Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1611 (2003) 204–216
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Transfection properties of stabilized plasmid–lipid particles containing cationic PEG lipids

Lorne R. Palmer[a,b], Tao Chen[a], Angela M.I. Lam[a], David B. Fenske[a], Kim F. Wong[a], Ian MacLachlan[b], Pieter R. Cullis[a,c,*]

[a] *Liposome Research Unit, Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3*
[b] *Protiva Biotherapeutics Inc., 150-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8*
[c] *Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8*

Received 7 February 2003; accepted 14 February 2003

Abstract

Recent work has shown that plasmid DNA can be efficiently encapsulated in well-defined "stabilized plasmid–lipid particles" (SPLP) that have potential as systemic gene therapy vehicles [Gene Ther. 6 (1999) 271]. In this work, we examine the influence of ligands that enhance cellular uptake on the transfection potency of SPLP. The ligand employed is a cationic poly(ethylene glycol) (PEG) lipid (CPL) consisting of a lipid anchor and a PEG_{3400} spacer chain with four positive charges at the end of the PEG (CPL_4). It is shown that up to 4 mol% CPL_4 can be inserted into preformed SPLP, resulting in up to 50-fold enhancements in uptake into baby hamster kidney (BHK) cells. The addition of Ca^{2+} to SPLP-CPL_4 (CPL_4-incorporated SPLP) results in up to 10^6-fold enhancements in transgene expression, as compared to SPLP in the absence of either CPL_4 or Ca^{2+}. These transfection levels are comparable to those observed for plasmid DNA–cationic lipid complexes (lipoplexes) but without the cytotoxic effects noted for lipoplex systems. It is concluded that in the presence of Ca^{2+} and appropriate ligands to stimulate uptake, SPLP are highly potent transfection agents.

Keywords: Liposome; Intracellular delivery; Gene therapy; SPLP; Calcium

1. Introduction

Work in this laboratory has focused on developing well-defined non-viral vectors for systemic gene therapy applications. This work has been guided by studies on liposomal systems containing anticancer drugs which have shown that small (diameter <100 nm), long circulating systems give rise to preferential delivery to distal tumor sites following intravenous (i.v.) injection [1–3]. We have therefore focused on the development of small, long circulating liposomal systems containing plasmid DNA. Techniques have been developed that allow the efficient encapsulation of plasmid DNA in well-defined "stabilized plasmid–lipid particles" (SPLP) of ~ 70 nm diameter that contain one plasmid per vesicle [4] and exhibit extended circulation lifetimes and preferential accumulation of intact plasmid at distal tumour sites following i.v. injection [5,6].

The levels of transgene expression observed at the tumour site following i.v. injection of SPLP containing the luciferase marker gene are superior to the levels that can be achieved using either plasmid DNA–cationic liposome complexes (lipoplexes) or naked DNA. However, for therapeutic benefits, SPLP-mediated expression may still need to be improved [5]. SPLP also exhibit low transfection potencies in vitro [4]. These poor transfection properties may be due to intrinsically low transfection potencies of the SPLP system or may reflect low levels of uptake into cells [7]. Here, we examine whether the insertion of ligands into SPLP that leads to enhanced cell uptake can also enhance in vitro transfection properties. Certain features of lipoplexes guided the choice of the ligand to stimulate non-specific uptake into cells. Cationic lipids in these lipoplexes facilitate electrostatic interactions between the negatively charged plasmid DNA and the positively charged liposome to form the

* Corresponding author. Liposome Research Unit, Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3. Tel.: +1-604-822-4144; fax: +1-604-822-4843.
E-mail address: pieterc@interchange.ubc.ca (P.R. Cullis).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00058-0

plasmid DNA–liposome complex. Moreover, the cationic lipids also provide an overall positive charge that enhances association of the complex with the negatively charged cell membrane [8]. The absence of a positive charge on the surface of the SPLP likely results in the reduced cellular uptake as compared to lipoplexes. We have therefore synthesized a series of cationic poly(ethylene glycol) (PEG) lipids (CPL) designed for post-insertion into preformed SPLP to impart a positive charge to the particle [9]. Previous work has shown that CPL containing distearoyl-PE (DSPE) coupled to PEG_{3400} containing a distal positive charge can be post-inserted into preformed large unilamellar vesicles (LUV) with the same lipid composition as SPLP, and that a CPL containing four positive charges (CPL_4) gives rise to maximum uptake of LUV-CPL into baby hamster kidney (BHK) cells [10]. We show here that CPL_4 can be inserted into preformed SPLP and that the resulting SPLP-CPL_4 exhibit improved uptake and markedly improved transfection potency in vitro.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Burnaby, BC). 1,2-Dioleoyl-3-phosphatidylethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl) (Rh-PE) and PicoGreen were obtained from Molecular Probes (Eugene, OR). *n*,*n*-Dioleoyl-*n*,*n*-dimethyl ammonium chloride (DODAC) was synthesized and supplied by Dr. S. Ansell of Inex Pharmaceuticals (Vancouver, BC). The PEG-$CerC_{20}$ was synthesized as indicated elsewhere [11] and was supplied by Dr. Z. Wang of Inex. The pCMVLuc plasmid encodes the *Photinus pyralis* luciferase gene under the control of the human cytomegalovirus (CMV) early promoter and was supplied by Dr. P. Tam of Inex. The pCMVGFP plasmid contains the gene for the green fluorescent protein from *Aequorea victoria* and was also supplied by Dr. P. Tam. DEAE-Sepharose CL-6B, Sepharose CL-4B, octyl-β-D-galactoside, and HEPES were obtained from Sigma-Aldrich (Oakville, ON). Lipofectin was obtained from Gibco BRL (Burlington, ON). BHK cells were obtained from Dr. R. MacGillivray of the Department of Biochemistry and Molecular Biology, UBC.

2.2. Preparation of SPLP-CPL_4

SPLP composed of DOPE:DODAC:PEG-$CerC_{20}$ (84:6:10; mol%) and containing the plasmid pCMVLuc (or pCMVGFP) were prepared according to the detergent dialysis method of Wheeler et al. [4] using purification by anion exchange (DEAE-Sepharose CL-6B) chromatography and sucrose density gradient centrifugation to remove unencapsulated plasmid and empty vesicles, respectively. SPLP containing Rh-PE were prepared by dissolving Rh-PE with other component lipids in $CHCl_3$ at a molar ratio of 83.5:10:6:0.5 (DOPE:DODAC:PEG-$CerC_{20}$:Rh-PE) before forming the lipid film.

CPL_4 was inserted into preformed SPLP by incubating SPLP (500 nmol lipid) with CPL_4 (12.5, 19, and 30 nmol) at 60 °C for 2–3 h in HEPES-buffered saline (HBS), pH 7.5, unless otherwise indicated. The integrity of plasmid DNA following CPL_4 insertion was assayed by Southern analysis as described below. Unincorporated CPL_4 was removed by gel filtration chromatography on a Sepharose CL-4B column equilibrated in HBS. Fractions (1 ml) were collected and assayed for CPL4, phospholipid, and DNA content. Fractions containing all three components were pooled and concentrated. CPL_4 content was determined by the fluorescence of the dansyl labeled CPL at λ_{em} 510 nm following excitation at λ_{ex} 340 nm employing a Perkin Elmer LS52 Luminescence spectrophotometer. A standard curve was derived from a stock solution of dansylated CPL in HBS. For SPLP containing Rh-PE, the phospholipid content was determined from the fluorescence of the Rh label measured at λ_{em} 590 nm following excitation at λ_{ex} 560 nm, using excitation and emission slit widths of 10 and 20 nm, respectively. For SPLP that did not contain the Rh label, phospholipid was determined using the method of Fiske and Subbarow [12], following lipid extraction according to Bligh and Dyer [13]. Plasmid DNA was determined using the PicoGreen Assay kit (Molecular Probes) as previously described [16]. For the Rh-PE-containing systems, the incorporation of CPL_4 was determined by dividing the dansyl to rhodamine ratio before the Sepharose column by the ratio after the column multiplied by 100%. For the other systems, incorporation was determined by dividing the CPL_4 content by the total lipid content and multiplying by 100%.

Lipoplexes were prepared at a charge ratio of 1.5:1 (positive-to-negative) by adding 25 μl of 88 μg/ml plasmid DNA (pCMVLuc or pCMVGFP) to 25 μl of DOPE:DODAC LUV (0.8 mM) in distilled water while vortexing, followed by incubation at room temperature for 30 min before addition to cells. Lipofectin lipoplexes were similarly prepared.

Mean vesicle diameters were determined by quasi-elastic light scattering (QELS) using a Nicomp Model 270 Submicron Particle Sizer (PSS, Santa Barbara, CA). Freeze-fracture electron microscopy studies were performed as described by Wheeler et al. [4]. SPLP diameters were obtained from the population of particles exhibiting equatorial fracturing [14], or from all the particles using the size correction method of Hallett et al. [15].

DNA for Southern analysis was extracted using a phenol/chloroform extraction following incubation of SPLP systems with 50% mouse serum. The resulting DNA was then subjected to electrophoresis through a 1% agarose gel, transferred to a nylon membrane (Amersham, Piscataway, NJ) and subjected to Southern analysis. The membrane was exposed to random-primed ^{32}P-labelled PvuII restriction fragment from the luciferase gene according to current protocols. Hybridization intensities were quantified using a

Phosphorimager™ SI from Amersham Biosciences (Piscataway, NJ). The data were converted to give amounts of intact DNA relative to undigested DNA.

