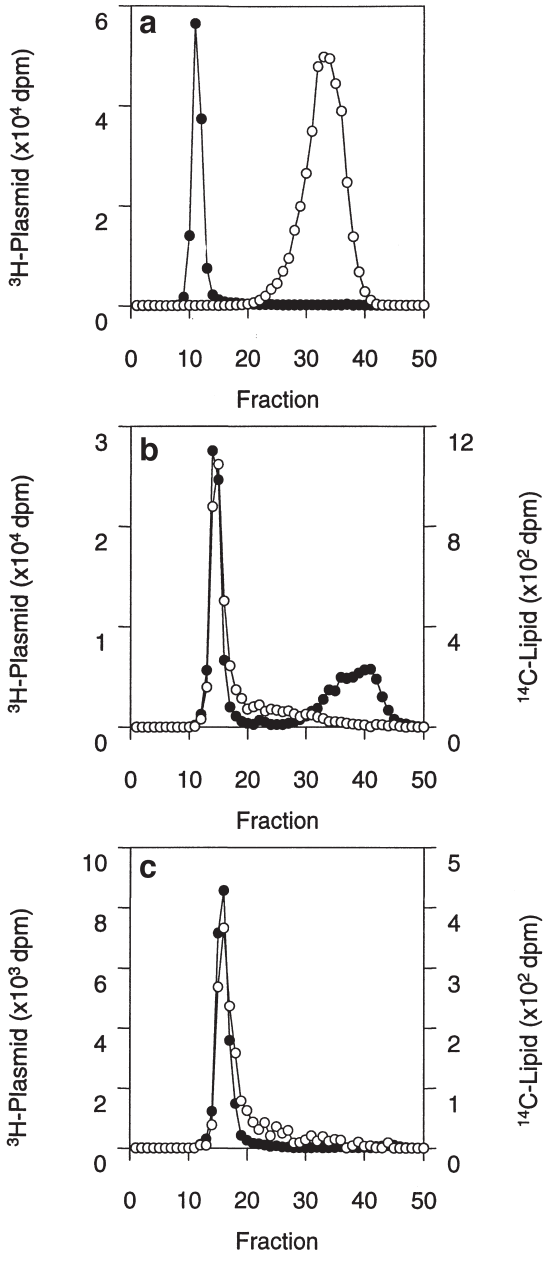

Figure 3 *Plasmid in SPLP is protected from serum nuclease cleavage. The stability of plasmid (pCMVCAT) in the free form or encapsulated in SPLP was determined in the presence of serum. The SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1 and 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°C 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 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 before the serum treatment.*

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.

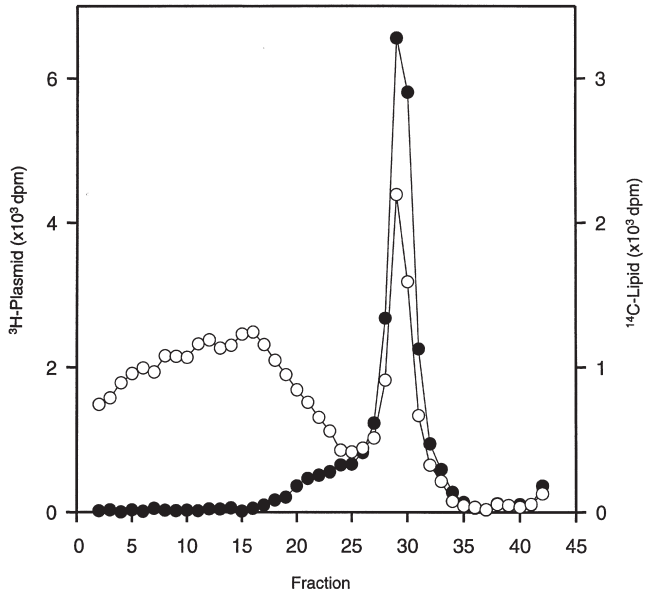

Figure 4 *Separation of SPLP from empty vesicles by discontinuous sucrose density gradient centrifugation. The solid circles indicate the behavior of the ^{3}H-labeled plasmid (pCMVLuc), whereas the open circles indicate the distribution of lipid as reported by the ^{14}C-labeled CHE lipid marker. SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1, 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.*

A final test of the stability of the SPLP formulation is given in Figure 3c, which shows 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°C). 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$_{20}$, exhibited similar plasmid protection properties.

