Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties

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Abstract

Previous work has shown that plasmid DNA can be encapsulated in small 'stabilized plasmid-lipid particles' (SPLP) composed of 1,2-dioleyl-3-phosphatidylethanolamine (DOPE), the cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) and poly(ethylene glycol) (PEG) conjugated ceramides (PEG-Cer), employing a detergent dialysis procedure. These SPLP have potential as vectors for in vivo gene therapy. This study is aimed at characterizing the influence of the cationic lipid and PEG-Cer species on SPLP formation and in vitro transfection properties. It is shown that the transfection potency of SPLP is sensitive to the cationic lipid species employed, the size of the PEG polymer incorporated in the PEG-ceramide and the length of the acyl chain contained in the ceramide anchor. With regard to the influence of cationic lipid, the transfection levels achieved were highest for SPLP containing N-[2,3-(dioleyloxy)propyl]-N,N-dimethyl-N-cyanomethylammonium chloride (DODMA-AN) and lowest for SPLP containing 3-β-[N-(N',N'-dimethylaminoethyl)carbamoyl]-cholesterol (DC-CHOL), according to the series DODMA-AN > N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) > DODAC > N,N-distearyl-N,N-dimethylammonium chloride (DSDAC) > DC-CHOL. Incorporation of short (PEG_{750}) PEG polymers in the PEG-ceramide components resulted in modest improvements in transfection levels over PEG_{2000} and PEG_{5000} polymers, however variation of the length of the acyl chain contained in the hydrophobic ceramide anchor from octanoyl ($PEG\text{-}CerC_8$) to myristoyl ($PEG\text{-}CerC_{14}$) to arachidoyl ($PEG\text{-}CerC_{20}$) had the most dramatic effects. Transfection levels achieved for SPLP containing $PEG\text{-}CerC_8$ were substantially larger than observed for SPLP containing $PEG\text{-}CerC_{14}$ or $PEG\text{-}CerC_{20}$, consistent with a requirement for the PEG-ceramide to dissociate from the SPLP surface for maximum transfection potency. It is also shown that the ability of SPLP to be accumulated into cells is

a dominant factor influencing transfection potency, and that the transfection potency of SPLP that are accumulated is at least equivalent to that of cationic lipid-plasmid DNA complexes. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plasmid DNA-cationic liposome complexes have considerable utility as non-viral gene transfer systems [1,2]. However, complexes are large, charged, heterogeneous structures that have limited utility in certain applications. For example, the large size and positive charge of complexes results in rapid clearance from the circulation following intravenous injection, largely limiting transfection to 'first pass' organs such as the lung, liver or spleen [1–6]. In this regard recent work employing a detergent dialysis technique [7] has shown that plasmid DNA can be encapsulated in 'stabilized plasmid-lipid particles' (SPLP) that are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. These particles are small (diameter approximately 70 nm), contain high (>80 mol%) levels of the 'fusogenic' lipid dioleylphosphatidylethanolamine (DOPE), low (<10 mol%) levels of the cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), protect encapsulated plasmid from degradation by serum nucleases, and are well-defined and stable systems containing one plasmid per particle [7].

The transfection potency of SPLP as generated by Wheeler et al. [7] is limited. However, SPLP are flexible systems that can potentially be constructed from a variety of compounds, the choice of which may strongly affect transfection properties. Such compounds include the cationic lipid contained in the SPLP and the PEG coating surrounding the SPLP. For example, plasmid DNA-cationic lipid complexes which contain different cationic lipids can exhibit markedly different transfection properties both in vitro and in vivo [6,8–12]. Alternatively, it has been shown that the presence of PEG coatings on vesicles can dramatically inhibit intervesicular contact and fusion [13]; and the presence of PEG-PE in DNA-cationic lipid complexes can dramatically reduce in vivo transfection activity [14].

In this work we investigate the influence of different species of cationic lipid and PEG coatings on the formulation and in vitro transfection properties of SPLP. It is shown that whereas the SPLP formulation process is relatively independent of the (monovalent) cationic lipid species employed, the highest levels of expression are observed for N-[2,3-(dioleyloxy)propyl]-N,N-dimethyl-N-cyanomethylammonium chloride (DODMA-AN). Alternatively, incorporation of shorter acyl groups in the ceramide which 'anchors' the PEG to the SPLP surface, which results in rapid dissociation of the PEG from the SPLP surface, dramatically improves transfection levels. Finally, it is shown that low levels of cellular uptake is a dominant parameter modulating the transfection potential of SPLP, and that the intrinsic transfection potency of SPLP that are taken up is at least equivalent to that exhibited by complexes.

2. Materials and methods

2.1. Lipids and chemicals

N,N-Dioleyl-N,N-dimethylammonium chloride (DODAC), N-[2,3-(dioleyloxy)propyl]-N,N-dimethyl-N-cyanomethylammonium chloride (DODMA-AN), N,N-distearyl-N,N-dimethylammonium chloride (DSDAC), 3-β-[N-(N',N'-dimethylaminoethyl)-carbamoyl]-cholesterol (DC-CHOL) were obtained from Dr. S. Ansell (Inex Pharmaceuticals, Burnaby, B.C., Canada). 1-O-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-N-octanoylsphingosine (PEG-CerC$_8$), 1-O-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)-succinoyl)-2-N-myristoylsphingosine (PEG-CerC$_{14}$), 1-O-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-N-arachidoylsphingosine (PEG-CerC$_{20}$), 1-O-(2′-(ω-methoxypolyethyleneglycol$_{(750)}$)succinoyl)-2-N-myristoylsphingosine (PEG$_{750}$-CerC$_{14}$), 1-O-(2′-(ω-methoxypolyethyleneglycol$_{(5000)}$)succinoyl)-2-N-myristoylsphingosine (PEG$_{5000}$-CerC$_{14}$) were obtained from Dr. Z. Wang (Inex), and N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) was synthesized as described in previous

work [15]. 1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). ^{14}C-labeled cholesteryl hexadecyl ether (^{14}C-CHE) was obtained from DuPont NEN Products (Boston, MA, USA). Spectra/Por 2 molecular-porous membrane tubing (MW 12 000–14 000) was purchased from VWR Scientific (Edmonton, Alta, Canada). Magnesium sulfate, β-mercaptoethanol, potassium acetate, potassium chloride, sodium acetate, sodium phosphate, sodium chloride, sodium hydroxide, and sucrose were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ampicillin, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), lithium chloride (LiCl), lysozyme, *n*-octyl-β-D-glucopyranoside (OGP), poly(ethylene glycol) (PEG$_{8000}$), sodium dodecyl sulfate, tris(hydroxymethyl)aminomethane (Tris), diethylaminoethyl (DEAE) Sepharose CL-6B anion exchanger, and Sepharose CL-4B resins were obtained from Sigma (St. Louis, MO, USA). Bactotryptone and bacto-yeast extract were purchased from Difco (Detroit, MI, USA). All organic solvents were purchased from Fisher Scientific (Nepeau, Ont., Canada). Agarose was purchased from Bio-Rad (Richmond, CA, USA). Aquacide II was purchased from Calbiochem (La Jolla, CA, USA). Glycerol and glucose were obtained from BDH (Toronto, Ont., Canada). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Burlington, Ont., Canada). Chlorophenol red galactopyranoside (CPRG) was purchased from Boehringer Mannheim (Germany). Scintillation fluid (Ultima Gold grade) was obtained from Packard Instrument (Downers Grove, IL, USA). *Xho*I and *Hin*dIII restriction endonuclease and ribonuclease I 'A' from bovine pancreas (RNase) were purchased from Pharmacia Biotech (Uppsala, Sweden). BHK cells (BHK 21) were obtained from American Tissue Culture Collection (ATCC CCL-10). Distilled water was purified from Corning Mega-Pure MP-4S system.

