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# A novel cationic cardiolipin analogue for gene delivery

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The optically active *R* and *S* isomers of cationic cardiolipin analogues (CCA) were synthesized and evaluated as a liposome based transfection reagent. Both isomers form stable liposomes with mean diameters of about 120 nm without any additional lipid ingredients. No significant change in particle size distribution profile was observed over one-month storage at room temperature (20–25 °C). The gel to liquid crystalline phase transition temperature ($T_m$) of cationic liposomes comprised of both *R* and *S* isomers was approximately 2 °C, as measured by differential scanning calorimetry (DSC). Both isomers also formed stable liposomes when combined with DOPE. *In vitro* transfection efficiency of the CCA/DOPE liposomes complexed to plasmid DNA was evaluated using a luciferase reporter gene. Both liposomes composed of *R* and *S* isomers of the cationic cardiolipin displayed higher transfection efficiency than commercially available Lipofectin®. Further *in vivo* studies are warranted.

## 1. Introduction

Gene therapy is considered a promising approach for the treatment of a wide variety of diseases. Among DNA delivery systems, the attenuated viruses have shown the greatest efficiency to date (Cavazzana-Calvo et al. 2000), but numerous concerns over the safety of these vectors has led to the development of nonviral vectors in gene therapy (Temin 1990; Gunter et al. 1993; O'Neal 2000; Yang 1994 and 1995). Since positively charged liposomes were found to form complexes with DNA and subsequently transfer DNA into various cells, considerable effort has been devoted to synthesizing new cationic lipids. The advantages of using cationic liposomes in gene transfection are ease of large scale production, simplicity in preparation and administration, relatively low toxicity and immunogenicity (Felgner et al. 1987; Miller 1998; Wang 1998; Gao and Hui 2001; Hyvonen et al. 2000) however, the transfection achieved with these nonviral vectors is significantly lower than that achieved with viral vectors. The low transfection efficiencies are due to the multiple biological barriers encountered during their delivery from extracellular locations into the cell's nucleus (Zabner 1995). One of the major problems with systemic nonviral gene delivery is that the particles are endocytosed when they reach the target cell and the resulting endosomes are generally trafficked to lysosomes causing degradation of the DNA in lysosome before reaching target (Fig. 1). Therefore, it is important to release the gene from the endosomes before it sequesters to the lysosomes. It has been suggested that the ability of cationic lipids to facilitate intracellular delivery is related to their ability to induce the nonbilayer hexagonal $H_{II}$ phase in combination with anionic lipids at endosomal stage, and that the most potent cationic lipids are those that are the most effective inducers of $H_{II}$ phase organization (Hafez 2001). It is known

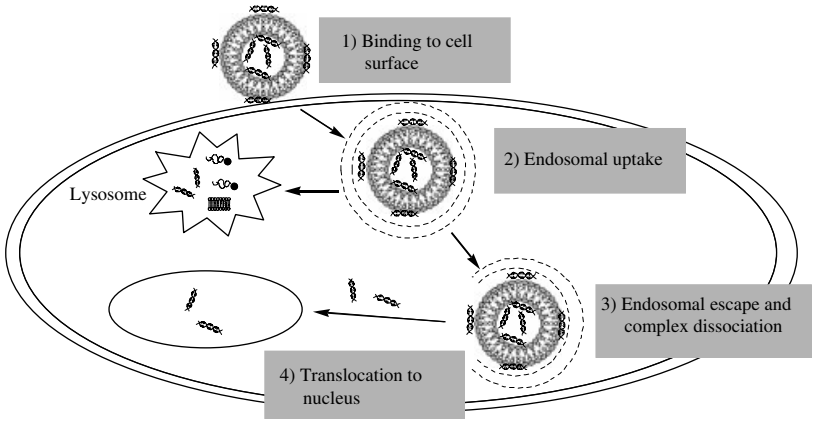
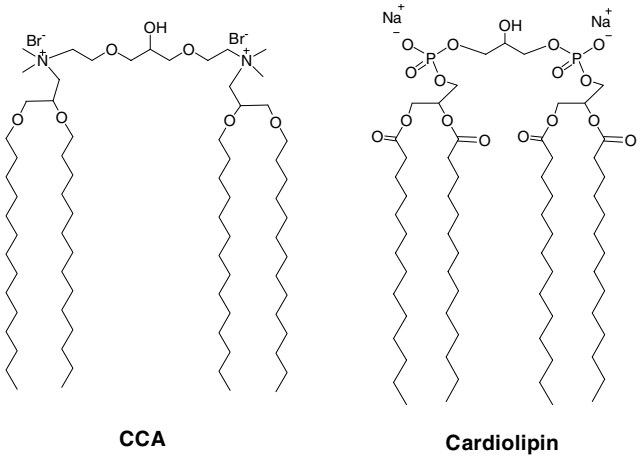