Stabilized plasmid-lipid particles can be isolated by density centrifugation

The detergent dialysis process clearly results in plasmid-containing particles where the plasmid is protected from the external environment. However, it is likely that empty vesicles are also produced, as detergent dialysis of lipids (in the absence of plasmid) is well known to result in the formation of small lipid vesicles.[17] These empty vesicles may be expected to be less dense than SPLP. The density gradient profile of a DOPE / DODAC / PEG-CerC$_{20}$ (84:6:10; mol:mol:mol) SPLP preparation (plasmid-to-lipid ratio of 200 μg DNA to 10 mg lipid) was therefore examined employing sucrose density step gradient centrifugation. As shown in Figure 4, after centrifugation at 160 000 *g* for 2 h, the encapsulated DNA is present as a band which was 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 plasmid DNA, which corresponds to 55% of the total DNA. The plasmid-to-lipid ratio in these

purified SPLP was determined (as indicated in Materials and methods) to be 62.5 μg plasmid per μmol lipid. It was found that SPLP generated by detergent dialysis and purified by density gradient centrifugation may be concentrated by dialysis against carboxymethyl cellulose to achieve plasmid concentrations of 1 mg/ml or higher.

Stabilized plasmid-lipid particles exhibit a narrow size distribution

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 Figure 5, 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 (Figure 6). A size analysis of the particles in these micrographs indicated a size of 36 ± 15 nm for the empty vesicles and 64 ± 9 nm for the isolated SPLP.

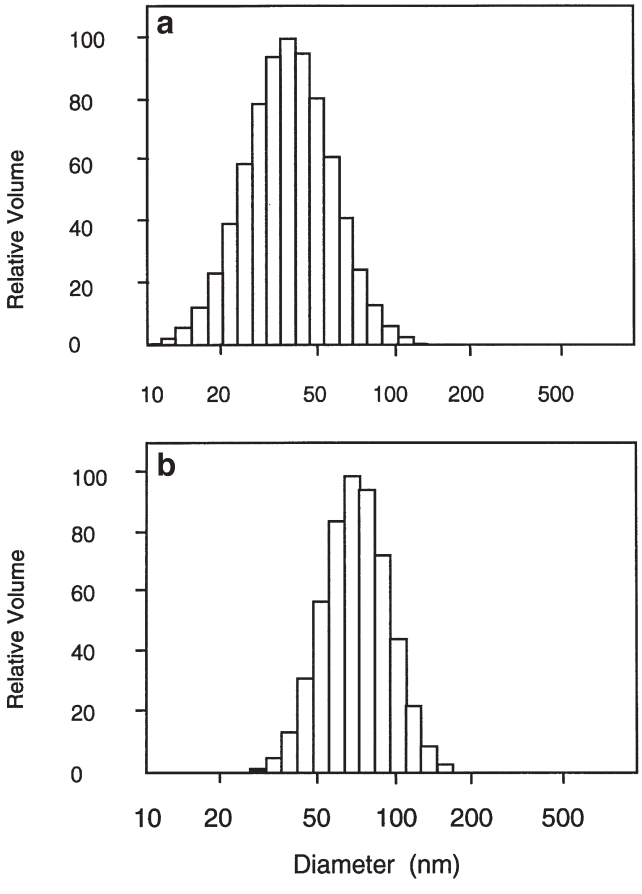

Figure 5 *Size distribution of SPLP and empty vesicles as determined by QELS. SPLP were prepared containing pCMVLuc as indicated in the legend to Figure 1, and separated from empty vesicles by discontinuous sucrose density gradient centrifugation. (a) Size distribution for empty vesicles (upper band). (b) Size distribution for SPLP (lower band). The sizes were determined by quasi-elastic light scattering using a Nicomp (Santa Barbara, CA, USA) model 370 sub-micron particle sizer operating in the solid particle mode.*