2.2. Plasmid

pCMVCAT plasmid, initially obtained from Inex, and pCMVβgal plasmid, initially purchased from Clontech, were amplified in *Escherichia coli* (DH5α)

with the selection of resistance to ampicillin, and were isolated by alkali lysis and purified by PEG precipitation as described elsewhere [15,16]. The purity of pCMVCAT and pCMVβgal was confirmed by 1% agarose gel electrophoresis with restriction endonuclease *Xho*I or *Hin*dIII digest, respectively. The plasmid concentration was determined by using standard phosphorus assays [17] and was expressed as phosphorus concentration of plasmid DNA. ^{3}H-labeled pCMVCAT was obtained from A. Annuar (Inex).

2.3. Preparation of vesicles

Mixtures of lipids dispensed in chloroform were dried under a stream of nitrogen gas with continuous vortex mixing. The residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with 150 mM NaCl, 20 mM HEPES (pH 7.4) buffer and then freeze-thawed five times to produce multilamellar vesicle (MLV) systems. Large unilamellar vesicle system (LUVs) were obtained by extruding MLVs 10 times through two 100-nm pore size polycarbonate filters (Costar Nuclepore) under nitrogen at a pressure of 300–400 psi [18]. The size of the LUVs was checked by quasi-elastic light scattering (QELS) employing a Nicomp model 270 submicron particle sizer operating in the vesicle mode. Phosphorus assays were used to quantify phospholipid concentration [17].

2.4. Preparation of plasmid DNA-cationic lipid complexes

Plasmid DNA-cationic lipid complexes were formed by incubating appropriate amounts of preformed DOTMA/DOPE (1:1) LUVs with pCMVβgal to obtain the desired charge ratio (positive-to-negative) of 1.0 in 100 μl distilled water. The resulting mixture was incubated at room temperature for 20–30 min, and was then mixed with an equal volume of culture media (see below).

2.5. Preparation of stabilized plasmid-lipid particles (SPLP)

SPLP were prepared employing the method of Wheeler et al. [7] with modifications. Briefly, for

each preparation, appropriate amounts of cationic lipid dissolved in chloroform were dried under a stream of nitrogen gas. Similarly, appropriate amounts of DOPE, PEG-ceramide and trace amounts of ^{14}C-CHE (as the lipid marker) were mixed in chloroform and dried. Residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated separately. *n*-Octyl-β-D-glucopyranoside (OGP, 50 µl of a 1.0 M solution) was added to the dried cationic lipid followed by brief vortexing. Subsequently 200 µl of an aqueous solution containing appropriate amounts of NaCl, pCMVCAT and trace amounts of ^{3}H-pCMVCAT was added. Unless indicated otherwise, 25 µg pCMVCAT and 5.0 µmol total lipids in 0.5 ml total volume were used for each preparation in the entrapment studies. After thorough mixing, the clear homogenous mixture was incubated at room temperature for 30 min. A final concentration of 200 mM OGP in 150 mM NaCl was used for each preparation. The lipid-containing medium was made by adding 250 µl of 200 mM OGP in 150 mM NaCl to the dried DOPE/PEG-Cer/^{14}C-CHE. After mixing and incubating at room temperature for 30 min, a clear homogeneous mixture was obtained. The two mixtures were then mixed together and transferred into a 6 cm Spectra/Por 2 molecular-porous membrane tubing (MW 12 000–14 000) for dialysis for 40 h against two changes of a 150 mM NaCl, 5 mM HEPES (pH 7.4) buffer. The SPLP thus formed were then purified by DEAE anion exchange chromatography and sucrose density gradient centrifugation as indicated below, and characterized with respect to plasmid entrapment and size. All experiments involved triplicate samples. For the transfection studies, samples of SPLP containing pCMVβgal were prepared similarly with the exception that 400 µg pCMVβgal, 10 µmol total lipids, and a total volume of 1.0 ml were used initially.

2.6. Quantification of DNA entrapment and lipid recovery using anion exchange column chromatography

A column chromatography procedure employing DEAE-Sepharose CL-6B to remove free DNA was utilized [7]. The average of two 40 µl aliquots of the SPLP dialysate radioactivity was used as a reference

for ^{3}H and ^{14}C radioactivity. The SPLP solution (100 µl of the dialysate) was applied to the DEAE-Sepharose column (diameter 1.0 cm, height 1.5 cm) and eluted using 150 mM NaCl, 20 mM HEPES (pH 7.4) buffer. Six fractions of 10 droplets each were collected in scintillation vials and were counted for ^{3}H and ^{14}C radioactivity. The percentage of recovery was obtained by comparing the total eluant radioactivity with the reference radioactivity after background correction. Thus the ^{3}H recovery represents the fraction of DNA associated with the SPLP and sequestered from the anion exchanger, whereas the ^{14}C recovery represents the fraction of lipid in the void volume. Free plasmid DNA bound to the column could be washed out using 10 ml of a 5.0 M NaCl solution. A Beckman LS3801 scintillation counter was used for all radioactivity measurements.

2.7. Purification of SPLP using sucrose density gradient centrifugation

All samples of SPLP used for transfection were further purified using sucrose density gradient centrifugation [7]. SPLP were prepared as outlined above with 400 µg pCMVβgal and 10 µmol total lipids in a total volume of 1.0 ml and free DNA was removed by passing through a DEAE column. The diluted eluant (3 ml) was transferred to a dialysis bag and briefly dried by placing Aquacide II around the bag. After the desired volume was reached, the contents were dialyzed overnight in 150 mM NaCl, 5 mM HEPES (pH 7.4) buffer. The concentrated eluant (800 µl) was then subjected to sucrose density gradient centrifugation (160 000×*g* for 2.5 h). The gradient was formed by loading 3.67 ml each of 1.0%, 2.5%, and 10% sucrose in 150 mM NaCl, 20 mM HEPES, pH 7.4 buffer into a centrifuge tube (Beckman Ultra-Clear Tubes) using a drawn out glass pipette. The crude SPLP preparation was then applied to the top of the gradient and centrifuged using a swinging bucket rotor (SW-41Ti) in an ultracentrifuge (Beckman L2-65B). Following centrifugation, a band of concentrated SPLP with high DNA content was observed at the interface between the 2.5% and 10% sucrose gradient levels. The fractionation profile was achieved by removing 250 µl fractions from the top of the gradient, and these fractions were counted for ^{3}H and ^{14}C radioactivity. An ali-

quot (100 μl) of the sample before density gradient separation was also counted as a reference. For the transfection studies, the band corresponding to the SPLP with high DNA content was isolated using needle suction. The resulting purified SPLP were concentrated by Aquacide II treatment as outlined above, and then dialyzed against 150 mM NaCl, 5 mM HEPES (pH 7.4) with one change of buffer. Quantification of DNA and lipid was performed by comparing the ^{3}H and ^{14}C radioactivity of the purified SPLP against the radioactivity of crude SPLP before sucrose density gradient. The overall recoveries of DNA and lipid were computed by multiplying the recovery from the DEAE column by the recovery from sucrose density gradient. For size analysis, purified SPLP were transferred into a 6×50 mm borosilicate glass tube and placed in a Nicomp Model 270 submicron particle sizer, using the particles mode of QELS.