Fig. 1:
Cationic liposome-mediated gene delivery mechanism

that the ability of lipids to adopt the $H_{II}$ phase is related to their dynamic molecular shapes. Generally, a lipid with a small cross-sectional area in the headgroup region and a larger acyl chain cross-sectional area exhibit a "cone" shape compatible with $H_{II}$ phase organization (Cullis and de Kruijff 1979). Recently, Gaucheron et al. used this strategy to increase the cone-shaped character of a given lipid by increasing the number of alkyl chains associated with the headgroup. The resulting dimer lipids exhibited higher transfection efficiency than monomer. A novel cationic cardiolipin analogue (CCA) was synthesized in this laboratory for drug delivery and to achieve better transfection efficiency. Cationic cardiolipin analogue (CCA) is an ether analogue of naturally occurring cardiolipin where the fatty acid chains were replaced with alkyl chains. In addition, the phosphate groups were replaced with quaternary ammonium groups resulting in positive charge. A spacer was introduced between quaternary ammonium groups and central glycerol unit (Kasireddy 2004). Little is known about the influence of backbone chirality of cationic lipid on gene transfection therefore, we synthesized both *R* and *S* forms of cationic cardiolipin aiming at understanding how lipid stereochemistry could affect gene transfection efficiency. In this work, we conducted physical characterization of the liposomes formed by these two novel cationic cardiolipin analogues and evaluated their efficiency in gene transfection *in vitro*.




## 2. Investigations, results and discussion

### *2.1. Characterization of R-CCA and S-CCA*

The characterization results for *R*-CCA are as follows: $^1$H NMR ($CDCl_3$, 500 MHz): δ 0.88 (t, J = 6.7 Hz, 12 H), 1.25 (s, 88H), 1.52–1.71 (m, 8 H), 3.41–3.68 (m, 30 H), 3.95–4.19 (m, 13 H), 4.63 (brs, 1 H, OH), IR ($cm^{-1}$): 3409 (br, OH), 2918 (s), 2873 (s), 1468 (s), 1124 (s),

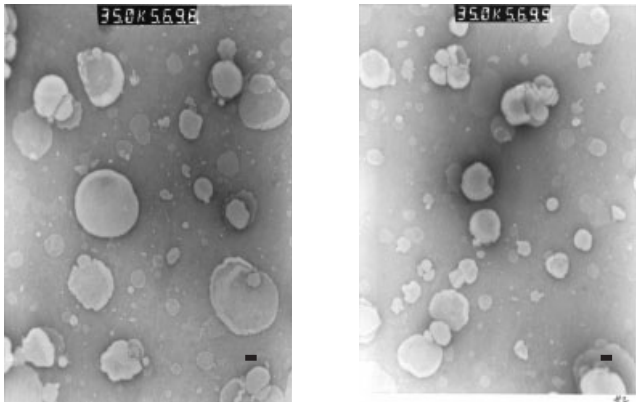


Fig. 2: TEM of *R*-CCA liposomes (2a) and *S*-CCA liposomes (2b) (bar = 100 nm)

ESI-MS 1248.5 [M+1−Br], 584.3 [M+1−2 Br/2]. The characterization data for *S*-CCA are as follows: $^1$H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, J = 6.6 Hz, 12 H), 1.25 (s, 88 H), 1.52–1.71 (m, 8 H), 3.40–3.71 (m, 36 H), 3.92–4.11 (m, 7 H), 4.74 (d, J = 5.7 Hz, 1 H, OH). IR ($cm^{-1}$): 3397 (br, OH), 2917 (s), 1467 (s), 1122 (s) $cm^{-1}$, Mass (ESI+ve mode): 1248.5 [M+1−Br], 584.4 [M+1−2 Br/2].