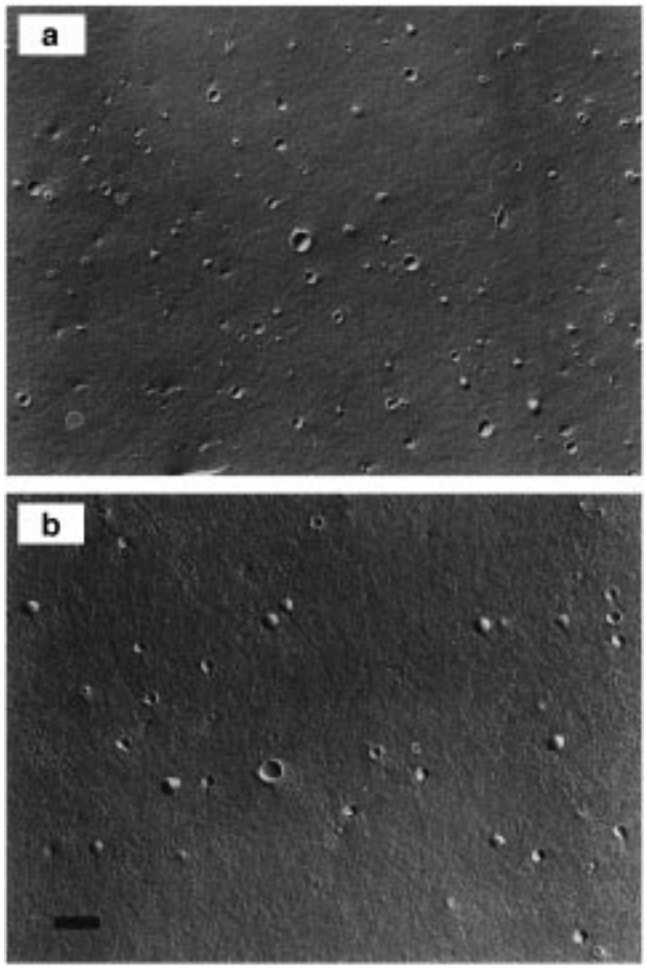

Figure 6 *Freeze–fracture electron microscopy of purified SPLP and empty vesicles. SPLP containing pCMVLuc were prepared as indicated in the legend to Figure 1 and separated into (a) empty vesicles and (b) SPLP employing discontinuous sucrose density gradient centrifugation. The bar indicates 200 nm. For details of sample preparation and electron microscopy, see Materials and methods.*

In vitro transfection properties of stabilized plasmid-lipid particles

SPLP consisting of DOPE/DODAC/PEG-CerC_{20} (84:6:10) containing pCMVLuc coding for the luciferase reporter gene were prepared for transfection studies. As shown in Figure 7, after incubation of these SPLP with COS-7 cells for 24 h, little if any transfection activity was observed. It is probable that the presence of the PEG coating on the SPLP inhibits the association and fusion of the SPLP with cells in the same manner that PEG coatings inhibit fusion between lipid vesicles,[13] and thus inhibit intracellular delivery of the encapsulated plasmid. In this regard, previous studies[13] on LUVs with PEG coatings attached to phosphatidylethanolamine (PE) anchors have demonstrated that, for PE anchors containing short acyl chains, the PEG-PE can rapidly exchange out of the LUV, rendering the LUVs increasingly able to interact and fuse with each other. The transfection properties of SPLP containing PEG-CerC_{20} were therefore compared to SPLP containing PEG-CerC_{14}, which has a shorter acyl chain. As shown in Figure 7, after incubation with COS-7 cells for 24 h, the SPLP containing PEG-CerC_{14} exhibits substantially higher levels of transfection compared with the

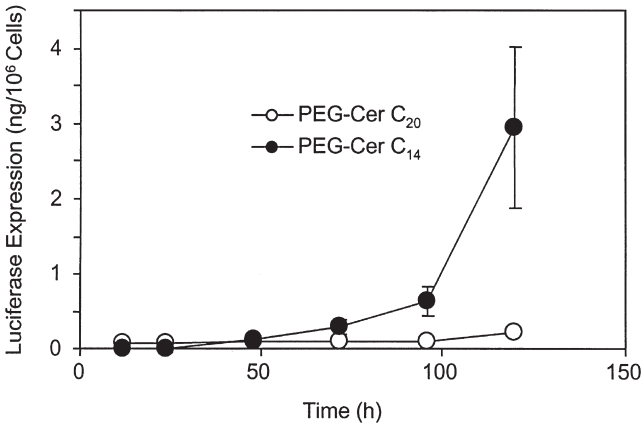

Figure 7 *Effect of PEG-Cer coating of SPLP on transfection activity in vitro. Plasmid (pCMVLuc) was encapsulated in SPLP (DOPE/DODAC/PEG-Cer; 84:6:10; mol/mol/mol) containing PEG-CerC$_{20}$ (○) or PEG-CerC$_{14}$ (●). Non-encapsulated plasmid was removed by anion exchange chromatography, as indicated in Materials and methods. The SPLP preparation (1 μg plasmid) was then added to COS-7 cells at a density of 2 x 10^4 per 24-well plate. The cells were incubated with the SPLP for the times indicated, and luciferase activity was measured as indicated in Materials and methods.*

system containing PEG-CerC$_{20}$. This is consistent with the ability of the PEG-CerC$_{14}$ coating to diffuse away from the SPLP surface. The SPLP containing either PEG-CerC$_{14}$ or PEG-CerC$_{20}$ exerted no apparent toxic effects on the cells as evaluated by monitoring protein content in the cell extract.