2.8. Transfection studies employing BHK cells

The transfection protocol of Felgner et al. [11] was employed with certain modifications. Purified SPLP were prepared as described above. Transfection studies employing SPLP were conducted on samples containing 2.0 μg pCMVβgal. This high amount of plasmid could be employed due to the low toxicity of SPLP. Complexes (charge ratio of 1.0) were formed by incubating DOTMA/DOPE (1:1) vesicles with appropriate amounts of pCMVβgal for 30 min before transfection, as outlined in previous work [15]. Transfection studies employing complexes used 0.5 μg plasmid. All transfection studies were performed in triplicate. Standards of β-galactosidase (β-gal) were prepared by two-fold serial dilutions of 200 mU β-gal with 0.5% BSA in phosphate buffered saline (PBS) (pH 8.0). First, BHK 21 cells cultured in DMEM with 10% fetal bovine serum (FBS) and 100 units of penicillin and 100 μg streptomycin were plated onto a 96 well plate with 2×10^4 cells per well. Wells used for the DNA standards were not plated with cells. The plate was then incubated for 20 h at 37°C with 5% CO_2. For each well containing the cells, appropriate amounts of complexes or SPLP were diluted with DMEM/FBS, and aliquots of 100 μl were used for transfection at 37°C, 5% CO_2 with incubation time of 4 or 24 h, respec-

tively. For the 4 h transfection studies, the transfected media was removed, and 100 μl of DMEM/FBS was added. The cells were further incubated for 20 h at 37°C, 5% CO_2. After incubation, the incubated media was removed, and the cells were lysed by adding 50 μl of lysis buffer containing 0.1% Triton X-100 in 250 mM phosphate buffer (pH 8.0) and were freeze-thawed (−70°C) to ensure complete lysis. After thawing, aliquots of 10 μl of the lysis buffer was transferred to another 96 well plate for protein analysis. The remaining samples were assayed for β-gal activity. Briefly, 50 μl of PBS containing 0.5% BSA or 50 μl of appropriate β-gal standard (0–100 mU) was added. Color development was induced by adding 150 μl of the substrate buffer containing chlorophenol red galactopyranoside (CPRG) (1 mg/ml), 60 mM Na_2HPO_4, 1 M $MgSO_4$, 10 mM KCl, and 50 mM β-mercaptoethanol. For the protein assay, 50 μl of BSA standard (0–20 μg) was prepared by serial dilution in lysis buffer. An additional 40 μl of lysis buffer was added to the 10 μl sample aliquot. The BCA reaction mixture was prepared according to established protocols from Pierce (Rockford, IL, USA) prior to the assay, and 100 μl of this mixture was added to each well, including the protein standards. The plate was incubated at 37°C for 2 h or until the color was developed. The amounts of β-gal and protein were quantified against the standard, after volume adjustment, and the β-gal activity was expressed as mU of β-gal per mg of protein. All absorbance readings were measured at 540 nm using a Microplate Autoreader EL-309 (Bio-Tek Instruments). For the transfection of SPLP formed with different PEG-Cer, 1×10^5 BHK cells were plated in a 24 well plate. Aliquots of 60 μl and 10 μl were used for the BSA and BCA assay, respectively.

2.9. Cellular uptake studies of plasmid DNA

BHK cells were plated at a density of 5×10^5 cells per 25 cm^2 T-flask the day before transfection. For each transfection, complexes containing 2 μg pCMVβgal at a charge ratio of 1.0 were formed by incubating DOTMA/DOPE (1:1) LUVs with pCMVβgal for 20–30 min at room temperature before transfection. Purified SPLP samples (DOPE/DOTMA/PEG$_{2000}$-CerC$_8$, 73:7:20) containing 2 μg pCMVβgal were also used for each transfection.

Both plasmid DNA-cationic lipid complexes and SPLP samples were made up to a final volume of 2 ml with DMEM containing 10% FBS before applying to BHK cells. For the cells transfected with complexes, the transfection medium was replaced with complete DMEM after the 4 h incubation time point. Cells transfected with the SPLP samples were incubated in the transfection medium until the specific time point. All transfection studies were performed in triplicate. The cellular uptake kinetics of the plasmid DNA were analyzed by terminating the transfection process at 4, 8, 24, and 48 h. At each time point, the transfection medium was removed. The cells were washed twice with PBS, and then treated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na from Gibco BRL). The cells were then washed with an isotonic buffer (250 mM sucrose, 50 mM HEPES, pH 7.2, 3 mM $MgCl_2$) and were centrifuged at 2000 rpm for 2 min in a Sorvall MC 12 V centrifuge. The resulting cell pellets were resuspended in the isotonic buffer, and the number of cells was counted using a hemacytometer. Then, the cells were centrifuged at 2000 rpm for 2 min, and the pellets were treated with the lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E (1 mg/ml) at 37°C overnight.

2.10. Southern blot analysis of delivered plasmid DNA

Genomic DNA was isolated from the BHK cells transfected with DNA-cationic lipid complexes and SPLP [16]. Briefly, the cell lysates were extracted twice with phenol/chloroform (1:1), then the DNA was precipitated with 95% ethanol and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 200 μl per 1×10^6 cells. The recovery of total genomic DNA was determined by measuring the absorbance at 260 nm of the resuspended samples. Genomic DNA (5 μg) from each sample was loaded into a 1% agarose gel with a set of plasmid DNA standards (0–5 ng). After size fractionation, the agarose gel was incubated in a denaturing buffer (1.5 M NaCl, 0.5 N NaOH) for 1 h, and then in a neutralizing solution (1.5 M NaCl, 1 M Tris, pH 7.4) for 45 min. The DNA fragments were transferred to a nylon membrane by capillary blotting overnight with 3 M NaCl, 0.3 M sodium citrate at pH 7.0. The nylon membrane was then baked at 80°C for

1 h prior to the hybridization procedure. A ^{32}P-labeled plasmid DNA probe was prepared using the ^{T7}QuickPrime kit (Pharmacia Biotech) with *Bam*HI cut pCMVβgal. This probe was then added to the DNA blot and allowed to hybridize overnight at 68°C. The blot was washed 3 times with 300 mM NaCl, 30 mM sodium citrate (pH 7.0) containing 0.1% SDS and once with 30 mM NaCl, 3 mM sodium citrate (pH 7.0) containing 0.1% SDS. The blot was then exposed for 2–4 h on a PhosphoImager screen and subsequently scanned (Molecular Dynamics-PhosphoImager SI). The amount of plasmid DNA taken up into cells was normalized by dividing the total plasmid DNA (pg) recovered by the total genomic DNA (μg).