Levels of PEG-CerC_{20} and DOPE were determined by HPLC analyses performed by Northern Lipids Inc.

2.3. Uptake and transfection studies

A transformed BHK cell line (tk^-) was used for all uptake and transfection studies. To determine the cellular uptake of SPLP, 1×10^5 BHK cells were seeded in each well of a 12-

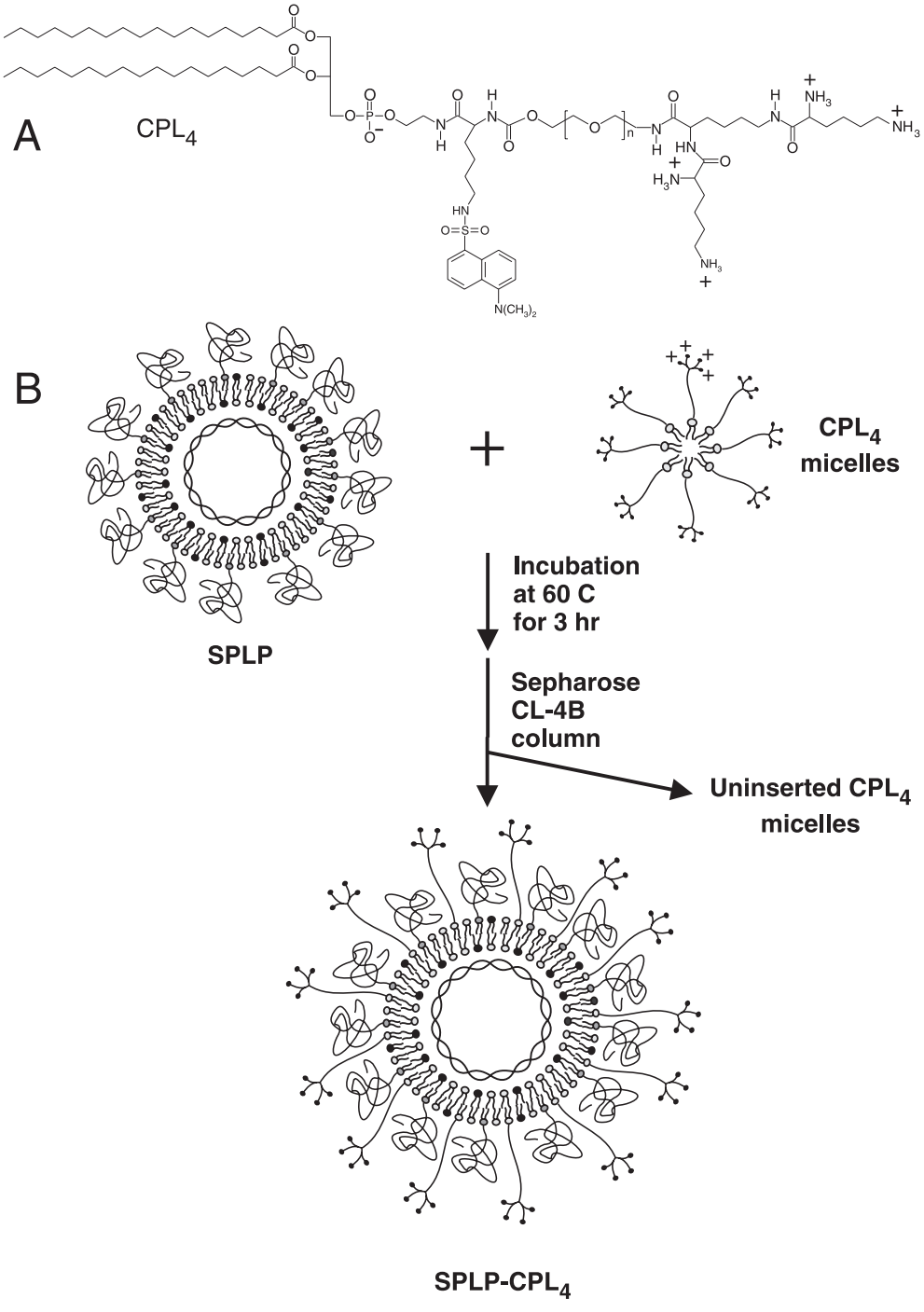

Fig. 1. Insertion protocol for the production of SPLP-CPL_4. (A) Structure of dansylated CPL_4. CPL_4 possesses four positive charges at the end of a PEG_{3400} molecule attached to a lipid anchor, DSPE. (B) Protocol for insertion of CPL_4 into preformed SPLP. The SPLP are composed of DOPE (light headgroups), DODAC (black headgroups), and PEG-CerC_{20} (lipids with attached polymer). The SPLP and CPL_4 are incubated together at 60 °C for 3 h, during which time CPL_4 monomers transfer from micelles and insert into the external monolayer of SPLP. Unincorporated CPL_4 is subsequently removed by Sepharose CL-4B column chromatography. For further details, see Materials and methods. Reproduced from D.B. Fenske, I. MacLachlan, P.R. Cullis, Stabilized plasmid–lipid particles: a systemic gene therapy vector, Methods Enzymol. 346 (2001) 36–71. Copyright 2001 Academic Press.

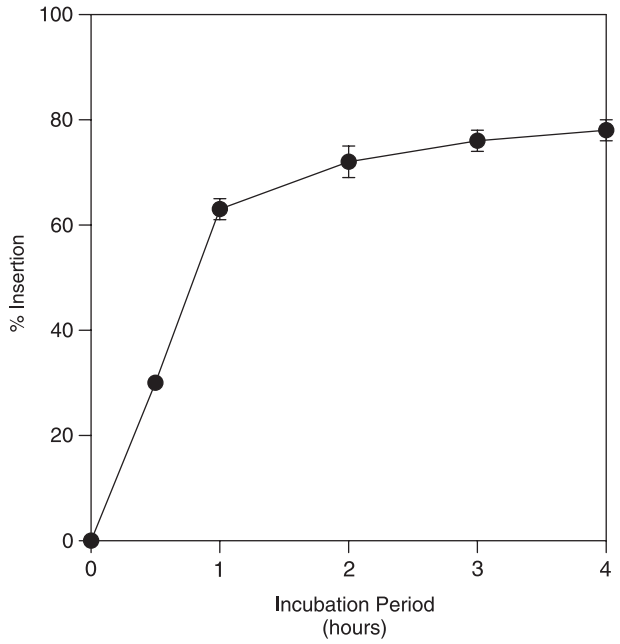

Fig. 2. Time course for the insertion of CPL_4 into SPLP at 60 °C. Dansylated CPL_4 (0.3 μmol) was added to SPLP composed of 6 μmol DOPE:PEG-$CerC_{20}$:DODAC:Rh-PE (83.5:10:6:0.5; mol%) containing 360 μg pCMVLuc in a total volume of 1.5 ml and incubated at 60 °C. Aliquots (250 μl) of the mixture were taken at the times indicated and unincorporated CPL_4 subsequently removed by Sepharose CL-4B column chromatography. CPL_4 incorporation was determined as described in Materials and methods.

well plate and incubated overnight in 2 ml of complete media [Dulbeco's modified Eagle's media (DMEM) containing 10% fetal bovine serum (FBS)] at 37 °C in 5% CO_2. SPLP and SPLP-CPL_4 were prepared in HBS containing 40 mM $CaCl_2$ and 200 μl of these preparations was mixed with 800 μl of complete media at a final lipid dose of 20 μM and added to the cells. DOPE:DODAC lipoplexes were prepared as indicated above and aliquots of 200 μl were again mixed with 800 μl of complete media (final lipid dose 20 μM) and added to the cells. Final plasmid DNA concentrations were 1.4 and 2.2 μg/ml for the SPLP systems and the lipoplexes, respectively. Cells were incubated at 37 °C for the indicated period, washed twice with phosphate-buffered saline (PBS), and lysed with 600 μl of lysis buffer (0.1% Triton X-100 in 250 mM sodium phosphate, pH 7.5). Rhodamine fluorescence was determined using a λ_{ex} of 560 nm and a λ_{em} of 600 nm. An emission filter of 530 nm was also used. Lipid uptake was determined by comparison of the fluorescence in the lysate to that of a lipid standard and normalized to the cell number as determined by the BCA protein assay (Pierce, Rockford, IL). Where indicated, fluorescence micrographs were obtained using an Axiovert 100 Zeiss fluorescent microscope (Carl Zeiss, Jena) using a rhodamine filter from Omega Opticals (Brattleboro, VT).

The effects of Ca^{2+} and Mg^{2+} on lipid uptake were determined as described above with minor modifications. BHK cells (5×10^4 per well) were seeded in a 24-well plate in 1 ml of complete media and incubated overnight at 37 °C. SPLP-CPL_4 (40 nmol) were prepared in HBS and appropriate amounts of $CaCl_2$ or $MgCl_2$ added (total volume 100 μl). Complete media (400 μl) were added to the SPLP-CPL_4 resulting in final cation concentrations of 4–14 mM. This mixture was then added to the cells and incubated for 4 h at 37 °C. Cells were then washed twice with PBS and lysed in 600 μl of lysis buffer (0.1% Triton X-100 in 250 mM sodium phosphate, pH 7.5).