### *2.2. Characterization of CCA lipid dispersions*

Transmission electron microscope (TEM) images obtained for these lipid dispersions are shown in Fig. 2. The micrographs revealed bilayer vesicle structures of the lipid dispersion formulated by both *R* and *S* form of the cationic cardiolipin analogue (CCA). The average particle size observed by TEM ranged from 100 to 200 nm, whereas, the mean diameters of *R* and *S* CCA dispersion measured by dynamic light scattering method were 115 and 128 nm, respectively (Fig. 3). Both lipid dispersions showed unimodal distribution. No significant change in particle size was observed over one-month storage at room temperature (Table 1) indicating that the lipid dispersions were physically stable. The thermotropic properties of *R* and *S* CCA dispersions were studied by DSC (Fig. 4 and Table 2). A temperature lag was observed in the thermograms of the cooling scans (liquid crystalline to gel phase transition) for both lipids. Both lipid dispersions showed two similar

**Table 1: One-month stability of *R* and *S* isomers of cationic cardiolipin analogue dispersions**

| Lipids | Conc. (mg/mL) | Mean diameter (nm) | |
|---|---|---|---|
| | | 0-month | 1-month |
| CCA(R) | 30 | 115 (99% < 242) | 117 (99% < 283) |
| CCA(S) | 30 | 128 (99% < 317) | 125 (99% < 306) |

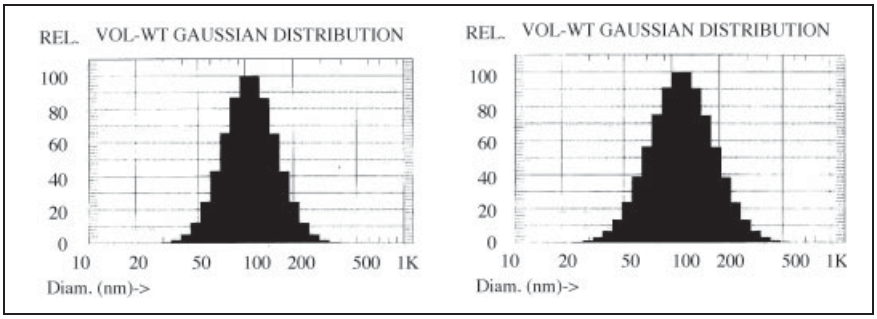



Fig. 3:
Size distribution of *R* and *S* isomers cationic cardiolipin analogues (left *R*-CCA, right *S*-CCA)

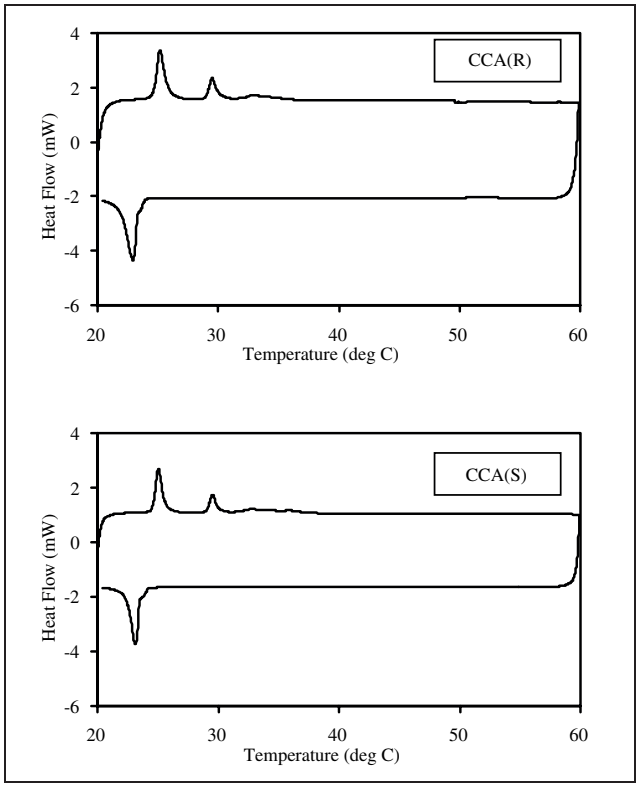



Fig. 4: Thermograms of CCA liposomes (upper *R*-CCA, lower *S*-CCA)

