Cationic Lipids with Increased DNA Binding Affinity for Nonviral Gene Transfer in Dividing and Nondividing Cells

ARTICLE *in* BIOCONJUGATE CHEMISTRY · JANUARY 2005

Impact Factor: 4.51 · DOI: 10.1021/bc049818q · Source: PubMed

4 AUTHORS, INCLUDING:

Ajit S Narang
Bristol-Myers Squibb
69 PUBLICATIONS **1,184** CITATIONS
SEE PROFILE

Duane D Miller
University of Tennessee
436 PUBLICATIONS **7,831** CITATIONS
SEE PROFILE

Ram I Mahato
University of Nebraska Medical Center
147 PUBLICATIONS **5,009** CITATIONS
SEE PROFILE

Cationic Lipids with Increased DNA Binding Affinity for Nonviral Gene Transfer in Dividing and Nondividing Cells

Ajit S. Narang,[†] Laura Thoma,[†] Duane D. Miller,[†] and Ram I. Mahato[†,‡,*]

Departments of Pharmaceutical Sciences and Biomedical Engineering, University of Tennessee Health Science Center, Memphis, Tennessee 38163. Received July 27, 2004; Revised Manuscript Received September 30, 2004

Effect of headgroup structure on catonic lipid-mediated transfection was investigated with either a (i) tertiary amine, (ii) quaternary amine with a hydroxyl, or (iii) quaternary amine with mesylate as headgroups. Liposomes were formulated using cholesterol or dioleoyl phosphatidyl ethanolamine (DOPE) as colipids, and transfection efficiencies were determined in rapidly dividing colon carcinoma (CT 26) and rat aortic smooth muscle (RASM) cells as well as in nondividing human pancreatic islets using luciferase and green fluorescent protein expression plasmids, pcDNA3-Luc and pCMS-EGFP, respectively. Liposome/pDNA complexes were evaluated for DNA conformational state by circular dichroism (CD), DNA condensation by electrophoretic mobility shift assay (EMSA), particle size and zeta potential by laser diffraction technique, and surface morphology by transmission electron microscopy (TEM). Encouraging transfection results were obtained with the mesylate headgroup based lipid in liposome formulations with DOPE as a colipid, which were higher than the commercially available Lipofectamine formulation. We hypothesize that the additional hydrogen bonding or covalent interactions of the headgroup with the plasmid DNA, leading to higher binding affinity of the cationic lipids to pDNA, results in higher transfection. This hypothesis is supported by TEM observations where elongated complexes were observed and more lipid was seen associated with the DNA.

INTRODUCTION

Cationic lipid-based nonviral gene delivery system is an attractive approach for therapeutic gene transfer. Most cationic lipids have three parts: (i) a hydrophobic lipid anchor group, (ii) linker group, such as an ester, amide, or carbamate, and (iii) a positively charged headgroup, which interacts with pDNA, leading to its condensation (*1*, *2*). Among all the basic components of the cationic lipid, the type of headgroup has been shown to have a dominant role in transfection efficiency and toxicity. Three functional properties characterize the activity of any cationic liposome formulation: (i) efficient DNA condensation, (ii) increased cellular uptake due to interaction of positively charged complexes with negatively charged biological surfaces, and (iii) membrane fusion or transient membrane destabilization with the plasma membrane or the endosome to achieve delivery into the cytoplasm while avoiding degradation in the lysosomal compartment (*3*).

Since the introduction in 1987 of the transfection reagent Lipofectin, a 1:1 w/w mixture of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) and dioleoyl phosphatidyl ethanolamine (DOPE) (*4*), many cationic lipids bearing either a single tertiary or quaternary ammonium headgroup or which contain protonatable polyamines linked to dialkyl or cholesterol anchors have been designed for transfection of a variety of cell types in culture (*5*, *6*). Commercially available cationic lipids used as liposome formulations or alone for gene delivery include DOTMA; 2,3-dioleyloxy-*N*-[2-spermine carboxamide] ethyl-*N*,*N*-dimethyl-1-propanammonium trifluoroacetate (DOSPA), 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), *N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium bromide (DMRIE), *3β*-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), and dioctadecyl amidoglyceryl spermine (DOGS, Transfectam). A few such compounds have also been used in initial clinical studies (*7*). However, many of the cationic lipids used in early clinical trials such as DC-Chol (*8*), DMRIE (*9*), and GL-67 (*10*), although effective in vitro, have proven inefficient in vivo, especially when compared with viral vectors.