Unless otherwise indicated, transfection studies were performed employing 1×10^4 BHK cells plated in each well of a 96-well plate in 150 μl complete media before overnight incubation at 37 °C in 5% CO_2. SPLP and SPLP-CPL_4 corresponding to 0.5 μg of pCMVLuc in 20 μl HBS (SPLP), or HBS containing 40 mM $CaCl_2$ (SPLP-CPL_4) were added to 80 μl of complete media for a plasmid concentration of 5.0 μg/ml. A transfection time of 4 h with a total incubation time of 24 h was used routinely. The transfection time is defined as the time the cells are incubated with the plasmid-containing particles, whereas the total incubation time includes the transfection time (after which the transfection media is replaced) plus the subsequent time for which the cells are incubated before assaying for transgene expression. After 24 h, the cells were lysed with 100 μl of lysis buffer, and 40 μl of the lysate was transferred to a 96-well luminescence plate. Luciferase activity was determined using a Luciferase reaction kit (Promega, Madison, WI), a luciferase standard (Boehringer-Mannheim), and a ML3200 microtiter plate luminometer from Molecular Dynamics (Chantilly, VA).

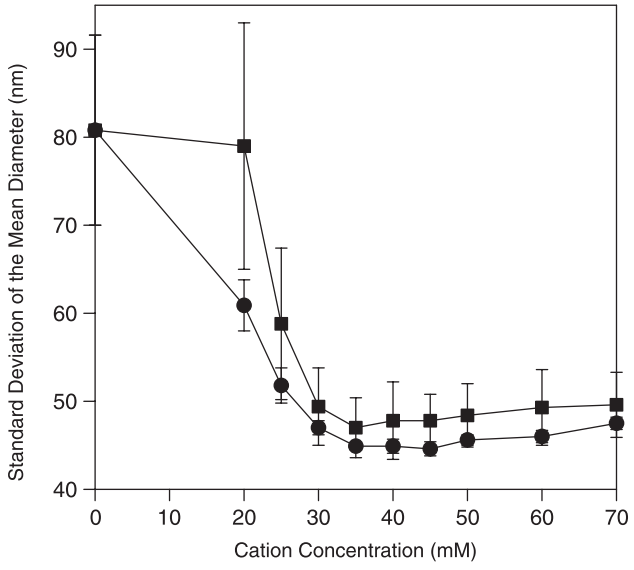

Fig. 3. Effect of cation concentration on the de-aggregation of SPLP following insertion of CPL_4. SPLP were prepared and 4 mol% CPL_4 was inserted as described in Materials and methods. The mean diameter and standard deviation of the mean diameter of the SPLP-CPL_4 in the presence of increasing concentrations of Ca^{2+} (●) and Mg^{2+} (■) were determined by QELS. $CaCl_2$ or $MgCl_2$ from 500 mM stock solutions was added to SPLP-CPL_4 (180 nmol in 400 μl). The addition of Ca^{2+} or Mg^{2+} results in a more monodisperse preparation as indicated by a reduction in the standard deviation of the mean diameter at cation concentrations above 30 mM.

Activity was normalized to the number of cells as determined by the BCA protein assay.

The transfection time course study included SPLP, SPLP-CPL_4, and Lipofectin (Gibco BRL) and DOPE/DODAC lipoplexes containing pCMVLuc. After transfection times of 4, 8, and 24 h, the transfection media were removed and, in the case of the 4- and 8-h transfections, replaced with complete media for a total incubation time of 24 h. At 24 h, all cells were lysed and assayed for luciferase activity and protein content (BCA assay).

SPLP-CPL_4, DOPE:DODAC lipoplexes, and Lipofectin lipoplexes containing pCMVGFP were prepared as described for pCMVLuc. The transfections were performed as described above at a plasmid DNA dose of 5.0 μg/ml. Following incubation of the samples for 24 and 48 h, the transfection media were removed, the cells were washed, and fresh media added. The cells were then viewed under the Zeiss fluorescence microscope. The number of cells expressing GFP were counted using a fluorescein filter (Omega Opticals). The transfection efficiency was expressed as percentage of cells expressing GFP.

3. Results

3.1. Cationic PEG lipids can be inserted into preformed SPLP

Previous work has shown that SPLP exhibit lower uptake into cells and much lower transfection potencies than lipoplexes [16]. It has also been shown that surface-associated CPLs, particularly those containing four charges at the end of the PEG molecule (CPL_4; for structure, see Fig. 1A), can dramatically enhance the uptake of LUV into cells [10].

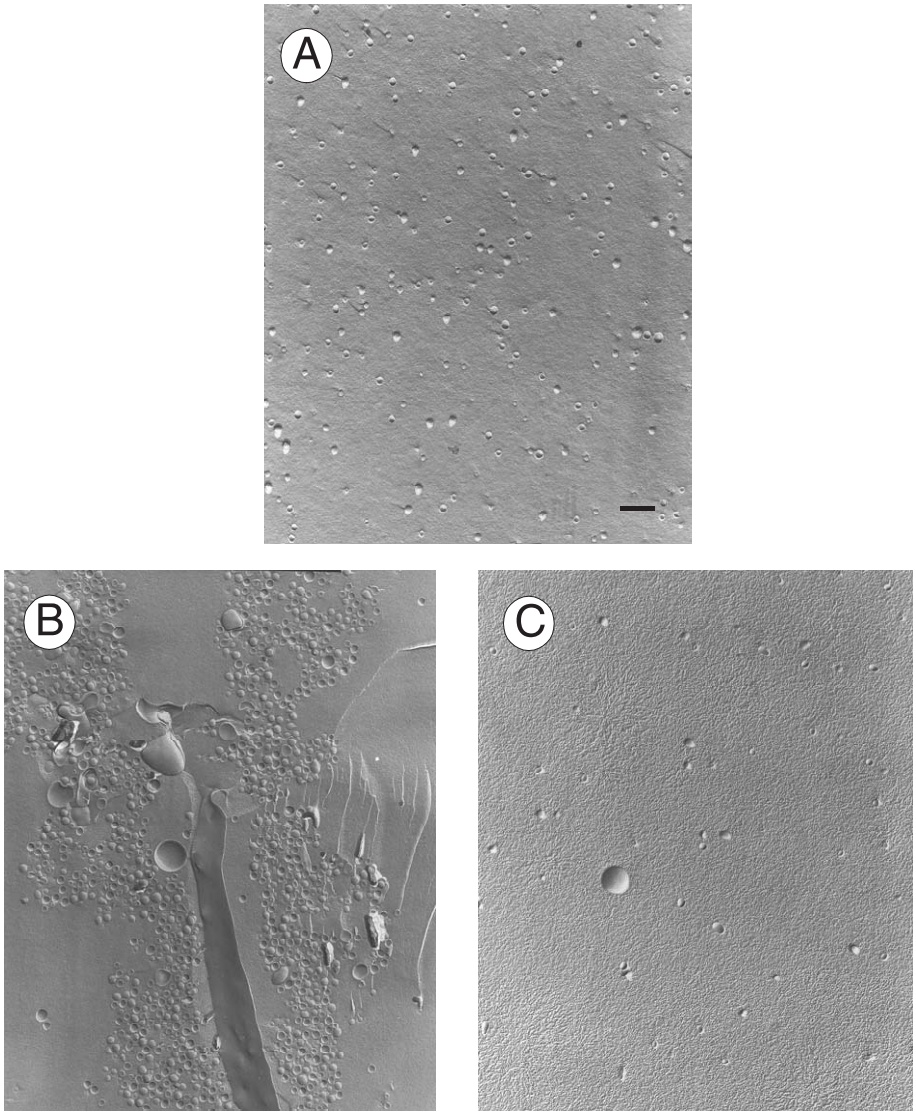

Fig. 4. Freeze-fracture electron micrographs of (A) SPLP, (B) SPLP-CPL_4, and (C) SPLP-CPL_4 in the presence of 40 mM $CaCl_2$. The SPLP-CPL_4 was prepared as described in Materials and methods, and contained 4 mol% CPL_4. The bar in plate A corresponds to 312 nm.

Further, CPL can be inserted into preformed LUV with lipid compositions similar to SPLP employing a straightforward incubation protocol [10]. We first examined whether a similar procedure could be developed to insert CPL_4 into SPLP. SPLP containing pCMVLuc were prepared by the detergent dialysis procedure of Wheeler et al. [4] from a lipid mixture containing 6 mol% of the cationic lipid DODAC, 84 mol% of the fusogenic helper lipid DOPE, and 10 mol% of a stabilizing lipid consisting of PEG_{2000} attached to a ceramide (Cer) anchor (PEG-Cer). The ceramide anchor of the PEG-Cer contained a C_{20} acyl chain (PEG-$CerC_{20}$) that does not readily exchange out of the vesicle, thus contributing to a highly stable SPLP system [4]. The detergent dialysis procedure results in the formation of a mixture of SPLP containing one plasmid per vesicle, free plasmid, and empty vesicles. SPLP were purified by removing free plasmid and empty vesicles by DEAE column chromatography and density centrifugation, respectively, as described elsewhere [4].