3. Results

3.1. Influence of cationic lipid species on formation of SPLP

Previous work has shown that incubation of plasmid DNA with the lipid mixture DOPE, PEG_{2000}-$CerC_{14}$ and the cationic lipid DODAC (84:10:6; molar ratios) in the presence of OGP followed by dialysis results in the formation of SPLP that are capable of low levels of transfection in vitro [7]. It is of interest to determine whether SPLP can be formed using other cationic lipids, and whether this influences transfection potency. Here we characterize SPLP formation and plasmid entrapment achieved using DOTMA, DODMA-AN, DSDAC, DODAC, and DC-CHOL (for structures see Fig. 1) using a total of 5.0 μmol lipid and 25 μg pCMVCAT plasmid. In these systems the PEG-$CerC_{14}$ content was held constant at 10 mol% of total lipid, and the cationic lipid content varied over the 0–20 mol% range. Plasmid entrapment was assayed employing the DEAE anion exchange column procedure detailed Section 2. As shown in Fig. 2, optimum entrapment levels of approximately 60% were achieved for each of the cationic lipids used, the only difference being that this maximum entrapment was observed at slightly different cationic lipid content depending on the cationic lipid species. The optimal lipid composition (DOPE/cationic lipid/PEG-$CerC_{14}$; molar ratios) for plasmid encapsulation was 83:7:10 for DOTMA and DOD-
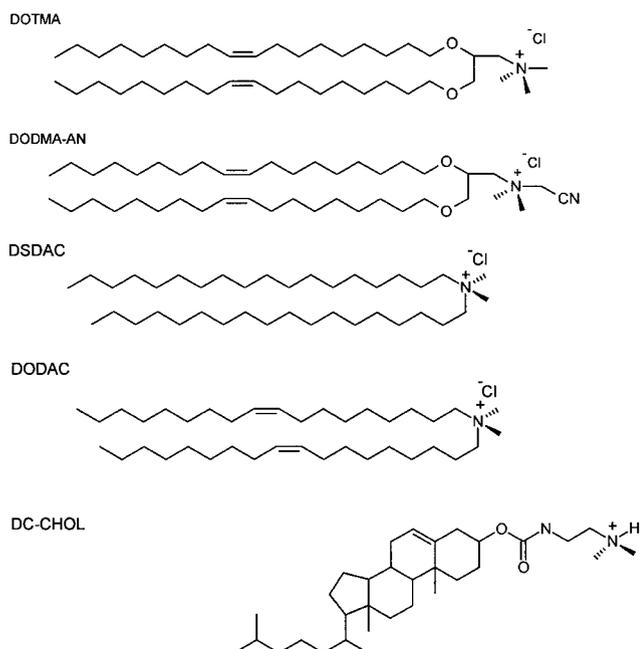
DOTMA

DODMA-AN

DSDAC

DODAC

DC-CHOL

Fig. 1. Structures of the cationic lipids used in this study.

MA-AN, 82.5:7.5:10 for DSDAC and DODAC, and 81:9:10 for DC-CHOL. These SPLP exhibited particle sizes of 90 ± 20 nm as measured by QELS.

3.2. Influence of the PEG polymer anchor on formation of SPLP

The PEG coating of SPLP is likely to inhibit association with cells, thus reducing transfection efficiency. Previous work has shown that SPLP constructed from PEG-CerC$_{20}$ did not result in appreciable transfection in vitro, whereas limited transfection was observed for SPLP containing PEG-CerC$_{14}$ [7]. This was attributed to an ability of the PEG-CerC$_{14}$ to dissociate from the SPLP surface. Here, we also examine the formulation and transfection properties of SPLP containing PEG$_{2000}$ polymers linked to ceramide anchors containing octanoyl acyl groups (PEG-CerC$_8$). Results for SPLP containing PEG-CerC$_{14}$ and PEG-CerC$_{20}$ are also presented for comparison (for structures see Fig. 3). Preliminary experiments suggested that more than 10 mol% of the PEG-CerC$_8$ was required to achieve optimal plasmid entrapment and, therefore, the entrapment profile was generated as a function of PEG-ceramide content rather than cationic lipid content. The detergent dialysis protocol was then applied
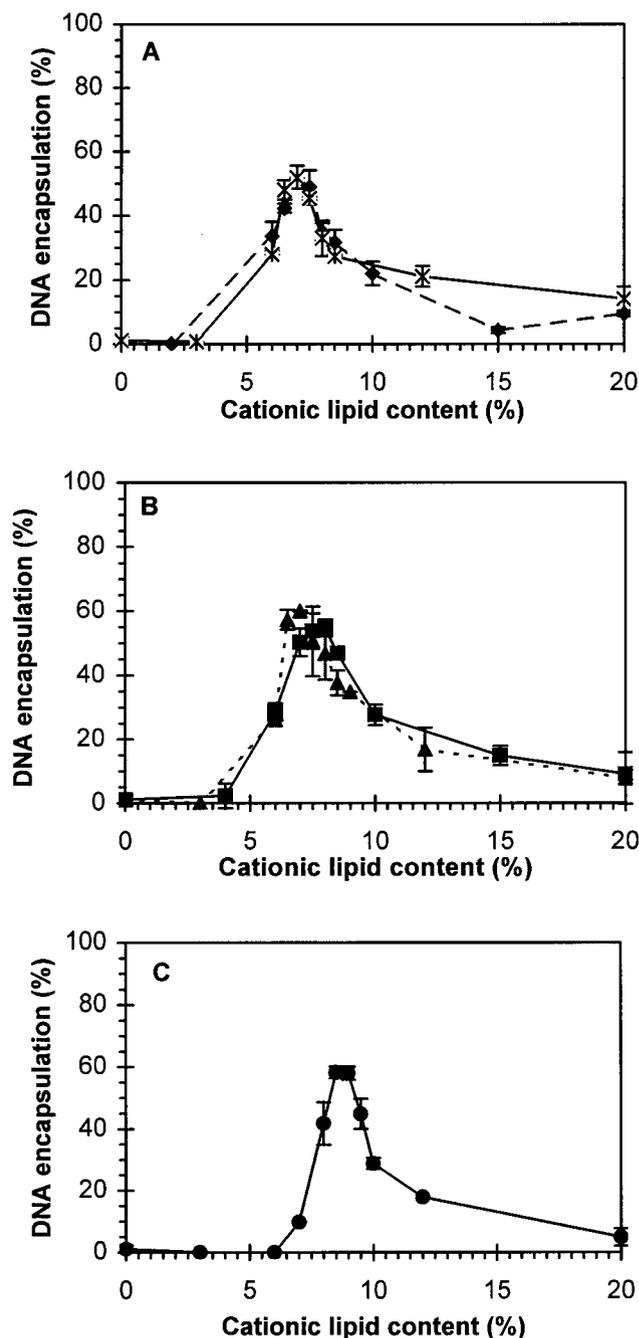
Fig. 2. Plasmid encapsulation efficiency for the SPLP detergent dialysis procedure as a function of cationic lipid content for a variety of cationic lipids: (A) DOTMA ($\blacklozenge$), DODMA-AN ($*$), (B) DSDAC ($\blacktriangle$), DODAC ($\blacksquare$), and (C) DC-CHOL ($\bullet$). SPLP were prepared as described in Section 2, employing 25 μg pCMVCAT plasmid with 5 μmol total lipid consisting of 10 mol% PEG-CerC$_{14}$, x mol% cationic lipid, and $90-x$ mol% DOPE. Encapsulation was assayed by measuring DNA recovery after passage of the dialysate through a DEAE Sepharose CL-6B column (see Section 2). The average and standard deviation from three individual experiments are shown.