**Table 2: Thermotropic parameters from DSC studies of CCA**

| Lipids | Tm (°C) | | ΔH (kcal/mol) | |
|---|---|---|---|---|
| | Upscan | Down-scan | Upscan | Down-scan |
| CCA(R) | 25.17<br>29.53 | 22.91 | 13.64<br>4.95 | 18.70 |
| CCA(S) | 25.03<br>29.56 | 23.13 | 13.97<br>4.95 | 19.69 |

peaks in their heating scan (upscan) and two peaks (not well separated) in their cooling scan (downscan) which suggested that the chirality of CCA had no effect on the phase transition temperature of the lipids. It was found that the thermal behaviors of both *R* and *S* forms of CCA were reversible. As indicated in subsequent scans, the same transition temperature appeared as the previous one.

### *2.3. Characterization of CCA-DOPE liposomes*

Multilamellar vesicles (MLV) of *R or S* CCA and DOPE 1 : 1 wt/wt were prepared by a thin film hydration method as described in the Experimental section. After size reduction by extrusion, both *R* and *S* forms of the liposome became translucent. The mean diameters measured by dynamic light scattering were 123 and 116 nm, respectively (Fig. 5 and Table 3). The particle size distributions of both *R* and *S* forms of the liposome were uniform (unimodal distribution).

**Table 3: Parameters for 1 : 1 wt/wt CCA-DOPE liposomal formulations**

| Formulation | Molar ratio | Conc. (mg/mL) | Mean diameter |
|---|---|---|---|
| CCA-DOPE(R) | 1 : 1.8 | 10 | 123 nm (99% < 249) |
| CCA-DOPE(S) | 1 : 1.8 | 10 | 116 nm (99% < 249) |

### *2.4. In vitro gene transfection*

The *in vitro* transfection efficiency of 1 : 1 wt/wt CCA-DOPE liposomes were evaluated using CHO cells and compared with that of commercially available Lipofectin®. DNA was held constant at one microgram and the amount of liposomes were varied to achieve different charge ratios (Table 4). Both CCA-DOPE liposomes and Lipofectin® showed much higher transfection efficiency than vector-free sample (Fig. 6). CCA-DOPE liposomes were at least 10-fold more efficient than Lipofectin®. Charge ratio had a less effect on transfection efficiency for CCA-DOPE liposomes than that for Lipofectin®.

## 3. Discussion

In this work, we characterized and evaluated formulations containing novel cationic cardiolipin analogues for gene transfection effciency. Cationic lipids having ether linkages were shown to have much greater *in vivo* transfection efficiency than corresponding cationic lipids with ester linkage. For example, it was reported that *N*-[1-(2,3-dioleyloxy)propyl}-*N,N,N*-trimethylammonium chloride (DOTMA) was more efficient than 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) in gene transfection (Song 1997). In addition, ether linkage is also resistant to hydrolysis. CCA is an ether analogue of naturally occurring cardiolipin where fatty acid chains were replaced by alkyl chains. The positive charge was created

**Table 4: Lipid/DNA complex composition and charge ratios of liposomal formulations used for *in vitro* gene transfection studies**

| Cationic Liposomes | Lipid/DNA charge ratio | | | | Lipid/DNA weight ratio | | | |
|---|---|---|---|---|---|---|---|---|
| Lipofectin® | 2.7 | 5.4 | 8.0 | 10.7 | 10 | 20 | 30 | 40 |
| 1 : 1 wt/wt PCL-2(R)-DOPE | 2.7 | 5.4 | 8.0 | 10.7 | 10 | 20 | 30 | 40 |
| 1 : 1 wt/wt CCA(S)-DOPE | 2.7 | 5.4 | 8.0 | 10.7 | 10 | 20 | 30 | 40 |

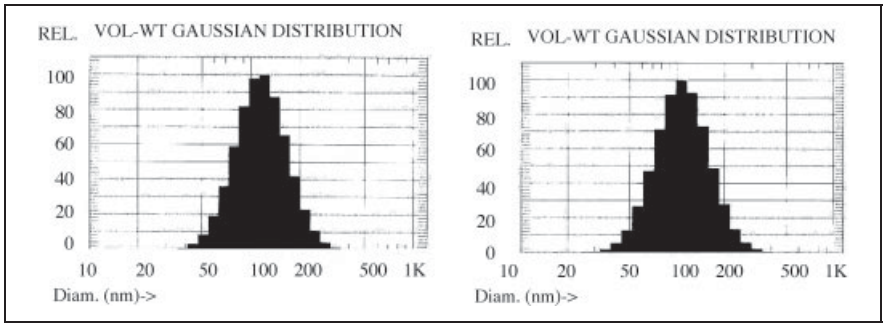



Fig. 5:
Size distribution of *R*-CCA-DOPE and *S*-CCA-DOPE liposomes (5a: *R*-CCA-DOPE, 5b: *S*-CCA-DOPE)