In order to determine whether the improved transfection properties of SPLP containing PEG-CerC$_{14}$ as compared with SPLP containing PEG-CerC$_{20}$ could be related to a faster dissociation rate from the SPLP surface, the dissociation rates at 37°C of radiolabeled PEG-CerC$_{14}$ and PEG-CerC$_{20}$ from 100 nm diameter large unilamellar vesicles (LUV) composed of egg phosphatidylcholine (EPC) were measured as indicated in Materials and methods. It should be noted that it is difficult to measure the dissociation rate of PEG-Cer from the surface of SPLP containing DOPE as the stability of these SPLP is dependent on the presence of the PEG-Cer coating. It was found that PEG-CerC$_{20}$ dissociated very slowly, with more than 90% remaining with the SPLP after 48 h incubation, corresponding to a half-time for dissociation of $t_{1/2} > 13$ days. In contrast, PEG-CerC$_{14}$ rapidly dissociated from the outer monolayer of the LUV with $t_{1/2} = 1.1 \pm 0.3$ h.

Discussion

This study presents a new method of encapsulating plasmid DNA in small, stable particulate systems that may find utility as gene delivery vehicles. Of particular interest are the relationship between properties of SPLP and other lipid-based systems containing plasmids, the structure of SPLP and the potential utility of SPLP with exchangeable PEG coatings. We discuss these areas in turn.

The SPLP protocol for plasmid entrapment allows trapping efficiencies of up to 70% and results in stable particles containing low levels of cationic lipids and high levels of fusigenic lipids, such as DOPE. These particles are small (<100 nm diameter), are resistant to external nucleases, exhibit high DNA-to-lipid ratios (62.5 μg/μmol) and can be concentrated to achieve high plasmid DNA concentrations (1 mg/ml). Furthermore, the detergent dialysis procedure is a gentle procedure that results in little, if any, plasmid degradation.

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,[18–20] ether injection,[21,22] detergent dialysis in the absence of PEG stabilization,[20,21] lipid hydration and dehydration–rehydration techniques[25–27] and sonication,[28–30] among others. The characteristics of these protocols are summarized in Table 1. 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 which can shear nucleic acids.[31] 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, 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.

With regard to the structure of SPLP, any model must take into account two important observations. First, SPLP form only at a critical cationic lipid content of approximately 6 mol%. At higher cationic lipid contents, aggregation is observed, whereas lower cationic lipid contents lead to little or no plasmid encapsulation. Second, purified SPLP exhibit a plasmid DNA-to-lipid ratio of 62.5 μg/μmol. For a 4.49 kbp (pCMVCAT) plasmid, this corresponds to a plasmid-to-particle ratio of 0.97 for an SPLP diameter of 70 nm (the average of the freeze–fracture electron microscopy and QELS results), assuming a lipid molecular area[32] of 0.67 nm^2 and an average nucleotide molecular weight of 330. It may therefore be concluded that SPLP contain one plasmid per particle.

The model that guided the construction of SPLP relied on the hypothesis that the plasmid combines with the cationic lipid to form a hydrophobic 'inverted micellar' structure that is stabilized in aqueous media by the detergent. In this model the addition of DOPE and PEG-Cer and subsequent dialysis results in deposition of a monolayer of DOPE and PEG-Cer around the hydrophobic intermediate, resulting in a stabilized plasmid-lipid particle. It is instructive to perform some simple calculations to see whether this model is consistent with experimental observations. In particular, if each negative charge on the plasmid has a cationic lipid associated with it, the total volume of each hydrophobic plasmid-cationic lipid particle can be calculated to be approximately 1.35 x 10^4 nm^3 for a 4.49 kbp plasmid. This calculation assumes that plasmid DNA has a density of 1.7 g/ml, the molecular weight of each base is 330, and that, as an upper limit, the volume per molecule of the cationic lipid is 1.5 nm^3, which is the volume of a liquid crystalline bilayer-forming lipid such as dioleoylphosphatidylcholine (lipid length 2.2 nm and area per molecule 0.67 nm^2).[32] Thus, if each SPLP contained one pCMVCAT plasmid completely neutralized by associated cationic lipid and arranged in a spherical conformation, the predicted diameter would