PEG$_{2000}$	**Cer C$_8$**	**n = 45; x = 6**
PEG$_{2000}$	**Cer C$_{14}$**	**n = 45; x = 12**
PEG$_{2000}$	**Cer C$_{20}$**	**n = 45; x = 18**
PEG$_{750}$	**Cer C$_{14}$**	**n = 17; x = 12**
PEG$_{5000}$	**Cer C$_{14}$**	**n = 114; x = 12**

Fig. 3. Structures of poly(ethylene glycol)-ceramides (PEG-Cer) used in this study.

to systems containing 25 μg pCMVCAT and 5.0 μmol total lipids where the cationic lipid content was maintained at 7.5 mol% DODAC. As shown in Fig. 4, maximum entrapment levels are observed at approximately 20 mol% PEG-CerC$_8$, whereas the maximum plasmid entrapment for the PEG-CerC$_{14}$ and PEG-CerC$_{20}$ systems are in the range of 10–12 mol% of the PEG-Cer.

3.3. Influence of the PEG anchor on transfection properties of SPLP

The transfection properties of SPLP were investigated employing SPLP with entrapped pCMVβgal to allow a convenient assay for transfection. Further, the transfection protocol involved using purified SPLP, where the empty vesicles produced during the detergent dialysis procedure were removed by density gradient centrifugation as detailed in Section 2. The initial set of experiments was designed to ascertain appropriate transfection conditions for the SPLP (DOPE/DOTMA/PEG-CerC$_8$; 73:7:20; molar ratios) system. As shown in Fig. 5A, protocols employing SPLP containing 0.5 μg pCMVβgal gave little or no transfection; however significant transfection was observed for SPLP containing 1.0 or 2.0 μg of plasmid DNA at transfection times of 24 h or longer. Thus, a 24 h incubation time and 2.0 μg plasmid DNA was utilized for the standardized SPLP transfection protocol in subsequent experiments. It is of interest to compare the transfection properties of SPLP with that achieved employing plasmid DNA-cationic lipid complexes. As shown

in Fig. 5B, transfection of BHK cells employing pCMVβgal-DOTMA/DOPE (1:1) complexes gave transfection levels which are approximately an order of magnitude higher than observed for the SPLP preparation. In addition, good transfection activity was observed at low (0.5 μg pCMVβgal) plasmid levels and at short (4 h) incubation times.

In order to characterize the influence of the ceramide anchor on the transfection properties, SPLP containing pCMVβgal were generated by using 400 μg pCMVβgal and 10 μmol lipid mixtures. Lipid compositions of DOPE/DOTMA/PEG-Cer (83.5:6.5:10; molar ratios) using the amide chain lengths of C$_8$, C$_{14}$, and C$_{20}$ were prepared. These systems were then purified to remove empty vesicles employing the density centrifugation protocol, and the transfection properties of the purified SPLP containing PEG-CerC$_8$, PEG-CerC$_{14}$ and PEG-CerC$_{20}$ in BHK cells under standard transfection conditions (2 μg pCMVβgal; 24 h incubation) are illustrated in Fig. 6. A correlation between the transfection activities and the length of the ceramide anchor is observed. SPLP formed with PEG-CerC$_8$ give rise to 30-fold higher transfection activity than systems formed with PEG-CerC$_{14}$, which in turn results in 8-fold higher transfection activity than SPLP formed with PEG-CerC$_{20}$.

3.4. Influence of cationic lipid species on transfection properties of SPLP
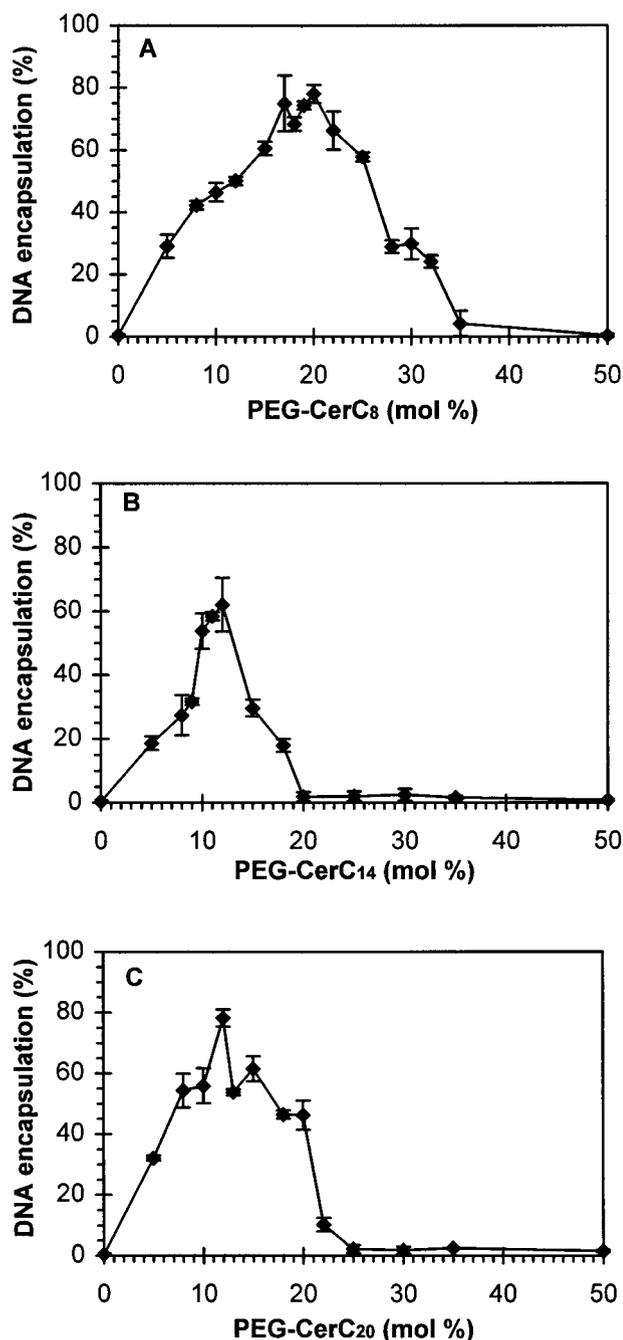
The transfection properties of SPLP containing the different cationic lipids with 20% PEG-CerC$_8$ are il-

Fig. 4. Plasmid encapsulation efficiency for the SPLP detergent dialysis procedure utilizing (A) PEG-CerC$_8$, (B) PEG-CerC$_{14}$ and (C) PEG-CerC$_{20}$. SPLP were prepared as described in Section 2 employing 25 µg pCMVCAT formulated with 5.0 µmol total lipids containing 7.5 mol% DODAC, x mol% PEG-Cer, and 92.5−x mol% DOPE. Encapsulation was assayed by measuring plasmid recovery after passage of the dialysate through a DEAE Sepharose CL-6B column. The average and standard deviations calculated from three individual experiments are shown.