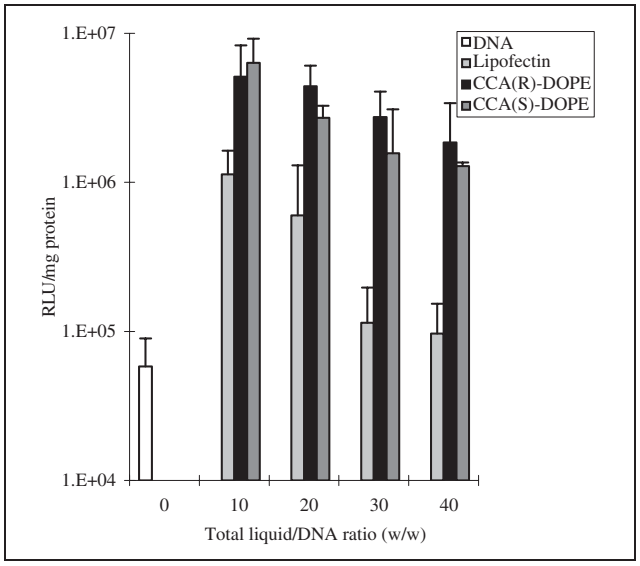



Fig. 6: Transfection results in CHO cells

by replacing phosphate groups by quaternary ammonium groups. HPLC data showed that CCA was chemically stable in the lipid dispersion (Ugwu 2005). The DSC thermograms of both isomers showed double peaks in both their heating and cooling scans. These thermal behaviors were found to be completely reversible. This suggests that a stabilized liquid crystal and gel phase might exist in the lipid aggregates of CCA. Likely, hydrogen bonding between water and the oxygen, as well as hydroxyl group in the central core of the molecule, caused some areas of CCA to aggregate therefore, heating of the lipid aggregate may not immediately allow those areas to melt and additional energy is needed to reach liquid crystal state. Alternatively, cooling of the melted lipid aggregate may not immediately allow the release of the water molecules prior to solidification to gel state. Similar behavior was previously observed by Bhattacharya et al. (2003) in their studies on oxyethylen-based cationic lipids. In addition, both isomers exhibited a temperature lag of about 2 °C, indicating first-order phase transitions in these assembles (Marsh 1991).

Based on the model for DNA delivery mechanism proposed by Xu and Szoka (1996), the cell surface associated complex should be internalized into endosomes. Subsequently, the complex would initiate a destabilization of the endosome membrane that resulted in flip-flop of anionic lipids that were predominately located on the cytoplasmic face of the membrane. In fact, the anionic lipid composition of early endosomes has been reported to be 18 mol% (Kobayashi et al. 1998). In addition, Zelphati and Szoka (Zelphati and Szoka 1996) used PG or PS-containing liposomes as an endosome model to examine how much of anionic lipids were necessary to release oligonucleotide from its complex with DOTAP at +/− ratio of 1 : 1. Their data suggested that 15% or 25% of PG or PS in model membranes were able to release oligonucleotide. Based on the above facts, 18 mol% of anionic lipids in endosomal membranes should be sufficient to neutralize the positive charges of CCA and release DNA. Although CCA forms bilayer structures as revealed in this work, the formation of charge-neutralized ion pairs would substantially reduce its net headgroup area similar to the case discussed by Hafez et al. (2000). This will increase the possibility of forming "cone" shaped lipid ion pairs and thus favor the formation of a hexagonal $H_{II}$ phase to cause endosomal membrane disruption. In addition, CCA has a larger cross-sectional area in the hydrocarbon chain region than monomer lipid DOTMA. This will further increase the formation of "cone" lipid ion pairs and therefore enhance endosomal membrane disruption as described above. Overall, CCA has much more "cone" shape character than DOTMA upon formation of ion pairs with anionic lipids from endosomal membrane and thus efficiently induce the nonbilayer hexagonal $H_{II}$ phase to facilitate membrane fusion and DNA release. As the ratio of total lipid/DNA in the transfection samples was increased from 10 to 40, the luciferase activity decreased for both CCA-DOPE liposomes and Lipofectin® due to the likelihood of lipid-related cell toxicity however, there were only 2.8 fold and 4.9 fold decrease in activity for *R*-CCA-DOPE and *S*-CCA-DOPE formulations, respectively. By contrast, for Lipofectin®, there was an 11.7 fold decrease in activity indicating CCA was less toxic than Lipofectin®. This result was also confirmed by a separate study, which showed that CCA containing liposomes were less toxic than most of the commercially available cationic lipids (Chien 2005). The influence of backbone chirality of cationic lipid on gene transfection has not been thoroughly investigated. To the best of our knowledge, there has been only one report in the literature which addressed this issue. Wang et al. (1998) investigated cationic L- and DL-derivatives of carnitine. Their data indicated that the L-derivative mediated the similar level of transfection as racemic mixture at the same optimal charge ratios (+/−). Since natural phospholipids in cell membrane have *R* configuration at the C-2 carbon of glycerol moiety, we assume that the *S*-isomer may exhibit lower transfection efficiency than the *R*-isomer due to its poor cooperativity with natural phospholipids in cell membrane during fusion at endosomal stage. However, both isomers showed a similar level of transfection at the same optimal charge ratios (+/−) (Fig. 6). Overall, our results together with Wang et al.'s have revealed that lipid chirality may have significant impact on gene transfection efficiency.