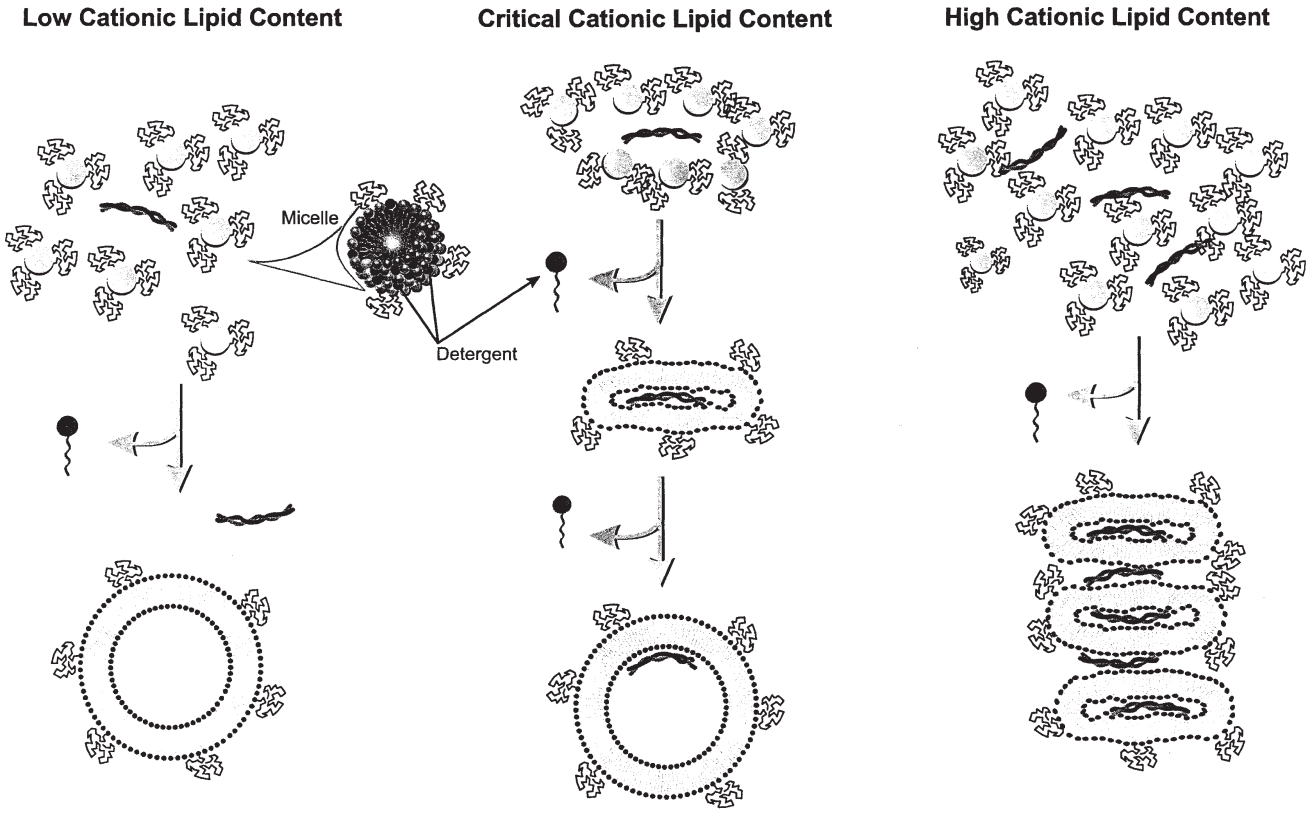

Figure 8 *Model of the formation and possible structure of SPLP. The first stage of dialysis is proposed to result in formation of macromolecular lipid intermediates, which may be in the form of lamellar sheets, cylindrical micelles or leaky vesicles.*[33,34] *If the cationic lipid content is too low (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.*

be approximately 30 nm. The freeze–fracture electron microscopy results presented here indicate that SPLP containing the pCMVCAT plasmid exhibit a diameter of approximately 70 nm, and are therefore too large to be composed solely of a plasmid-lipid particle with no interior aqueous volume.

An alternative working model for SPLP formation and structure is shown in Figure 8. It is unlikely that plasmid associates directly with the micelles, as the presence of high levels of detergent may be expected to dilute the positive surface charge due to the cationic lipid to the extent that electrostatic association is reduced. A probable first step of the dialysis process is the formation of macromolecular lipid intermediates, which may include cylindrical micelles, lamellar sheets or leaky vesicles that form as detergent is removed. These structures have been observed as intermediates in the micelle to vesicle transition undergone by dispersions of egg phosphatidylcholine as detergent (OGP) is removed by dialysis.[33,34] These structures are represented in Figure 8 as lamellar sheets by way of example. As shown in the left panel of Figure 8, low concentrations of cationic lipid would result in little association of plasmid with these intermediate structures, which is consistent with little or no plasmid entrapment following detergent dialysis. At high concentrations of cationic lipid, intermediate structures may be expected to associate with the plasmid and, if the cationic lipid content is too high, plasmid-lipid-plasmid association could dominate as dialysis proceeds, leading to formation of aggregates (Figure 8, right panel).

If the cationic lipid content is at a critical level (Figure 8, central panel), the positive surface charge on the plasmid-associated intermediates will be reduced below that needed to associate with other plasmids, due to charge neutralization. This would mitigate against further aggregation. Further dialysis will result in fusion between intermediates eventually to produce empty vesicles or in fusion between intermediates and the plasmid-lipid particle. Fusion with the particle will result in the deposition of excess bilayer lipid, leading to the formation of an associated vesicle in the final SPLP. In the structure presented, the plasmid is associated with the inner monolayer of the vesicle that is produced as more lipid is deposited in the particle. It should be noted that the forces driving a partial removal of the plasmid lipid coat are not clear, and it is possible that the plasmid resides in a hydrophobic domain inside the particle.