The procedure for post-insertion of CPL_4 into the preformed SPLP is illustrated in Fig. 1B. Purified SPLP were incubated with CPL_4 ($\sim$ 5 mol%) at 60 °C for up to 3 h and then separated from non-incorporated CPL_4 by column chromatography. As shown in Fig. 2, this resulted in association of up to 80% of the available CPL_4 with the SPLP, corresponding to 4 mol% of the total lipid in the SPLP-CPL_4 system.

3.2. SPLP-CPL_4 aggregate following insertion of CPL_4 and de-aggregate following addition of divalent cations

Previous work has shown that vesicles containing CPL tend to aggregate and that this aggregation can be inhibited by increasing the ionic strength of the medium [10]. It was found that SPLP-CPL_4 were also susceptible to aggregation, and that this aggregation could be reversed by adding NaCl, $CaCl_2$, or $MgCl_2$ to the SPLP-CPL_4 formulation. This effect is illustrated in Fig. 3, which shows the effect of the addition of $CaCl_2$ or $MgCl_2$ on aggregation of SPLP-CPL_4 as monitored by the change in the standard deviation of the mean diameter of the particles, as measured by quasi-elastic light scattering (QELS). For both cations the standard deviation decreased with increasing cation concentration, with optimal de-aggregation occurring above 30 mM. This behaviour could also be visualized by freeze-fracture electron microscopy. As shown in Fig. 4A, freeze-fracture micrographs of SPLP reveal small monodisperse particles, whereas SPLP-CPL_4 prepared in the absence of $CaCl_2$ are highly aggregated (Fig. 4B). As shown in Fig. 4C, the addition of 40 mM $CaCl_2$ reverses this aggregation to produce monodisperse particles similar to the SPLP preparation. It may be noted that this process is reversible in that the subsequent removal of the $CaCl_2$ by dialysis results in re-aggregation (data not shown).

The sizes of SPLP and SPLP-CPL_4 in the presence of $CaCl_2$ were compared using QELS and freeze-fracture electron microscopy. QELS studies revealed the mean diameter of SPLP and SPLP-CPL_4 to be 80 ± 19 and 76 ± 15 nm, respectively, whereas the freeze-fracture studies indicated diameters of 68 ± 11 and 64 ± 14 nm. These values for SPLP diameters are in close agreement with previous studies [4].

3.3. PEG-$CerC_{20}$ content and stability of SPLP-CPL_4

The observation that CPL_4 can be inserted to achieve levels as high as 4 mol% of the total SPLP lipid indicates that the level of CPL_4 in the outer monolayer of the SPLP-CPL_4 is 8 mol%. Given that the initial concentration of PEG-$CerC_{20}$ is 10 mol%, this suggests that the total levels of PEG-lipids in the outer monolayer of the SPLP-CPL_4 can

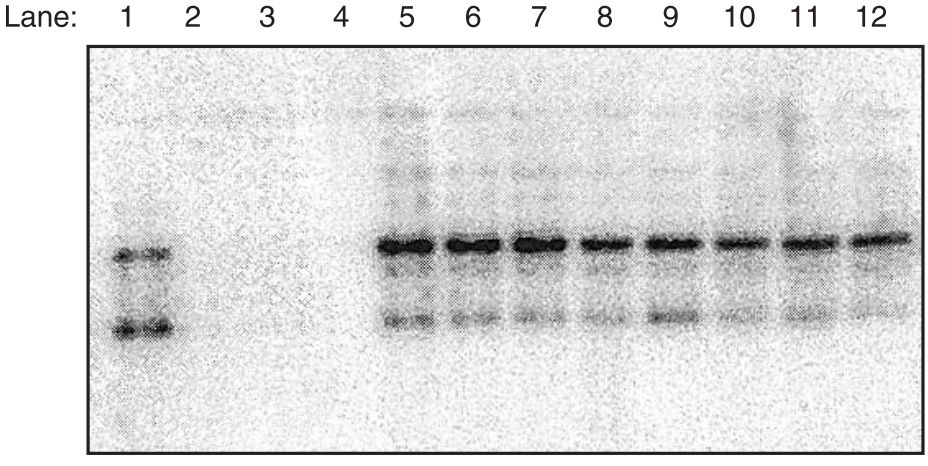

Fig. 5. Serum stability of SPLP-CPL_4 as assayed by Southern analysis of encapsulated plasmid. SPLP were prepared as indicated in the legend to Figs. 2 and 4 mol% of CPL_4 inserted using the post-insertion protocol. SPLP-CPL_4 containing 5 μg pCMVLuc were incubated in the presence of 50% mouse serum at 37 °C for the times indicated, an aliquot of the mixture corresponding to 1 μg of plasmid DNA was removed and plasmid DNA was extracted and subjected to Southern analysis, as described in the Materials and methods. Lanes 1–4 indicate the behaviour of naked plasmid DNA following 0, 1, 2, and 4 h incubation times, respectively; Lanes 5–8 indicate the behaviour of plasmid extracted from SPLP following 0, 1, 2, and 4 h incubation times; Lanes 9–12 show the behaviour of plasmid DNA extracted from SPLP containing 4 mol% CPL_4 following 0, 1, 2, and 4 h incubation times.

approach 18 mol%. These levels are consistent with recent observations showing that PEG(2000)-lipids can reach concentrations as high as 17.5 mol% in DOPE bilayers [17]. Nevertheless, these levels are higher than the levels of PEG-lipids that can usually be incorporated into phosphatidylcholine lipid vesicles [18], leading to the possibility that some of the PEG-$CerC_{20}$ in the outer monolayer exchanged out as CPL_4 was inserted. This was examined by HPLC by measuring the ratio of PEG-$CerC_{20}$-to-DOPE for the SPLP before and after insertion of CPL_4. CPL_4 was inserted into SPLP as described previously. Analysis following removal of non-incorporated material determined that 4 mol% CPL_4 (normalized to the total SPLP lipid) was inserted into the SPLP. Before insertion of the CPL_4, the PEG-$CerC_{20}$-to-DOPE ratio was 0.091, corresponding to a PEG-$CerC_{20}$ content of 7.6 mol% (assuming that the DOPE constituted 84 mol% of the lipid content). Following insertion of the CPL_4, the PEG-$CerC_{20}$-to-DOPE ratio was found to be 0.072, indicating a PEG-$CerC_{20}$ content of 6.0 mol%. Assuming that all of the PEG-$CerC_{20}$ lost from the SPLP during insertion of the CPL_4 is lost from the outer monolayer, this indicates that the PEG-$CerC_{20}$ content of the outer monolayer decreases from 7.6 to 4.4 mol% during the insertion process. The total PEG-lipid content in the outer monolayer of the SPLP-CPL_4 can then be estimated to be 12.4 mol%.

The stability of SPLP and SPLP-CPL_4 following incubation in 50% mouse serum for up to 4 h is illustrated in Fig. 5. In all cases, the encapsulated plasmid DNA was fully protected from serum degradation. In contrast, essentially complete degradation of the plasmid in lipoplexes was observed within 30 min of incubation in serum (data not shown).

3.4. SPLP-CPL_4 exhibit enhanced uptake into BHK cells and dramatically enhanced transfection potency

The next set of experiments was aimed at determining the influence of incorporated CPL_4 on the uptake of SPLP into BHK cells and the resulting transfection potency of the SPLP-CPL_4 system. SPLP containing up to 4 mol% CPL_4 were prepared in the presence of 40 mM $CaCl_2$ and were added to BHK cells (final $CaCl_2$ concentration 8 mM) and