Based on the structures of their cationic headgroups, cationic lipids can be classified into quaternary ammonium lipids, lipopolyamines, cationic lipids bearing both quaternary ammonium and polyamine moieties, amidinium and guanidinium salt lipids, and heterocyclic cationic lipids. A quaternary ammonium headgroup is widely used in many of the established cationic lipids such as DOTAP, DOTMA, and DMRIE, with the exception of Lipofectamine, which is a combination of both a polyamine and a quaternary cation. There are fewer lipids containing a primary, secondary, or tertiary amine headgroup. An example is DC-Chol, with a tertiary amino headgroup linked to cholesterol (*5*).

We have earlier studied the effect of cationic lipid structures, cationic lipid to colipid molar ratios, liposome size, charge ratio, and DNA dose on in vivo gene expression (*11*). Since liposome/plasmid complexes did not disperse well inside the solid tumor after local intratumoral injection (*12*) but showed beneficial effect in tumor regression (*13*), we later developed water soluble lipopolymers for gene delivery (*14*, *15*). Although water

* Corresponding author. Ram I. Mahato, Ph.D., Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, 26 S. Dunlap Street, Feurt Building, Room 413, Memphis, TN 38163. Tel: (901) 449 6929. Fax: (901) 448 6092. E-mail: rmahato@utmem.edu.

[†] Department of Pharmaceutical Sciences.

[‡] Department of Biomedical Engineering.

10.1021/bc049818q CCC: $30.25
Published on Web 12/31/2004

soluble lipopolymers were effective in transfecting various cells, they were not effective in transfecting nondividing human pancreatic islets. Therefore, we decided to use Lipofectamine for gene delivery to human islets. We observed low transfection efficiency, possibly due to poor nuclear translocation, as the nuclear membrane remains intact in these cells at all times (*16*, *17*). This is also because islets are a compact cluster of ~1000 nondividing cells (*18*). To further enhance the transfection efficiency in both dividing and nondividing cells, we have designed novel cationic lipids based on C_{18} monounsaturated oleoyl esters of glycerol backbone to be used as liposomes. C_{18} chain length was selected based on the previous studies by various groups who have shown that carbon chain length significantly influences transfection efficiency, and C_{18} is the most commonly used and efficient in transfection (*3*, *11*, *19*). Moreover, smaller chain length lipids tend to provide more fluidity and weaker binding (*19*, *20*). Furthermore, introduction of unsaturation into carbon chain is known to give higher transfection (*21*).

MATERIALS AND METHODS

RPMI medium 1640 and trypsin−ethylenediaminetetraacetic acid (EDTA) were procured from Gibco, Invitrogen Corporation (Grand Island, NY). Dulbecco's phosphate-buffered saline (DPBS), heat-inactivated fetal bovine serum (FBS), and penicillin (10000 units/mL penicillin G sodium)−streptomycin solution (10000 μg/mL streptomycin sulfate) in 0.85% saline were purchased from Cellgro, Mediatech, Inc. (Herndon, VA). Luciferase Assay System, Luciferase Cell Culture Lysis Reagent 5X (CCLR), and Sca I restriction enzyme were procured from Promega Corporation (Madison, WI). QIAfilter plasmid maxi kit was purchased from Qiagen, Inc. (Valencia, CA); 96-well flat-bottom white LIA plate from Greiner Bio-One, Inc. (Longwood, FL); BCA protein assay kit from Pierce (Rockland, IL); Terrific Broth (Modified), Luria Agar (Miller's LB agar), glycerol, agarose, ethidium bromide aqueous solution (10 mg/mL), 2-propanol, absolute ethyl alcohol, sodium hydroxide pellets, oleoyl chloride, 2-bromoethanol, and various organic solvents including chloroform, acetone, ethanol, methylene chloride, phosphomolybdic acid spray reagent, and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich Co. (St. Louis, MO). SURE-2 supercompetent cells were purchased from Stratagene, Inc. (La Jolla, CA), SOC medium from Invitrogen (Carlsbad, CA), 3-(dimethylamino)-1,2-propanediol, silica gel, and methanesulfonyl chloride from Aldrich Chemical Co., Inc. (Milwakee, WI), deuterated chloroform from Cambridge Isotope Labs, Inc. (Andover, MA), and cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) from Avanti Polar Lipids, Inc. (Alabaster, AL). Carbon-coated 300-mesh copper grids were purchased from Electron Microscopy Sciences, Inc. (Fort Washington, PA). pCMS-EGFP plasmid was procured from BD Biosciences Clontech (Palo Alto, CA) and pcDNA3-Luc plasmid was a kind gift from Expression Genetics, Inc. (Huntsville, AL).