The final area of discussion concerns the potential util-

Table 1 Procedures for encapsulating plasmid in lipid-based systems

Procedure	*Lipid composition*	*Length of DNA*	*Trapping efficiency*[a]	*DNA-to-lipid-ratio*[a]	*Diameter*
Reverse phase evaporation[18]	PS or PS:Chol (50:50)	SV40 DNA	30–50%	<4.2 μg/μmol	400 nm
Reverse phase evaporation[19]	PC:PS:Chol (40:10:50)	11.9 kbp plasmid	13–16%	0.23 μg/μmol	100 nm to 1 μm
Reverse phase evaporation[20]	PC:PS:Chol (50:10:40)	8.3 kbp, 14.2 kbp plasmid	10%	0.97 μg/μmol	ND
Reverse phase evaporation[41]	EPC:PS:Chol (40:10:50)	3.9 kbp plasmid	12%	0.38 μg/μmol	400 nm
Ether injection[21]	EPC:EPG (91:9)	3.9 kbp plasmid	2–6%	<1 μg/μmol	0.1–1.5 μm;
Ether injection[22]	PC:PS:Chol (40:10:50) PC:PG:Chol (40:10:50)	3.9 kbp plasmid	15%	15 μg/μmol	ND
Detergent dialysis[23]	EPC:Chol:stearylamine (43.5:5:43.5:13)	sonicated genomic DNA (250 000 mw)	11%	0.26 μg/μmol	50 nm
Detergent dialysis, extrusion[24]	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[25]	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)[26]	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[27]	EPC	2.96 kbp, 7.25 kbp plasmid	35–40%	2.65–3.0 μg/μmol	1–2 μm
Sonication (in the presence of lysozyme)[28]	asolectin (soybean phospholipids)	1.0 kbp linear DNA	50%	0.08 μg/μmol	100–200 nm
Sonication[29]	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[30]	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[42]	PS:Chol (50:50)	42.1 kbp bacteriophage	52–59%	22 μg/μmol	ND
Freeze–thaw, extrusion[43]	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]Some values calculated based on presented data.
[b]ND, not determined.

ity of SPLP with exchangeable PEG coatings. As previously indicated, the SPLP system has been designed for systemic (intravenous) gene therapy applications. This places two potentially conflicting demands on the delivery system. First, the carrier must circulate long enough to achieve accumulation at disease sites, such as tumors, by taking advantage of the increased vascular permeability in these regions. Second, the carrier must be able to bind to target cells and to destabilize the plasma or endosomal membrane after arrival at the disease site in order to facilitate intracellular delivery of the enclosed plasmid. The first requirement implies a very stable carrier that does not interact with cells, whereas the second requirement necessitates a particle that can bind to cells and exhibit a membrane-destabilizing 'fusigenic' character.

PEG coatings that can dissociate from a carrier provide a potential solution to these demands. First, the presence of a PEG coating allows SPLP to be formed with a large proportion of DOPE in the outer monolayer. Previous work has shown that DOPE prefers the (non-bilayer) hexagonal H_{II} phase at temperatures above 10°C,[35] and that PEG lipids can stabilize DOPE in the bilayer organization.[36] Thus in the absence of the PEG-Cer the SPLP would be expected to be highly unstable and fusigenic. The detergent dialysis procedure therefore allows an intrinsically fusigenic plasmid-containing particle to be formed, where the stability of the particle is dependent on the presence of the PEG coating. As demonstrated here, these particles are stable in the presence of DNase I, as well as serum nucleases, consistent with an ability to protect encapsulated DNA in the circulation. In addition, the small size and presence of the PEG coating would be expected to promote the extended circulation life-times required to achieve preferential accumulation at disease sites such as tumors following intravenous administration.

The stability of the SPLP would, however, be expected to mitigate against uptake and intracellular delivery of the plasmid. The use of PEG coatings that dissociate from the SPLP after arrival at a disease site provides a potential solution to this problem. This is supported by the *in vitro* results presented here, which show that a PEG-$CerC_{20}$ coating, which has a long residence time in lipid bilayers, exhibits poor transfection properties, whereas improved transfection is observed for the SPLP containing a PEG-$CerC_{14}$ coating, which can dissociate from lipid bilayers more rapidly.

In summary, this study presents a method for encapsulating plasmid DNA in particulate systems that have the properties of small size, high plasmid-to-lipid ratio and high content of fusigenic lipid, and that can be concentrated to achieve high plasmid concentrations. These SPLP are stabilized by the presence of a PEG coating that can be designed to dissociate, thus increasing the transfection potency of the SPLP. It is expected that these systems will find utility as delivery systems for systemic gene therapy.