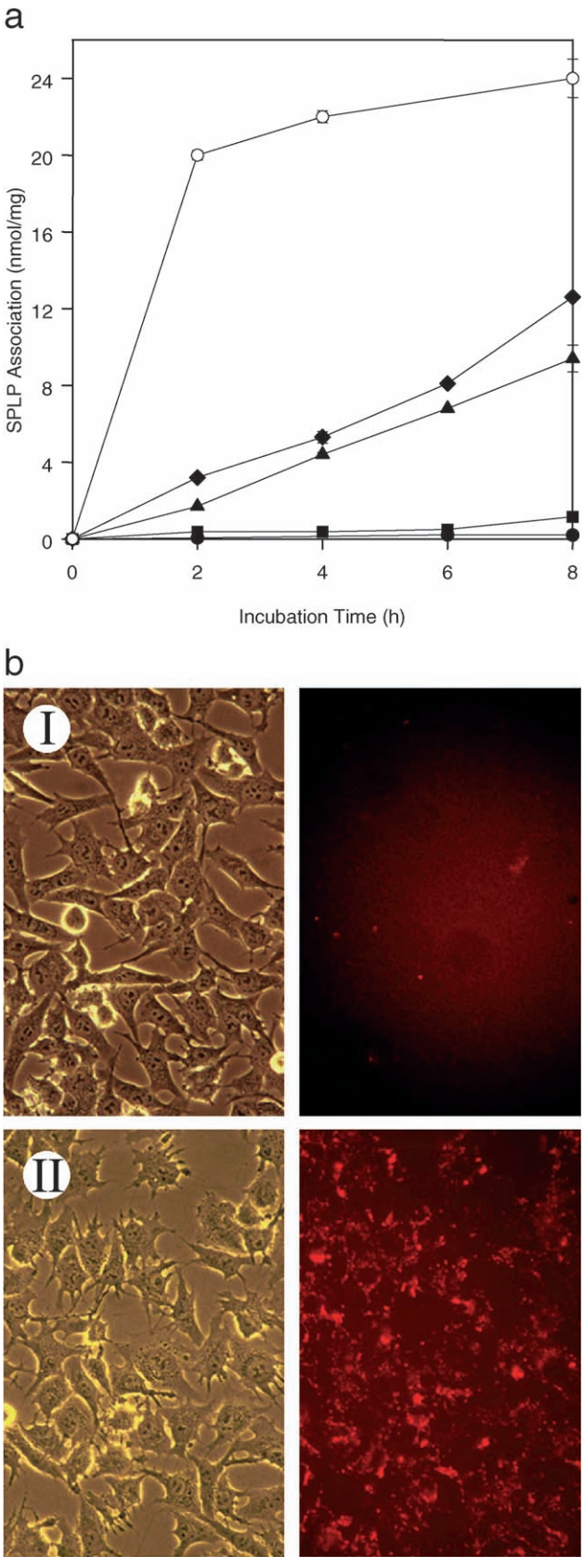

Fig. 6. (a) Influence of the amount of CPL_4 incorporated into SPLP on the association (binding/uptake) of SPLP-CPL_4 with BHK cells. Uptake of SPLP containing 0 (●), 2 (■), 3 (▲), or 4 (♦) mol% CPL_4 was investigated; the uptake of DOPE:DODAC lipoplexes (○) is given for comparison. The insertion of CPL_4 into SPLP and the preparation of lipoplexes was performed as described in Materials and methods. The SPLP-CPL_4 media contained 40 mM $CaCl_2$ to prevent aggregation, with addition to the BHK cells resulting in dilution of the $CaCl_2$ concentration to 8 mM. The uptake protocol involved incubation of SPLP-CPL_4 (20 μM total lipid) with 10^5 BHK cells in DMEM containing 10% FBS. Following incubation, the cells were lysed and uptake of rhodamine-PE was measured as described in Materials and methods. (b) Fluorescence micrographs of BHK cells following uptake of SPLP (Panel I) and SPLP containing 4 mol% CPL_4 (Panel II) following a 4-h incubation. The micrographs on the left were taken in the phase contrast mode and those on the right in the (rhodamine) fluorescence mode.

incubated for varying times. The cells were then assayed for associated SPLP-CPL_4 as indicated in Materials and methods. As shown in Fig. 6a, uptake of SPLP that contain no CPL_4 is minimal even after 8 h of incubation. However, uptake is dramatically increased for SPLP containing 3 mol% or higher levels of CPL_4. For example, SPLP containing 4 mol% CPL_4 exhibit accumulation levels at 8 h that are approximately 50-fold higher than those achieved by SPLP in the absence of CPL. This enhanced uptake is visually illustrated in Fig. 6b, which shows fluorescence micrographs of BHK cells following incubation with rhodamine-labeled SPLP and SPLP-CPL_4 for 4 h.

The transfection properties of SPLP, SPLP-CPL_4, and plasmid DNA-cationic liposome lipoplexes (DODAC/DOPE; 1:1) were examined using the transfection protocol described in Materials and methods. This protocol involves incubation of BHK cells with SPLP or lipoplexes for 4 h (the transfection time) followed by replacement of media and further incubation to maximize transgene expression. The total incubation time (transfection time plus time of incubation following the media change) was kept constant at 24 h. As shown in Fig. 7, the presence of increasing amounts of CPL_4 resulted in dramatic increases in the transfection potency of the SPLP system. SPLP-CPL_4 containing 4

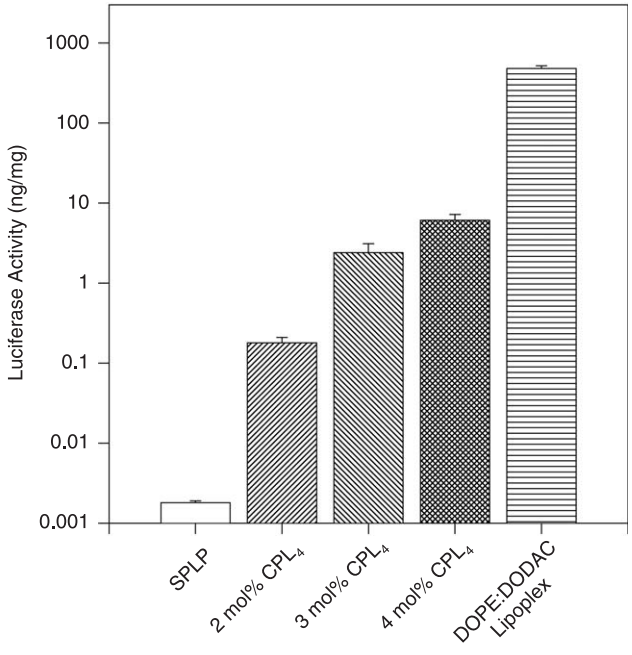

Fig. 7. Luciferase expression in BHK cells following transfection by SPLP containing various amounts of CPL_4. SPLP containing 2, 3, and 4 mol% CPL_4 were prepared employing the post-insertion process. BHK cells (10^4) were transfected with SPLP, SPLP-CPL_4, and DOPE:DODAC (1:1) lipoplexes containing 5.0 μg/ml pCMVLuc using a transfection time of 4 h and a complete incubation time of 24 h, as described in Materials and methods. The $CaCl_2$ concentration in the SPLP-CPL_4-containing systems following dilution with media and addition to the BHK cells was 8 mM. After transfection, the cells were lysed and the luciferase and BCA assays performed as described in Materials and methods.

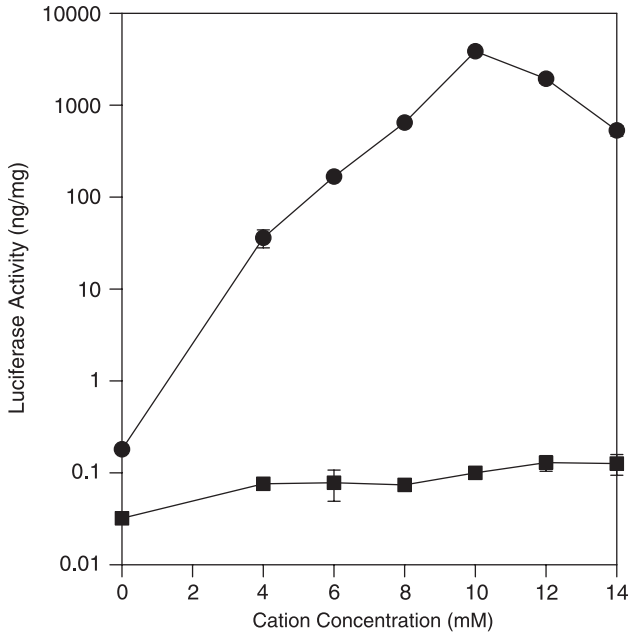

Fig. 8. Influence of Ca^{2+} (●) and Mg^{2+} (■) on the transfection potency of SPLP-CLP_4. SPLP-CPL_4 containing 4 mol% CPL_4 were prepared by the post-insertion process as described in Materials and methods. Increasing concentrations of $CaCl_2$ or $MgCl_2$ were added to the SPLP-CPL_4 (5.0 μg pCMVLuc/ml), transferred to BHK cells, and incubated for 48 h in DMEM containing 10% FBS. The cells were then lysed and the luciferase activity and protein content measured as described in Materials and methods.

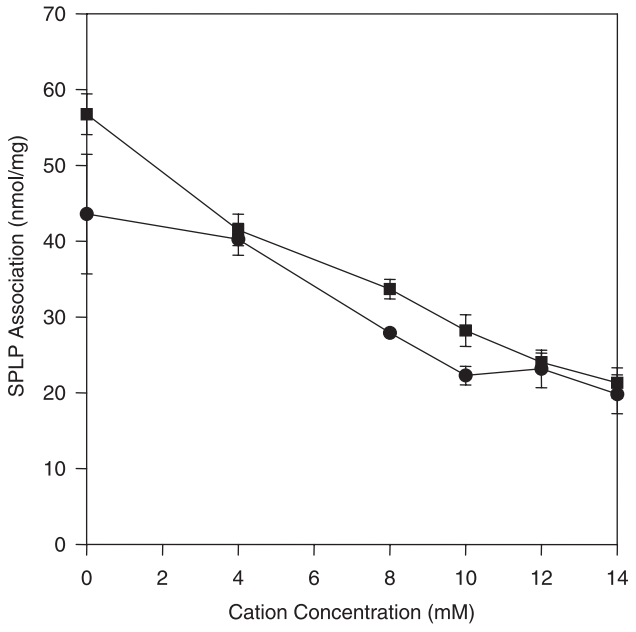

Fig. 9. Effect of Ca^{2+} (●) and Mg^{2+} (■) on the association (binding/uptake) of SPLP-CPL_4 with BHK cells. SPLP-CPL_4 were prepared with increasing cation concentration as indicated for Fig. 8 and incubated with BHK cells (~ 80 μM lipid and ~ 5.0 μg pCMVLuc/ml per well) for 4 h in DMEM containing 10% FBS. The cells were then lysed and the SPLP-CPL_4 content (as indicated by the Rh-PE lipid label) and cellular protein measured as described in Materials and methods.