Synthesis and Purification. The synthesis scheme of tertiary, quaternary, and mesylate cationic lipids is shown in Figure 1. 3-(Dimethylamino)-1,2-propanediol (I) was reacted with oleoyl chloride (II) in anhydrous chloroform overnight at room temperature. The reaction mixture was extracted sequentially with 0.1 N sodium hydroxide, water and brine followed by dehydration in sodium sulfate and evaporation of chloroform in a rotary evaporator. Complete removal of starting materials was ascertained by ^{1}H nuclear magnetic resonance (NMR) spectroscopy using Bruker ARX-300 (Bruker Biospin

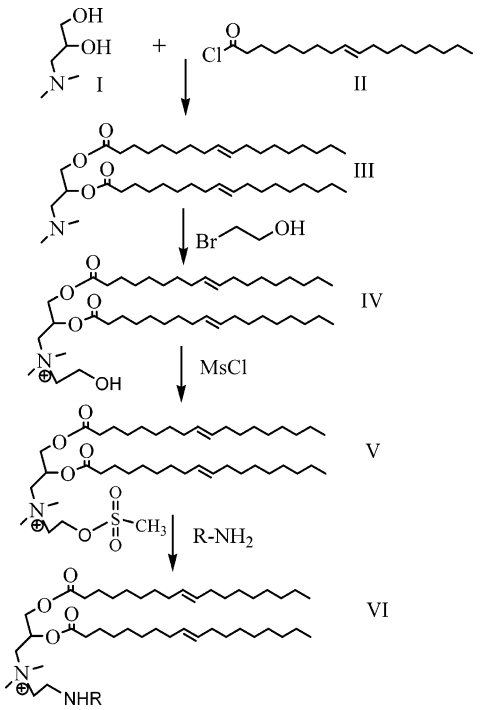

Figure 1. Synthesis scheme for 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP, III), 3-(*N*-2-hydroxyethyl dimethylamino)-1,2-dioleoylpropanediol (HE-DADP, IV), and 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP, V) cationic lipids starting with 3-(dimethylamino)-1,2-propanediol (I) esterification using oleoyl chloride (II), followed by quaternization using 2-bromoethanol and mesylation of the free hydroxyl group. EMS-DADP can be attached to various amine headgroups to yield transfection reagents with the structure (VI).

Corporation, Billerica, MA). The product, 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP, III), was dehydrated in a desiccator and stored at −20 °C (yield >90%). The quaternary amine compound, 3-(*N*-2-hydroxyethyldimethylamino)-1,2-dioleoylpropanediol (HE-DADP, IV), was synthesized by reacting (III) in 1:1 molar quatity in *N*,*N*-dimethylformamide at 105 °C for 24 h followed by solvent evaporation under high vacuum. Reaction conditions were optimized through ^{1}H NMR monitoring to minimize the formation of side products and maximize the reaction yield, which was 80%. The product was identified by both NMR and electrospray ionization-mass spectrometry (ESI-MS) using Bruker Esquire-LC Ion Trap LC/MS (Bruker Biospin Corporation, Billerica, MA). The mesylate derivative, 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP, V), was synthesized by reacting HE-DADP with methanesulfonyl chloride in the presence of triethylamine as the base, in methylene chloride on ice, followed by room temperature, for 3 h. Following characterization using thin-layer chromatography (TLC) using acetone/NaOH medium with spot visualization using phosphomolybdic acid/heat gun treatment, the reaction mixture was extracted using silica gel chromatography using acetone: 0.1 N aqueous NaOH (97/3) as the mobile phase. The Product eluted in the second fraction at an $R_f \sim 0.6$. purified product was identified by both NMR and ESI-MS.