Materials and methods

Materials

Dioleoylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). The lipids 1-O-(2′-(ω-methoxypolyethyleneglycol) succinoyl)-2-N-myristoylsphingosine (PEG-$CerC_{14}$) and 1-O-(2′-(ω-methoxypolyethyleneglycol) succinoyl)-2-N-arachidoylsphingosine (PEG-$CerC_{20}$) were synthesized as described elsewhere,[37] and dioleoyldimethylammonium chloride (DODAC) was kindly provided by Dr S Ansell (Inex Pharmaceuticals). Octylglucopyranoside (OGP), HEPES and NaCl were obtained from Sigma (St Louis, MO, USA). The plasmid pCMVCAT (4490 bp, coding for the chloramphenicol acyl transferase gene) was originally obtained from Dr K Brigham (Vanderbilt University, Nashville, TN, USA). The plasmid pCMVLuc (5650 bp, coding for the luciferase reporter gene) was provided by Dr P Tam (Inex Pharmaceuticals). All reporter genes were under the control of the human CMV immediate–early promoter-enhancer element. ^{3}H-cholesteryl hexadecyl ether (CHE) and ^{14}C-CHE were obtained from Mandel Scientific (Guelph, ON, Canada). Mouse serum was obtained from CedarLane (Mississauga, ON, Canada). Dialysis tubing (SpectraPor 12 000 to 14 000 mwco) was obtained from Fisher Scientific (Ottawa, ON, Canada), DEAE-Sepharose CL-6B column from Sigma, *E. coli* DNase I from Life Technologies (Mississauga, ON, Canada) and the luciferase assay kit from Promega (Madison, WI, USA).

Preparation of plasmids

Plasmid DNA was transformed into *E. coli* strain DH5α by electroporation. Plasmid DNA was then isolated from *E. coli* by alkaline lysis[38] followed by anion exchange chromatography (according to the manufacturer, Qiagen, Santa Clarita, CA, USA) or CsCl gradient centrifugation.[39] DNA was precipitated and dissolved in pyrogen-free water for formulation with lipids.

Radiolabeled ^{3}H-plasmid DNA was isolated from an *E. coli* JM101 strain bearing pCMVβ, pCMVCAT or pCMVLuc. Briefly, cultures were grown in supplemented minimal media (M9 salts with 0.1% thiamine, 1% glucose, 100 μg/ml ampicillin) to mid log phase. Ten mCi of 81.9 mCi/mmol tritiated thymidine (Mandel Scientific) was added, then the cultures were allowed to grow for a further 12–16 h. Plasmid DNA was isolated by alkaline lysis and anion exchange chromatography, as described above.

Encapsulation of plasmid DNA

Plasmid DNA (50–400 μg) was incubated with DODAC in 500 μl of 0.2 m octylglucoside, 150 mm NaCl, 5 mm HEPES pH 7.4 for 30 min at room temperature. The plasmid-DODAC mixture was then added to DOPE and PEG-$CerC_{14}$ or PEG-$CerC_{20}$ dissolved in 500 μl of 0.2 m OGP; 150 mm NaCl, 5 mm HEPES pH 7.4. The total lipid concentration was either 5 or 10 mg/ml with DOPE: DODAC:PEG-Cer at molar ratios of 84:6:10, unless otherwise indicated. The plasmid-lipid mixture was dialyzed against 5 mm HEPES in 150 mm NaCl pH 7.4 (HBS) for 36 to 48 h with two buffer changes. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE-Sepharose CL-6B column (1 × 4 cm). To determine the encapsulation efficiency, a 50-μl aliquot of each sample was loaded on to a DEAE-Sepharose CL-6B column (1 ml) equilibrated with HBS. The column was eluted with HBS and the fractions were assessed for ^{3}H-plasmid and ^{14}C-lipid by scintillation counting.

Isolation of encapsulated plasmid by sucrose density gradient centrifugation

The fractions from the DEAE column containing co-eluting lipid and plasmid were pooled and equal volumes were applied to the top of a discontinuous sucrose gradient in 12.5 ml ultracentrifuge tubes. The gradient was formed with 3 ml each of 10% sucrose, 2.5% sucrose and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 160 000 *g* for 2 h at 20°C and separated into aliquots (250 μl) removed from top to bottom. The fractions were assayed for ^{3}H-plasmid and ^{14}C-CHE by dual-label scintillation counting. The lipid encapsulated plasmid DNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. It was found that the isolated SPLP could be concentrated by dialysis against 500 000 molecular weight carboxymethyl cellulose (Aquacide II; Calbiochem, San Diego, CA, USA) in a 12 000–14 000 molecular weight cut-off dialysis tube. When the desired volume was reached, the formulation was transferred into a new dialysis bag and dialyzed overnight against HBS to adjust the NaCl concentration to 150 mm.