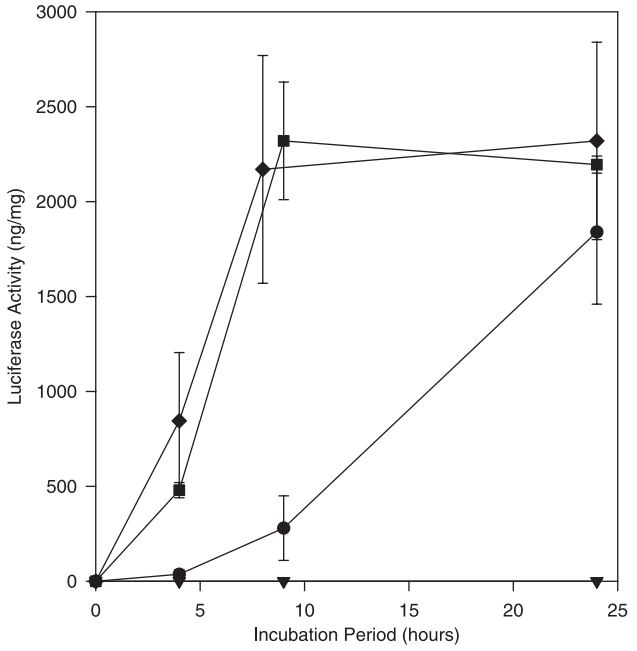

Fig. 10. Luciferase expression in BHK cells as a function of transfection time for SPLP, SPLP-CPL_4, and lipoplexes. SPLP-CPL_4 containing 4 mol% CPL_4 were prepared by the post-insertion process. BHK cells in DMEM and 10% FBS were incubated with SPLP, SPLP-CPL_4, and lipoplexes (5.0 μg/ml pCMVLuc) employing transfection times of 4, 8, and 24 h and total incubation times of 24 h. The final $CaCl_2$ concentration following addition of media was 8 mM. The cells were then assayed for luciferase activity and protein content. Luciferase activity following transfection with SPLP-CPL_4 (●), SPLP (▼), DOPE:DODAC lipoplexes (■), and Lipofectin lipoplexes (♦) is plotted as a function of transfection time. Lipoplexes were prepared at a charge ratio of 1.5:1.

mol% CPL_4 exhibited luciferase expression levels some 3×10^3 times higher than those attained with SPLP.

3.5. Ca^{2+} is required for transfection activity of SPLP-CPL_4

Previous work has shown that the transfection potency of SPLP is highly sensitive to the presence of Ca^{2+}, where the presence of ~ 10 mM Ca^{2+} enhances transfection potency several hundred-fold [19]. It was therefore of interest to determine the influence of Ca^{2+} on the transfection activity of SPLP-CPL_4. SPLP containing 4 mol% CPL_4 were incubated with BHK cells for 48 h in the presence of varying amounts of $MgCl_2$ or $CaCl_2$ and the luciferase activities were determined. As shown in Fig. 8, the transfection activity was almost completely dependent on the presence of Ca^{2+} in the transfection medium. At the optimum $CaCl_2$ concentration of 10 mM, SPLP-CPL_4 exhibited transfection potencies that were more than 10^5 times higher than if the corresponding amount of $MgCl_2$ was present.

To determine whether the different transfection properties of SPLP-CPL_4 in the presence of Ca^{2+} or Mg^{2+} could be accounted for by differences in cellular uptake, the accumulation of SPLP-CPL_4 into BHK cells was monitored following a 4-h incubation in the presence of $MgCl_2$ or $CaCl_2$. As shown in Fig. 9, uptake of SPLP-CPL_4 into BHK cells is the same for both Ca^{2+} and Mg^{2+}. It may be noted that SPLP-CPL_4 uptake decreases slightly as the concentration of divalent cations increases, likely due to the shielding of the negatively charged CPL_4 binding sites on the surface of BHK cells. These results are consistent with a previous study indicating that Ca^{2+} has little effect on the cellular uptake of SPLP [19].

3.6. SPLP-CPL_4 exhibit transfection potencies in vitro that are comparable to or greater than the transfection potencies of lipoplexes

The data presented in Fig. 7 indicate that DOPE/DODAC lipoplexes yield ~ 100-fold higher levels of gene expres-

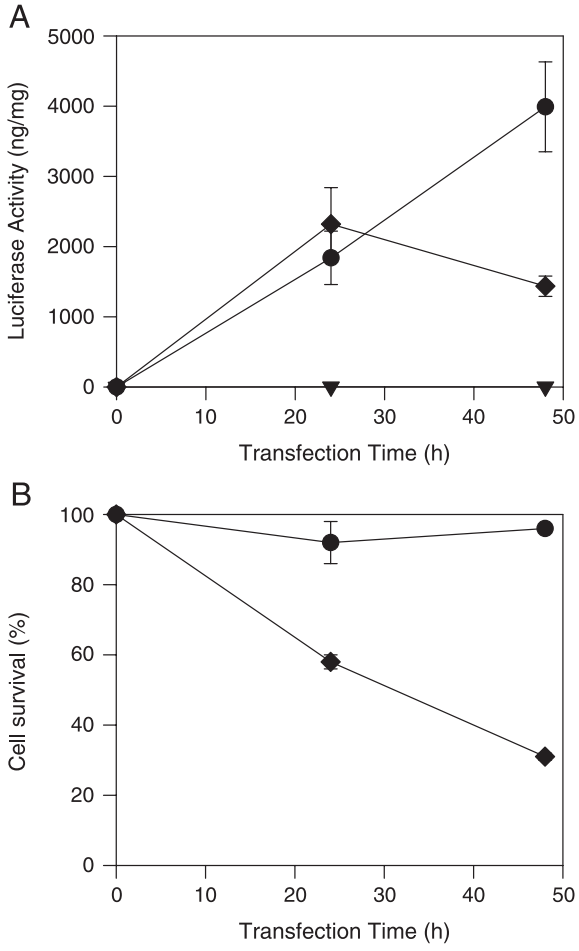

Fig. 11. (A) The transfection potency of SPLP (▼), SPLP-CPL_4 (●) containing 4 mol% CPL_4, and Lipofectin lipoplexes (♦) following extended transfection times with BHK cells. SPLP-CPL_4 and lipoplexes were generated as indicated for Fig. 10. BHK cells were transfected in DMEM containing 10% FBS for 24 and 48 h with SPLP-CPL_4 and Lipofectin lipoplexes (charge ratio of 1.5:1) containing 5.0 μg/ml pCMVLuc. Following transfection, the luciferase expression levels and cell protein levels were determined in the cell lysate. The luciferase activity was normalized for protein content in the lysate and plotted as a function of transfection time. (B) The toxicity of SPLP-CPL_4 (●) containing 4 mol% CPL_4 and Lipofectin lipoplexes (♦) as a function of transfection time, as assayed by cell survival based on the protein concentration in the cell lysate.

sion than SPLP-CPL_4 when applied to BHK cells for a period of 4 h. Given that SPLP-CPL_4 are stable systems, uptake can conceivably continue over extended time periods. We therefore examined the transfection levels achieved when SPLP-CPL_4 or the lipoplexes were applied to BHK cells for transfection times of 4, 8, and 24 h. Two types of lipoplexes were used, namely, DOPE:DODAC (1:1) lipoplexes (charge ratio 1.5:1) and lipoplexes generated using the transfection reagent Lipofectin, consisting of DOPE/DOTMA (1:1) lipoplexes at a charge ratio of 1.5. As shown in Fig. 10, the potency of SPLP-CPL_4 increases markedly with increased transfection times, suggesting that the rate of uptake of the SPLP-CPL_4 system is still a limiting factor for transfection. For the 24-h transfection time, where the cells are assayed for luciferase expression immediately after the transfection period, transfection levels are comparable to those achieved by Lipofectin or the DOPE/DODAC lipoplexes.

Further experiments were conducted to determine transfection levels after 24 or 48 h with SPLP-CPL_4 and lipoplexes where luciferase activities were assayed immediately following the transfection period. The activity of Lipofectin (DOPE:DOTMA) lipoplexes leveled off at ~ 2000 ng luciferase/mg cell protein after 24 h (Fig. 11A). Similar results were obtained for the DOPE:DODAC lipoplexes (data not shown). In contrast, the activity of the SPLP-CPL_4 formulation continued to increase as the incubation time was increased, achieving luciferase expression levels corresponding to 4000 ng/mg cell protein at 48 h. This activity is approximately 10^6 times higher than observed for SPLP (in the absence of Ca^{2+}) and almost double the levels that can be achieved by Lipofectin lipoplexes.