lustrated in Fig. 7. The standard SPLP transfection protocol (2.0 µg pCMVβgal; 24 h incubation time) was utilized. It may be observed that the inclusion of different cationic lipids in SPLP did lead to different transfection activity, leading to the transfection potency profile of DODMA-AN > DOTMA > DODAC > DSDAC > DC-CHOL.
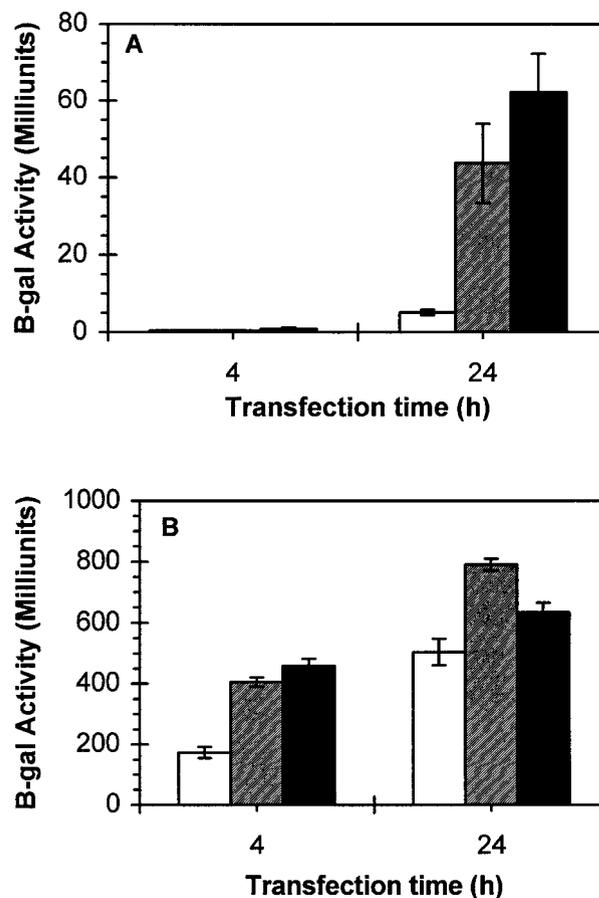
Fig. 5. Transfection properties of SPLP and plasmid DNA-cationic lipid complexes. (A) β-Gal expression in BHK cells resulting from incubation with SPLP containing 0.25 µg pCMVβgal (open bars), 1.0 µg pCMVβgal (shaded bars), and 2.0 µg pCMVβgal (solid bars) for 4 and 24 h is shown. (B) β-Gal expression in BHK cells resulting from incubation with complexes containing 0.25 µg pCMVβgal (open bars), 0.50 µg pCMVβgal (shaded bars), and 1.0 µg pCMVβgal (solid bars) for 4 and 24 h. SPLP (DOPE/DOTMA/PEG-CerC$_8$; 73:7:20; molar ratios) and plasmid DNA-cationic lipid complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0) were prepared from pCMVβgal as described in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.
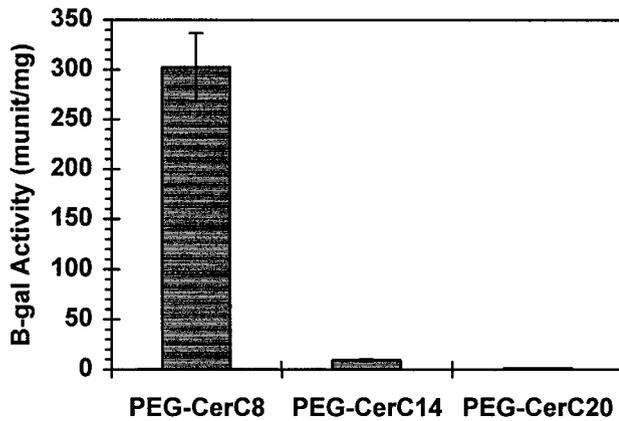
Fig. 6. Transfection properties of SPLP containing PEG-CerC$_8$, PEG-CerC$_{14}$ and PEG-CerC$_{20}$. BHK cells were transfected with SPLP composed of DOPE/DOTMA/PEG-Cer (83.5:6.5:10; molar ratios) and 2.0 µg pCMVβgal for 24 h as outlined in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.

3.5. Influence of PEG polymer length on formation and transfection properties of SPLP

An alternative approach to improve the transfection potency of SPLP is to reduce the length of the
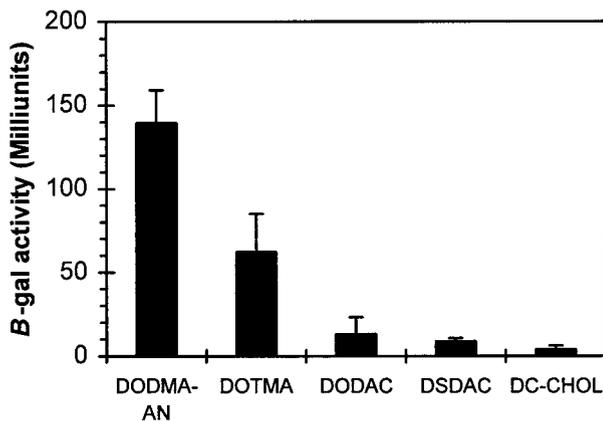
Fig. 7. Transfection properties of SPLP formed with the cationic lipids DODMA-AN, DOTMA, DODAC, DSDAC, and DC-CHOL and transfection achieved employing DOTMA-DOPE complexes. SPLP composed of cationic lipid (DODMA-AN and DOTMA, 7.0 mol%; DODAC and DSDAC, 7.5 mol%; DC-CHOL, 9.0 mol%), DOPE, and 20 mol% PEG-CerC$_8$ containing 2.0 µg pCMVβgal, and pCMVβgal-DOTMA/DOPE (1:1) complexes (charge ratio of 1.0; 0.5 µg pCMVβgal) were incubated with BHK cells and transfection assayed at 24 h. The average and standard deviation from triplicates are shown.

PEG polymer associated with the ceramide anchor. This was investigated for PEG-CerC$_{14}$ molecules containing PEG$_{750}$, PEG$_{2000}$ and PEG$_{5000}$. Relatively poor entrapment levels in the range of 40% were achieved for the PEG$_{750}$-CerC$_{14}$ and PEG$_{5000}$-CerC$_{14}$ systems as compared to nearly 60% for the PEG$_{2000}$-CerC$_{14}$ containing system (data not shown).

Transfection studies were performed on SPLP formed initially with 400 µg pCMVβgal and 10 µmol total lipids composed of DOPE, DOTMA, and PEG-CerC$_{14}$ (83.5:6.5:10; molar ratios) for all the PEG species. These preparations were then purified employing the density centrifugation procedure and used to transfect BHK cells according to the standard protocol. The transfection properties of these SPLP containing PEG$_{750}$-CerC$_{14}$, PEG$_{2000}$-CerC$_{14}$ and PEG$_{5000}$-CerC$_{14}$ are illustrated in Fig. 8. SPLP containing PEG$_{750}$ have slightly higher in vitro transfection potency than systems formed with PEG$_{2000}$ or PEG$_{5000}$.