In conclusion, we have developed novel, optically active *R* and *S* isomers of cationic cardiolipin. Both *R* and *S* isomers of cationic cardiolipin liposomes displayed higher gene transfection efficiency than commercially available Lipofectin®. The eventual utility of the novel cationic lipid reported in this study will depend on its ability to facilitate efficient gene transfer *in vivo*. In contrast to transfection studies *in vitro*, a multitude of factors, especially pharmacokinetic properties, will profoundly affect the efficiency and tissue specificity of *in vivo* gene delivery. Based on the promising preliminary data obtained from the current study, we believe further studies are warranted to evaluate the *in vivo* transfection efficiency of the liposome/DNA complex formulation.

## 4. Experimental

### *4.1. Synthesis of 1,3-bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammonium bromide)-propane-2-ol (CCA)*

R-CCA and *S*-CCA were synthesized according to procedure reported previously (Kasireddy et al. 2004). The structures of *R*-CCA and *S*-CCA were characterized by $^1$H NMR, IR and MS analyses.

### *4.2. Preparation of liposomes*

Appropriate amounts of lipids were dissolved in ethanol. The solvent was evaporated under vacuum using a rotary evaporator. The resulting thin film was further dried under vacuum overnight. The film was hydrated in deionized water with the help of vortexing and a warm water bath (45 °C) to form a homogenous lipid dispersion. The dispersion was sized by extru-

sion through double stacked 0.2 and 0.1 μm polycarbonate membranes (Whatman, Clifton, NJ) using a 10-mL extruder (Northern Lipids Inc., Vancouver, Canada). The mean particle size of the above liposomes was reduced to about 120 nm.

### *4.3. Electron microscopy*

The CCA liposomes (30 mg/mL) were diluted 1 : 4 (volume ratio) with distilled water and applied to the 200 mesh, Formvar-carbon coated grids. The sample drops on the electron microscopy grids were maintained for 2 min without disturbing to settle out the liposome particles onto the grid surface. The liposome particles were negatively stained with 2% uranyl acetate, pH 4.8, for 30 and were subsequently characterized using a Philip CM 12 transmission electron microscope. The grid openings were randomly selected and viewed. Micrographs were taken at magnifications of 10000 to 35000× and magnifications were calibrated with the standardized grating replica.

### *4.4. Differential scanning calorimetry (DSC)*

Thermograms were obtained from a differential scanning calorimetry, Model Q100 series (TA Instruments, New Castle, DE). For each measurement, one empty hermetically sealed aluminum pan/lid pair was used as reference, and another pair was used for the samples. The difference in weight of the two different pairs was ensured to be no more than 1 mg prior to each measurement. *R* or *S* CCA dispersions were freeze-thawed three times before measurement. Approximately 20 mg of sample were placed into the sample pan. After both reference and sample pans were crimped, they were placed on the heating block of DSC and heated from 20 to 60 °C at a rate of 1 °C/min.