3.7. SPLP-CPL_4 are non-toxic and efficient transfection agents

It is well known that lipoplexes can be toxic to cells. The SPLP-CPL_4 contain low levels of cationic lipid and are potentially less toxic than lipoplexes. The toxicities of SPLP-CPL_4 and lipoplexes were assayed by determining cell viability following a 24- or 48-h exposure to levels of

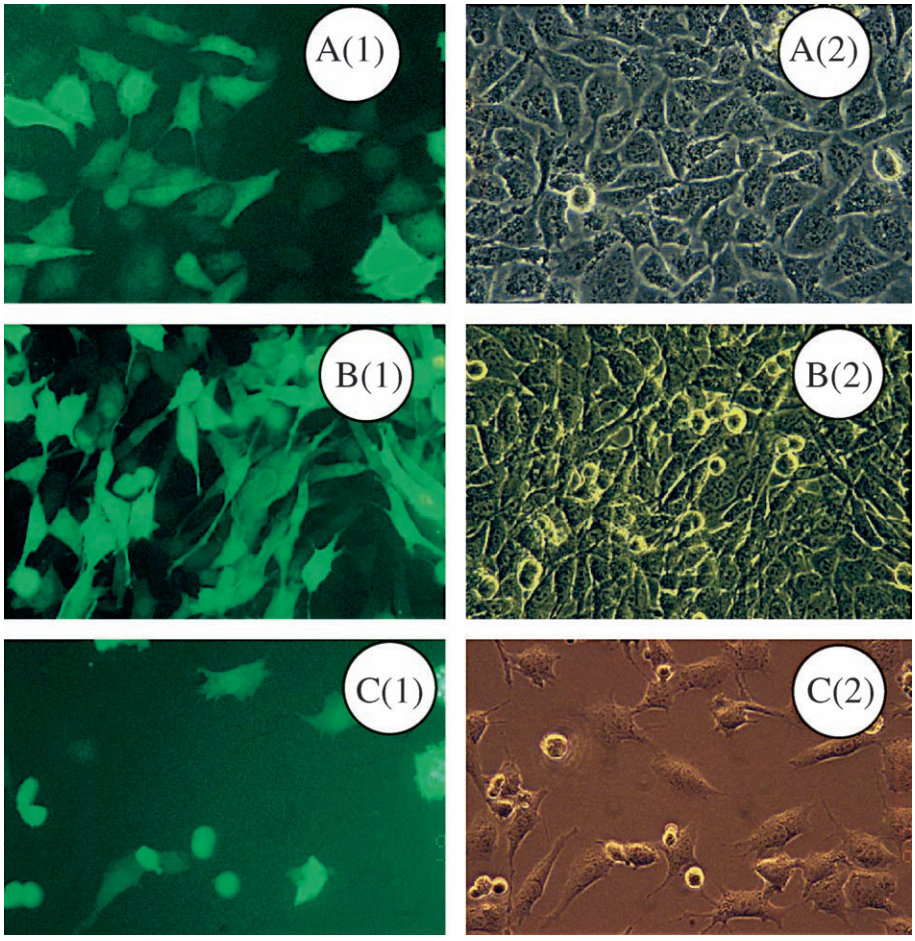

Fig. 12. Fluorescence and phase-contrast micrographs of BHK cells transfected with SPLP-CPL_4 and lipoplexes containing a plasmid coding for GFP. Cells were transfected with SPLP-CPL_4 for 24 h (A1, A2) and 48 h (B1, B2) and with Lipofectin for 24 h (C1, C2). SPLP and lipoplexes were prepared with pCMVGFP as described in Materials and methods. SPLP-CPL_4 containing 4 mol% CPL were prepared by the post-insertion process and contained $CaCl_2$, resulting in an 8-mM $CaCl_2$ concentration in the transfection medium. BHK cells (10^5) were incubated with SPLP-CPL_4 or Lipofectin (5.0 μg/ml) in DMEM containing 10% FBS for the 24- and 48-h transfection times and examined immediately after the transfection period.

SPLP-CPL_4 or lipoplexes corresponding to 5.0 μg/ml plasmid, and a total lipid dose of approximately 80 and 45 μM for SPLP-CPL_4 and lipoplexes, respectively. As shown in Fig. 11B, SPLP-CPL_4 exhibited little toxicity, whereas lipoplexes were highly toxic. Cell survival was only 30% after a 48-h incubation with Lipofectin lipoplexes, whereas ~ 95% of the cells were viable following a 48-h incubation with SPLP-CPL_4.

Studies were also conducted to determine the efficiency of transfection as indicated by the proportion of cells transfected by SPLP-CPL_4. The proportion of transfected cells was determined by employing plasmid containing the green fluorescent protein (GFP) gene. GFP expression was detected by fluorescence microscopy. As shown in Fig. 12A and B, approximately 55% of the cells at 24 h and 70% at 48 h were transfected by SPLP-CPL_4, with no apparent cell death. In contrast, Lipofectin lipoplexes exhibit maximum transfection efficiencies of about 35% with only ~ 50% cell survival after the 24-h transfection period (Fig. 12C). Similar low transfection efficiencies and high toxicities were also seen with DOPE:DODAC lipoplexes (data not shown).

4. Discussion

This study demonstrates that the incorporation of CPL_4 into preformed SPLP results in both improved uptake into BHK cells and dramatically enhanced transfection potencies when Ca^{2+} is present. There are three points of interest. The first concerns the post-insertion process itself and the chemical composition and structure of the SPLP-CPL_4 system following insertion of CPL into preformed SPLP. The second concerns the mechanism whereby the CPL increases the transfection potency of the SPLP vector. Finally, it is of interest to discuss the implications of these results for the design of SPLP that have improved properties as a systemic gene therapy system. We discuss these areas in turn.

The results presented here demonstrate that the cationic PEG lipid CPL_4 can be inserted into preformed SPLP employing a simple process involving incubation at 60 °C. It is likely that this procedure offers a general method for modifying the tropism and transfection potency of SPLP by post-insertion of PEG-lipids containing ligands that promote targeting and intracellular delivery. The ability to insert CPL_4 to levels corresponding to 8 mol% of the total lipid in the SPLP outer monolayer correlates well with results of Uster et al. [20]. They demonstrated that PEG-PE can be inserted into preformed LUV by employing a similar incubation protocol, resulting in systems that exhibit extended circulation lifetimes [20]. It is also consistent with previous results from this laboratory showing that CPL_4 can be inserted into preformed LUV with a lipid composition similar to the SPLP system [10]. The total levels of PEG-lipids achieved in the outer monolayer (12.4 mol%) are less than the maximum levels that can be accommodated by DOPE bilayers (17.5 mol%) [17]. Although the maximum levels of incorporation of PEG-lipids into LUVs containing phosphatidylcholine are somewhat lower, usually by 7–10 mol% [18], a number of authors have reported that levels as high as 15 mol% can be achieved before lytic effects are observed [21–23].

The tendency for the SPLP-CPL_4 system to aggregate following insertion of the CPL_4 is consistent with previous observations that LUV containing CPL_4 also aggregate [10]. The reason for this aggregation is not currently understood; however, two general points can be made. First, the interaction is likely due to electrostatic interactions between vesicles, given the inhibition of aggregation at higher ionic strengths. Second, the aggregation is not a consequence of the post-insertion process itself, because aggregation is also observed for LUV that contain CPL_4 in the original lipid mixture from which the LUV were formed [10]. One possibility is that H-bonding between the amino and carbonyl groups present in the distal headgroups leads to interactions between apposed membranes with subsequent aggregation.

The second point of discussion concerns the mechanism whereby CPL_4 increases the transfection potency of the SPLP system. The results presented here indicate that these improvements arise, at least in part, from dramatically improved uptake into cells. In this regard, a number of studies have indicated that the cationic lipids contained in lipoplex systems play a direct role in stimulating uptake into cells [24] and that this uptake arises due to the positive charge on the lipoplexes [25]. It has been suggested that heparin sulfonated proteoglycans on the cell surface play a primary role in this process [26,27]. Enhanced uptake of SPLP following addition of the CPL_4 could be attributed to similar mechanisms, although the increase in transfection potency is largely dependent on the additional presence of Ca^{2+}. Previous work has shown that the presence of Ca^{2+} results in a maximum increase in SPLP transfection potency of ~ 600-fold and that this increase is derived from the ability of Ca^{2+} to assist in destabilizing the endosomal membrane following uptake, rather than from an increase in uptake [19]. It could therefore be suggested that the observed improvements in transfection potency of SPLP-CPL_4 are the result of improvements in uptake mediated by the CPL_4 coupled with the enhanced ability to destabilize the endosomal membrane due to the presence of Ca^{2+}. In this regard, the transfection potency of SPLP-CPL_4 (in the presence of Ca^{2+}) is increased by a factor of ~ 10^4 (Fig. 7), for a 4-h incubation, in comparison to the transfection potency of SPLP in the absence of Ca^{2+}. This could be accounted for by an increase in uptake of SPLP into BHK cells by approximately 50-fold due to the presence of 4 mol% CPL_4 (Fig. 6A, 4 h incubation) multiplied by a factor of ~ 600 due to the presence of Ca^{2+}.