3.6. Comparison of intracellular delivery of plasmid by SPLP and complexes

The results to this point indicate that SPLP containing DODMA-AN, PEG anchors with shorter amide chains and PEG coatings composed of shorter
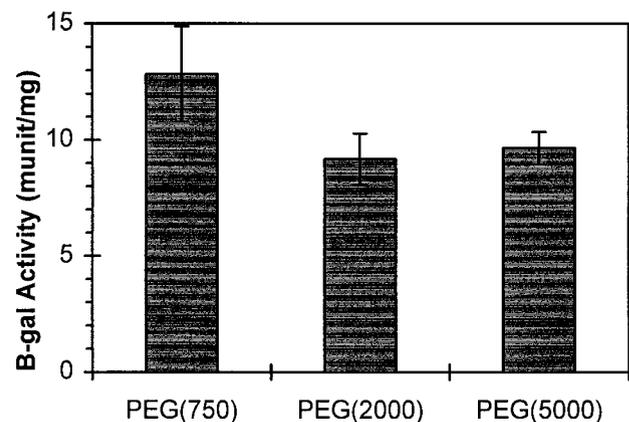

Fig. 8. Transfection properties of SPLP containing PEG-ceramides in which the size of the PEG polymer is varied (PEG$_{750}$, PEG$_{2000}$ and PEG$_{5000}$). β-Gal expression in BHK cells was assayed following incubation with SPLP containing 2.0 µg pCMVβgal for 24 h. SPLP (DOPE/DOTMA/PEG-CerC$_{14}$; 83.5:6.5:10; molar ratios) containing pCMVβgal were prepared as indicated in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.

PEG polymers result in improved transfection of BHK cells in vitro. However, in all cases the transfection levels achieved are substantially lower than those observed for plasmid DNA-cationic lipid complexes. This may result from a reduced affinity of SPLP for cells due to the presence of the PEG coating on the SPLP and the much reduced positive charge on the SPLP as compared to complexes. Both of these effects may act to substantially reduce the amount of plasmid that is delivered to the cell. In order to determine whether this could account for the reduced transfection potency of SPLP as compared to complexes, the time dependent cellular uptake of pCMVβgal in both lipid-based DNA carriers was investigated. SPLP were formed from 400 μg pCMVβgal and 10 μmol total lipids composed of DOPE/DOTMA/PEG-CerC$_8$ (73:7:20; molar ratios) and were purified by density centrifugation. Purified SPLP samples (2 μg pCMVβgal) and DNA-cationic lipid complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0; 2.0 μg pCMVβgal) were transfected as outlined in Section 2. Plasmid DNA delivered by the complexes demonstrate rapid, high cellular uptake and subsequent degradation with maximum plasmid levels at 4 h; whereas SPLP yield maximum plasmid delivery at a 8–24 h incubation (Fig. 9A). It may be noted that the maximum level of plasmid delivered by complexes is more than 30 times that delivered by SPLP.

The integrity of the delivered plasmid DNA over time is illustrated in Fig. 9B. Although the complexes delivered much more plasmid, this plasmid was degraded more rapidly than that delivered by the SPLP systems. This is indicated by the smears observed in the DNA delivered by the complexes as shown in the agarose gel electrophoretic pattern (Fig. 9B). This indicates that plasmid DNA entrapped within the particles is not as susceptible to degradation by cellular enzymes as compared to plasmid DNA associated with complexes.

4. Discussion

This work was aimed at characterizing factors that influence the transfection potency of stabilized plasmid-lipid particles with the aim of improving transgene expression. It is shown that three factors that
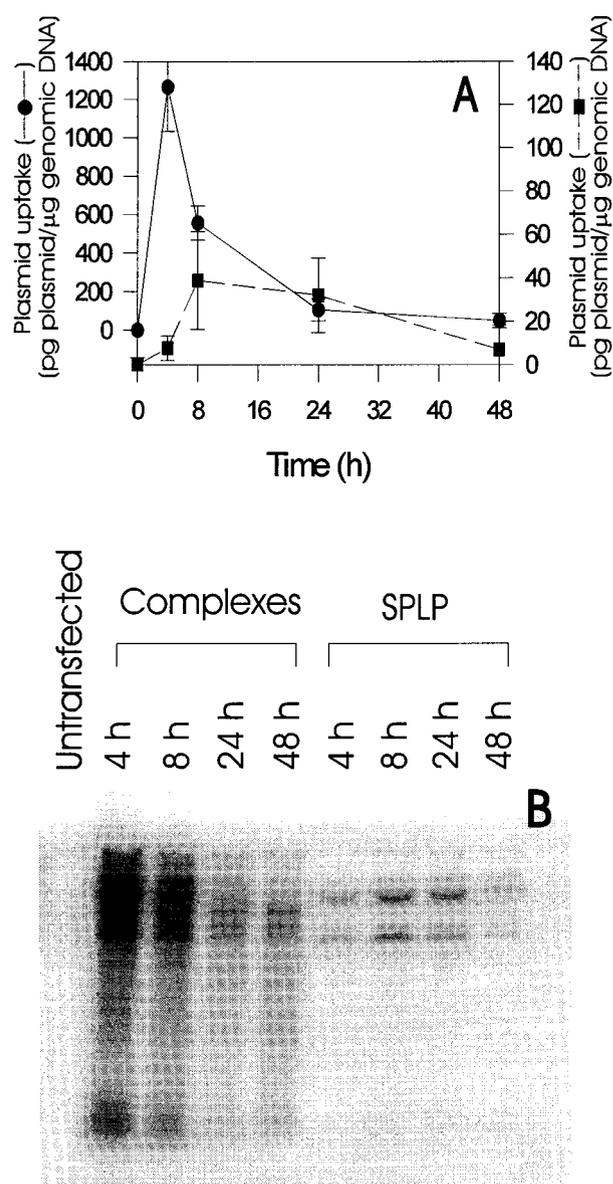

Fig. 9. Southern blot analysis of intracellular delivery of plasmid DNA by SPLP and plasmid DNA-cationic lipid complexes. SPLP composed of DOPE/DOTMA/PEG-CerC$_8$ (73:7:20; molar ratios) and complexes composed of DOTMA/DOPE (1:1)-pCMVβgal (charge ratio of 1.0) were used. DNA transfection and cellular uptake studies were performed as described in Section 2. Comparison of the cellular uptake of plasmid DNA using DNA-cationic lipid complexes (●) and SPLP (■) is shown in panel A. Southern blot analysis of the integrity of the plasmid DNA delivered by the two lipid-based DNA carriers is shown in panel B. The average and standard deviation from triplicates are shown.

can modulate SPLP transfection efficacy are the species of cationic lipid employed, the size of the PEG polymer coating the SPLP and the length of the acyl

chain contained in the ceramide 'anchor'. These factors are discussed in turn, followed by a discussion of the implications of the observation that the low levels of SPLP uptake into cells may be the primary parameter limiting transgene expression.