Freeze–fracture electron microscopy

Freeze–fracture was performed on a Balzers Freeze-Etching system, BAF 400D (Balzers, Lichtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid freon 22. The fractured surface was shadowed unidirectionally with platinum/carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a Jeol model JEM 1200 EX electron microscope (Jeol, Montreal, QC, Canada).

Serum stability assay

SPLP formulations were assayed for serum stability in the presence of 90% mouse serum *in vitro*. A 50 μl aliquot was added to 450 μl mouse serum and incubated at 37°C for 30 min. The sample was then loaded on to a Sepharose CL-4B column and eluted with HBS, pH 7.4. The fractions were analyzed for ^{3}H-plasmid and the lipid label ^{14}C-CHE.

Determination of DNase I stability

Three sets of samples were exposed to DNase I digestion, including naked plasmid DNA, plasmid complexed with DOPE:DODAC vesicles and SPLP. Plasmid–cationic lipid complexes were prepared by mixing 500 μl plasmid (pCMVLuc, 0.5 mg/ml) in 5% glucose with 500 μl DODAC:DOPE (1:1) 100 nm diameter LUVs (0.9 mm lipid) prepared by the extrusion method[40] in 5% glucose. This corresponds to a lipid-to-DNA charge ratio (positive-to-negative) of 3. The resulting solution was incubated at room temperature for 30 min before DNase I treatment. For the DNase digestion, samples (free plasmid, plasmid–lipid complex, encapsulated plasmid) containing 1 μg of DNA were incubated with 0, 100 or 1000 units of DNase I in a total volume of 100 μl of 5 mm HEPES, 150 mm NaCl, 10 mm $MgCl_2$ pH 7.4 in the presence or absence of 1.0% Triton X-100. After incubation at 37°C for 30 min, the DNA was isolated by adding 500 μl of DNAzol (Life Technologies) followed by 1.0 ml of ethanol. The samples were centrifuged for 30 min at 20 000 *g* in a tabletop microfuge. The supernatant was decanted and the DNA pellet was washed twice with 80% ethanol and dried. The DNA was dissolved in 30 μl of TE buffer and analyzed by agarose (1.0%) gel electrophoresis in TAE buffer.

In vitro transfection

COS-7 cells and 293 cells were grown at 37°C, 5% CO_2 in complete media consisting of T75 flasks in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) and 10% fetal bovine serum (FBS; Intergen, Purchase, MA, USA). Transfections were performed in the presence of cell culture media when the cells were 60–70% confluent. The plasmid (pCMVLuc) formulations were diluted in complete medium to give 0.5 μg DNA/ml. The cells were incubated in the presence of the plasmid formulations for up to 120 h and assayed for luciferase activity. Calcium phosphate-mediated transfection with plasmid extracted from SPLP was performed as follows. Plasmid (0.1–1 μg) in 50 μl 0.25 m $CaCl_2$ was slowly added to 50 μl HBS, and the resulting precipitate was added to 293 cells. Following incubation for 2 days at 37°C, the luciferase activity was determined.

Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay System reagent kit (Promega E1501) according to the manufacturer's instructions. Cell lysates were assayed for luciferase activity using a Dynex Technologies ML3000 microplate luminometer (Dynex Technologies, Ghentilly, VT, USA). Luminescence readings were calibrated according to a standard curve obtained using a *Photinunus pyralis* luciferase standard (Boehringer Mannheim, Laval, QC, Canada; 634 409).

PEG-Cer dissociation rates

The dissociation rates of ^{3}H-PEG-CerC_{14} and ^{3}H-PEG-CerC_{20} from EPC LUV using EPC multilamellar vesicles (MLV) as a 'sink'. The LUV were prepared containing 10 mol% PEG-Cer and a trace of ^{14}C-CHE (^{3}H/^{14}C ratio approximately 5) by detergent dialysis as described above for SPLP. MLV were prepared by hydration of EPC in HBS (250 mg/ml) at 65°C. The MLV were washed five times in HBS by centrifugation (2 min at 12 000 *g*) to remove any small vesicles. LUV (1 mg lipid) were mixed with 125 mg MLV to give a final volume of 1.5 ml and incubated at 37°C. At different time intervals, 100 μl of the mixture were transferred into 0.5 ml ice-cold HBS and the MLV pelleted by centrifugation. The LUV in the supernatant were analyzed for ^{3}H-PEG-Cer and ^{14}C-CHE and the ^{3}H/^{14}C ratio plotted as a function of time.

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