### *4.5. Particle size analysis*

The particle size was measured on a Nicomp™ 380 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with an auto dilution function. The laser in this equipment was operated at 632.8 nm using a 90° angle between incident and scattered beams. Polystyrene bead standards were used to verify the performance of the instrument prior to sample measurement. Data were analyzed automatically either by Gaussian analysis or Nicomp distribution analysis from the Nicomp™ CW380 software. Data were analyzed in terms of intensity, volume and number distributions assuming that particles are spheres of uniform density which scatter light according to classic Mie theory. Data were reported as volume weighted distribution and represented as mean of three measurements.

### *4.6. Transfection study*

pcDNA3-CMV-Luc (pLuc, 7.1 kb) plasmid DNA containing the firefly luciferase gene under the control of the cytomegalovirus enhancer/promoter was kindly provided by Dr. Leaf Huang at the University of Pittsburgh Department of Molecular Genetics and Biochemistry. Cloning and preparation of plasmid DNA were performed by propagating transformed DH5-α *E. coli* in LB media containing ampicillin (50 μg/mL), followed by isolation and purification with the Qiagen™ Plasmid Mega Kit (Qiagen, Valencia, CA). The concentration and purity of plasmid DNA was assessed by measuring absorbance at 260 and 280 nm ($OD_{260}/OD_{280} \sim 1.9$) on a Shimadzu UV-160U spectrophotometer. The size and integrity of the plasmid were confirmed by gel electrophoresis (0.9% agarose gel containing 0.5 μg/mL ethidium bromide) in Tris acetate-EDTA buffer (TAE, pH 8.0). Chinese hamster ovary (CHO) cells were cultured in RPMI 1640 media (GIBCO BRL, Rockville, MD) supplemented with 10% newborn calf serum (NCS), penicillin-streptomycin and 300 μg/mL L-glutamine at 37 °C in a 5% $CO_2$-humidified incubator.

Liposomes with the composition *R*-CCA:DOPE or *S*-CCA:DOPE (1 : 1 wt/wt) were prepared as described previously. Lipofectin® (Invitrogen, Carlsbad, CA) was used as the control. The total lipid content in these liposomal formulations was 1 mg/mL. Liposome/DNA complexes were prepared in PBS (Phosphate-buffered saline, pH 7.4) by rapidly adding liposomes to pcDNA3-CMV-Luc plasmid DNA in 150 μL of PBS to achieve the desired Lipid/DNA ratios (w/w) in a final volume of 300 μL. One microgram of pcDNA3-CMV-Luc plasmid DNA per well was used. Samples were incubated at room temperature for 20 min prior to transfection studies.

For transfection studies, CHO cells were seeded at a density of $1.0 \times 10^5$ cells per well on 24-well plates (Falcon, San Diego, CA) and incubated for 24 h in RPMI 1640 media supplemented with 10% NCS and antibiotics at 37 °C (~70% confluent). Prior to transfection, the medium was removed and the plates were rinsed with 0.5 mL of PBS. One hundred microliters of liposome/DNA complexes or naked DNA were then added to each well and incubated with cells in 0.5 mL serum-free media at 37 °C for 4 h. After incubation, the media were removed and the cells were rinsed with 0.5 mL of PBS followed by the addition of 0.5 mL of fresh medium containing 10% fetal bovine serum and antibiotics (Life Technologies, Rockville, MD). The cells were incubated for another 24 h and then harvested and assayed for luciferase activity. All transfection experiments were performed in duplicate.

To assay for the luciferase activity, the medium was removed from each well and cells were washed with 0.5 mL/well of cold PBS. Two hundred microliters of ice-cold lysis buffer (100 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, pH 7.8) was added to each well and incubated on ice for 20 min. The cell lysate was then transferred to 0.5 mL centrifuge tubes and centrifuged at 13,000 rpm for 2 min. The luminescence was measured on a Mini-Lum™ luminometer (Bioscan, Inc., Washington, DC) immediately after mixing 10 μL of cell lysate with 50 μL of luciferase substrate (Promega, Madison, WI). Relative light units were standardized for protein concentration determined by the bicinchoninic acid (BCA) protein assays using bovine serum albumin standards.

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