With regard to the influence of different cationic lipids, the results presented here demonstrate that plasmid encapsulation employing the detergent dialysis process is relatively independent of the species of monovalent cationic lipid employed. Although it is difficult to discern any definite trends, DODMA-AN and DOTMA appear to provide the maximum entrapment at the lowest cationic lipid content ($\sim 7\%$), followed by DODAC and DSDAC ($\sim 7.5\%$) and DC-CHOL ($\sim 9\%$). Interestingly, SPLP composed of DODMA-AN and DOTMA exhibit significantly higher transfection potencies than SPLP containing DODAC, DSDAC or DC-CHOL (Fig. 7). These results could be taken to suggest that cationic lipids with the greatest affinity for plasmid DNA under the conditions of detergent dialysis lead to the most potent transfection systems. In any event, it is clear that the species of cationic lipid does influence the transfection capability of the resulting SPLP, with DODMA-AN resulting in the highest transfection levels and DC-CHOL the lowest, with DODAC and DSDAC giving rise to intermediate transfection levels.

The second factor which clearly plays a major role in modulating the transfection potency of SPLP is the length of the acyl chain contained in the hydrophobic ceramide group which anchors the PEG coating to the SPLP surface. Previous work has shown that inclusion of PEG-CerC$_{14}$ in SPLP results in enhanced expression in vitro as compared to SPLP containing PEG-CerC$_{20}$, however the levels of gene expression were low in all cases [7]. The results presented here show that 30-fold higher transfection levels can be achieved for SPLP containing PEG-CerC$_8$ as compared to PEG-CerC$_{14}$ (Fig. 6). This improved transfection ability presumably reflects a faster leaving rate for the shorter chain PEG-ceramides from the SPLP surface in the sequence PEG-CerC$_8$ > PEG-CerC$_{14}$ > PEG-CerC$_{20}$, leaving the SPLP surface less shielded by PEG for the C$_8$ containing system. This effect is fully consistent with previous work showing that the half-time ($t_{1/2}$) for dissociation of PEG-CerC$_8$ from LUV is less than

1.2 min [19], whereas PEG-CerC$_{14}$ and PEG-CerC$_{20}$ exhibit $t_{1/2}$ values of 1.2 h and greater than 13 days, respectively [7]. It is also consistent with the observation that fusion between LUVs can be inhibited by the presence of a PEG coating anchored to PE molecules, and that fusogenicity could be restored by using PE anchors with short acyl chains [13]. Other workers have shown that the transfection potency of plasmid DNA-cationic lipid complexes is significantly reduced by the presence of PEG-PE [14]. The ability of the PEG coating on SPLP to inhibit transfection can arise due to reduced binding and therefore reduced uptake into target cells, or reduced efficiency in fusing with the endosomal membrane in order to achieve intracellular delivery of the plasmid.

The formulation properties of SPLP containing PEG-CerC$_8$ are clearly different from the properties of SPLP containing longer chain ceramides, in that optimum encapsulation is achieved at ~ 20 mol% PEG-CerC$_8$ as compared to ~ 10 mol% for SPLP containing PEG-CerC$_{14}$ or PEG-CerC$_{20}$ (Fig. 4). It is possible that the partition coefficient of the shorter chain PEG-CerC$_8$ for the SPLP outer monolayer is reduced, resulting in a requirement for a higher concentration of the PEG-CerC$_8$ to achieve adequate membrane levels for stabilization.

The third parameter, the length of the PEG polymer, appears to influence the formulation properties of the SPLP more than the transfection potency. The transfection potency of SPLP containing PEG$_{750}$, PEG$_{2000}$ and PEG$_{5000}$ coupled to CerC$_{14}$ are similar (Fig. 8), however the formulation properties differ significantly. In particular, the maximum efficiency for plasmid encapsulation that could be achieved for SPLP containing PEG$_{750}$ or PEG$_{5000}$ was $\sim 40\%$, as compared to $\sim 60\%$ for the PEG$_{2000}$ system (data not shown). Reduced plasmid entrapment for shorter PEG polymers would be expected due to the reduced steric stabilizing capacity of the shorter PEG polymers. The reason for the poorer entrapment for the SPLP containing PEG$_{5000}$-ceramide is not currently understood. It may result from a higher CMC of the PEG$_{5000}$-ceramide due to the larger size of the polar region, or to the interference of the longer PEG polymer with the association of plasmid with the lipid intermediates generated during formation of SPLP [7]. The fact that SPLP containing PEG-ceramides with shorter PEG polymers do not

exhibit significantly higher transfection potencies presumably reflects the fact that the presence of PEG-ceramides which are sufficient to stabilize formation of the SPLP during the detergent dialysis process are also sufficient to inhibit uptake into cells.

The final area of discussion concerns the observation that SPLP exhibit much lower levels of accumulation into target cells as compared to plasmid DNA-cationic lipid complexes, and that the plasmid delivered by SPLP remains intact in the cell for a longer time following cellular uptake. With regard to the first point, when BHK cells are presented with equivalent amounts of plasmid DNA in either the complex form or in SPLP (DOPE/DOTMA/PEG-CerC$_8$; 73:7:20; molar ratios) form, the maximum amount of plasmid that is delivered into the cells by the SPLP is less than 3% of the maximum amount delivered by the plasmid DNA-cationic lipid complexes. Thus even though the in vitro transfection potency of the complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0; 2 μg pCMVβgal) is at least 10-fold higher than the SPLP (DOPE/DOTMA/PEG-CerC$_8$; 73:7:20; 2 μg pCMVβgal; 24 h transfection), the reduced potency of the SPLP can be attributed to a 30-fold or more reduction in plasmid uptake as compared to complexes. This reduced plasmid uptake likely results from a low affinity of the SPLP particle for the cell membrane as compared to complexes, although breakdown of SPLP-associated plasmid could give similar results. The fact that previous studies [7] show SPLP plasmid to be highly stable and the results presented here showing that plasmid delivered to cells in SPLP is broken down more slowly than plasmid in complexes suggests that a low affinity of the SPLP for the cell is the dominant factor. In any event, the fact that the reduced potency of SPLP as compared to complexes can be attributed to low cellular uptake argues strongly that SPLP exhibit an intrinsic ability to transfect cells following uptake that is at least comparable to complexes. These results also suggest that the most direct way to improve the transfection properties of SPLP is to enhance cellular uptake. This could be accomplished in a number of ways, including incorporation of external targeting ligands to promote cell association and uptake.

The second observation that plasmid delivered to cells by SPLP is broken down at a much slower rate than plasmid delivered in complexes presumably reflects the resistance of the SPLP particle to breakdown by intracellular factors. It also points out the potential for more stable particles such as SPLP to extend the duration of transfection resulting from transfection by non-viral gene delivery systems.

In summary, this investigation characterizes factors that regulate the transfection potency of stabilized plasmid-lipid particles. It is shown that the transfection potency is sensitive to both the cationic lipid species and the species of PEG-ceramide employed to construct the SPLP. Improved transfection activity can be achieved by the use of the cationic lipid DODMA-AN, PEG-ceramides incorporating smaller PEG polymers and, most importantly, the use of PEG-ceramides containing shorter acyl groups in the ceramide anchor. Further, it is shown that the dominant factor leading to lower levels of transfection by SPLP is the low level of SPLP accumulation by the target cells. These observations point the way to achieve plasmid delivery systems that exhibit enhanced levels of gene expression for in vivo gene therapy.

Acknowledgements

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