The final area of discussion concerns the implications of the results presented here for the design of next-generation SPLP that exhibit improved transfection properties following i.v. administration. First, it should be noted that the SPLP-CPL_4 system described here is unlikely to be of direct utility

as a systemic gene therapy vector. This is because the CPL_4 employed contains a PEG_{3400} linker, and thus the cationic groups at the end of the PEG will extend beyond the PEG_{2000} 'cloud' provided by the PEG-Cer components of the SPLP. Charged liposomal systems are rapidly cleared from the circulation following i.v. administration [28], and it would therefore be expected that the SPLP-CPL_4 described here would not exhibit the long circulation lifetimes that lead to accumulation at disease sites such as tumours. Strategies that could overcome this difficulty include the possibility of inserting CPL containing a shorter PEG linker, such as PEG_{1000}. The positive charge should then be shielded by the PEG_{2000} of the PEG-Cer, leading to longer circulation lifetimes. A remaining problem concerns the need to achieve exposure of the CPL after arrival at the target site to stimulate uptake into cells. As we have noted elsewhere, the PEG cloud on the SPLP can be designed to dissociate over a time determined by the length of the acyl chain contained in the ceramide anchor [4]. If the SPLP PEG-Cer is designed to dissociate slowly, a situation can be envisioned where SPLP that have accumulated at a disease site such as a tumour will lose the PEG shield, thus exposing the CPL which promote uptake. An alternative possibility is to post-insert lipids containing specific targeting ligands, which do not stimulate clearance, at the end of the PEG linker.

The strong Ca^{2+} dependence of the transfection properties of the SPLP and SPLP-CPL systems also represents a challenge to translate into improved transgene expression in vivo, as it is unlikely that systemic Ca^{2+} levels of 8 mM can be achieved. Two possibilities are to generate local high concentrations of Ca^{2+} or to deliver factors with the SPLP that can substitute for Ca^{2+}. Local high concentrations of Ca^{2+} could theoretically be achieved by co-encapsulating Ca^{2+} in the SPLP system; however, initial attempts in this regard have not led to enhanced in vitro expression [19]. It is possible that higher entrapped Ca^{2+} levels or increased leakage rates may lead to improved properties. Alternatively, it may be possible to reduce the need for Ca^{2+} by using SPLP with increased levels of cationic lipid [29,30]. Ca^{2+} and cationic lipids act together to destabilize the endosomal membrane [19], and the magnitude of the Ca^{2+} effect is less pronounced for systems such as lipoplexes that contain higher levels of cationic lipids [31]. This difference in cationic lipid content is substantial as illustrated by a simple calculation, which assumes that lipoplexes have an internal structure consisting of lipid bilayers of 4 nm thickness sandwiching plasmid DNA in an interlamellar space 2.5 nm thick as indicated by X-ray studies [32]. If cationic lipids exhibit a surface area of 0.6 nm^2 (comparable to diacylphospholipids [33]), a 100-nm-diameter lipoplex particle contains $\sim 10^5$ cationic lipids, whereas an SPLP containing 6 mol% cationic lipid contains only $\sim 3 \times 10^3$ cationic lipids.

In summary the results presented here demonstrate that cationic PEG lipid can be post-inserted into SPLP, resulting in well-defined SPLP-CPL_4 systems that exhibit improved uptake into BHK cells in vitro. In the presence of Ca^{2+}, SPLP-CPL_4 systems give rise to transfection potencies that are increased by up to 10^6-fold as compared to SPLP in the absence of Ca^{2+}. These results indicate that the SPLP system is a highly transfection potent entity following uptake into cells and suggest methods for improving the properties of SPLP for systemic gene therapy applications.

Acknowledgements

These studies were supported by the Medical Research Council of Canada (now the Canadian Institutes of Health Research) and the Natural Sciences and Engineering Research Council of Canada.

References

[1] A. Gabizon, D. Papahadjopoulos, Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6949–6953.
[2] A. Chonn, P.R. Cullis, Recent advances in liposome drug delivery systems, Curr. Opin. Biotechnol. 6 (1995) 698–708.
[3] S. Kohn, J.A. Nagy, H.F. Dvorak, A.M. Dvorak, Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumor blood vessels, Lab. Invest. 67 (1992) 596–607.
[4] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid–lipid particles: construction and characterization, Gene Ther. 6 (1999) 271–281.
[5] M.A. Monck, A. Mori, D. Lee, P. Tam, J.J. Wheeler, P.R. Cullis, P. Scherrer, Stabilized plasmid–lipid particles: pharmokinetics and plasmid delivery to distal tumors following intravenous injection, J. Drug Target. 7 (2000) 439–452.
[6] D.B. Fenske, I. MacLachlan, P.R. Cullis, Long-circulating vectors for the systemic delivery of genes, Curr. Opin. Mol. Ther. 3 (2001) 153–158.
[7] P. Tam, M. Monck, D. Lee, O. Ludkovski, E.C. Leng, K. Clow, H. Stark, P. Scherrer, R.W. Graham, P.R. Cullis, Stabilized plasmid–lipid particles for systemic gene therapy, Gene Ther. 7 (2000) 1867–1874.
[8] P. Pires, S. Simoes, S. Nir, R. Gaspar, N. Duzgunes, M.C.P. de Lima, Interaction of cationic liposomes and their DNA complexes with monocyte leukemia cells, Biochim. Biophys. Acta 1418 (1999) 71–84.
[9] T. Chen, K. Wong, D.B. Fenske, L.R. Palmer, P.R. Cullis, Fluorescent-labeled poly(ethylene glycol) conjugates with distal cationic headgroups, Bioconjug. Chem. 11 (2000) 433–437.
[10] D.B. Fenske, L.R. Palmer, T. Chen, P.R. Cullis, Cationic PEG–lipids incorporated into pre-formed vesicles enhance binding and uptake to BHK cells, Biochim. Biophys. Acta 1512 (2001) 259–272.
[11] M.S. Webb, D. Saxon, F.M.P. Wong, H.J. Lim, Z. Wang, M.B. Bally, L.S.L. Choi, P.R. Cullis, L.D. Mayer, Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine, Biochim. Biophys. Acta 1372 (1998) 272–282.
[12] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorous, J. Biol. Chem. 66 (1925) 375–400.
[13] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[14] R. Van Venetie, J. Leunissen-Bijvelt, A.J. Verkleij, P.H.J.T. Ververgaert, J. Microsc. 118 (1980) 401–408.
[15] F.R. Hallett, B. Nickel, C. Samuels, P.H. Krygsman, Determination

of vesicle size distributions by freeze-fracture electron microscopy, J. Electron Microsc. Tech. 17 (1991) 459–466.
[16] K.W.C. Mok, A.M.I. Lam, P.R. Cullis, Stabilized plasmid–lipid particles: factors influencing plasmid entrapment and transfection properties, Biochim. Biophys. Acta 1419 (1999) 137–150.
[17] M. Johnsson, K. Edwards, Phase behavior and aggregate structure in mixtures of dioleoylphosphatidylethanolamine and poly(ethyleneglycol)–lipids, Biophys. J. 80 (2001) 313–323.
[18] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, Biochim. Biophys. Acta 1113 (1992) 171–199.
[19] A.M.I. Lam, L.R. Palmer, P.R. Cullis, Calcium dramatically enhances the transfection potency of stabilized plasmid–lipid particles, Molec. Ther. (2002) (submitted for publication).
[20] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time, FEBS Lett. 386 (1996) 243–246.
[21] K. Edwards, M. Johnsson, G. Karlsson, M. Silvander, Effect of polyethyleneglycol–phospholipids on aggregate structure in preparations of small unilamellar liposomes, Biophys. J. 73 (1997) 258–266.
[22] A.K. Kenworthy, S.A. Simon, T.J. McIntosh, Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol), Biophys. J. 68 (1995) 1903–1920.
[23] K. Hristova, A. Kenworthy, T.J. McIntosh, Effect of bilayer composition on the phase behavior of liposomal suspensions containing poly(ethylene glycol)–lipids, Macromolecules 28 (1995) 7693–7699.
[24] C.R. Miller, B. Bondurant, S.D. McLean, K.A. McGovern, D.F. O'Brien, Liposome–cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes, Biochemistry 37 (1998) 12875–12883.
[25] I. vanderWoude, H.W. Visser, M.B.A. terBeest, A. Wagenaar, M.H.J. Ruiters, J.B.F.N. Engberts, D. Hoekstra, Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system, Biochim. Biophys. Acta 1240 (1995) 34–40.
[26] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12349–12354.
[27] L.C. Mounkes, W. Zhong, G. Cipres-Palacin, T.D. Heath, R.J. Debs, Proteoglycans mediate cationic liposome–DNA complex-based gene delivery in vitro and in vivo, J. Biol. Chem. 273 (1998) 26164–26170.
[28] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes, J. Biol. Chem. 267 (1992) 18759–18765.
[29] Y.P. Zhang, et al., Stabilized plasmid–lipid particles for regional gene therapy: formulation and transfection properties, Gene Ther. 6 (1999) 1438–1447.
[30] E.G. Saravolac, et al., Encapsulation of plasmid DNA in stabilized plasmid–lipid particles composed of different cationic lipid concentrations for optimal transfection activity, J. Drug Target. 7 (2000) 423–437.
[31] A.M.I. Lam, P.R. Cullis, Calcium enhances the transfection potency of cationic liposome–plasmid DNA complexes, Biochim. Biophys. Acta 1463 (2000) 279–290.
[32] J.O. Radler, I. Koltover, T. Salditt, C.R. Safinya, Structure of DNA–cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, Science 275 (1997) 810–814.
[33] G.I. King, R.E. Jacobs, S.H. White, Hexane dissolved in dioleoyllecithin bilayers has a partial molar volume of approximately zero, Biochemistry 24 (1985) 4637–4645.