Plasmid Preparation and Purification. Luciferase expression plasmid, pcDNA3-Luc, driven by cytomegalovirus (CMV) promoter, and enhanced green fluorescent protein (EGFP) expression plasmid, pCMS-EGFP, were transformed in SURE-2 supercompetent cells. Colonies were grown in 50 μg/mL ampicillin containing LB Agar

plates, followed by growth of single colonies in ampicillin containing terrific broth media. After sufficient bacterial growth, the cells were pelleted by centrifugation at 5000*g* and 4 °C for 20 min followed by plasmid extraction using QIAfilter plasmid maxiprep kit as per vendor's protocol. Plasmid purity was assessed by UV spectrophotometry ($A_{260/280} > 1.75$), agarose gel electrophoresis, and restriction digestion using *Sca I* enzyme.

Preparation of Liposomes and Lipid/Plasmid Complexes. Liposomes were prepared from a cationic lipid and a colipid (cholesterol or DOPE) at various molar ratios. Briefly, lipids were mixed in chloroform at desired molar ratios and evaporated to dryness in a 50-mL round-bottom flask using a rotary vacuum evaporator. Ethanol was used in the case of EMS-DADP due to the limited solubility of the mesylate compound in chloroform. The lipid film was hydrated in purified water to produce multilamellar vesicles, which were passed through an extruder (Lipex Biomembranes, Vancouver, Canada) using 100, 400, and 800 nm pore size polycarbonate membrane 10 times. The liposomes were stored at 4 °C as colloidal dispersions until used.

Plasmid and cationic liposomes were diluted separately to a volume of 250 μL with a final glucose concentration of 5% w/v, and then the DNA was added to the liposomes with mild mixing by pipeting. Complex formation was allowed to proceed for 45 min at room temperature. Lipid/plasmid complexes of different charge ratios were prepared by adjusting the stoichiometry of plasmid and cationic liposomes. The complexes were characterized for condensation efficiency, particle size, and ξ-potential as described previously (*11*, *22*, *23*). Briefly, complex formation and DNA condensation were determined using 1% agarose gel electrophoresis at 60V. The mean particle size of lipid/plasmid complexes was determined at 90° by dynamic light scattering using 90Plus Particle Sizer (Brookhaven Instruments Corp., Holtsville, NY). Particle size data is reported as a mean ± SD of three samples. ξ-Potential was measured by measuring electrophoretic mobility in aqueous solution at 25 °C and pH 7 utilizing a 30 mW laser and 0 to 3.2 kV/m field strength. The ξ-potential is reported as an average of 10 separate readings for a single sample.

Cell Culture and Transfection. Colon carcinoma (CT-26) and rat aortic smooth muscle (RASM) cells were cultured in 162 cm^2 flasks in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic−antimycotic mixture. About 50 000 cells were plated in each well of 12-well cell culture plates. When the cells reached ~80% confluency (about 48 h after plating), cells were washed with cold Dulbecco's phosphate buffered saline (DPBS) and were incubated with cationic liposome/pDNA complexes in 500 μL serum free medium. At 6 h posttransfection, the culture media was replaced with serum containing media. In the case of EGFP expression plasmid, cells were incubated for additional 46−48 h and analyzed for transfection efficiency by fluorescence microscopy and flow cytometry. For luciferase expression plasmid, 48 h posttransfection the cells were washed with cold PBS and lysed in 500 μL of luciferase cell culture lysis reagent. Total protein was measured using BCA protein assay kit. Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate Labsystems Luminoskan RS luminometer (Albertville, MN). Human pancreatic islets were isolated, purified, counted, and cultured as described earlier (*16*, *17*). For transfection experiments, 1000 I.E. were suspended in 48-well plates and incubated for 12 h with pDNA/liposome complexes at the dose of 1 μg/well, followed by addition of more media and incubation at 37 °C in 5% CO_2 incubator for 48 h until cell lysis. $N = 6$ replicas were used for each sample.

Circular Dichroism. Circular dicrhroism (CD) spectra of plasmid DNA and pDNA/liposome complexes were obtaind using the Aviv 62A spectrometer (Lakewood, NJ). Samples were scanned through a wavelength range of 220−360 nm at 25 °C using a 0.5 mL quartz cuvette, with a path length of 1 cm. Each sample was scanned four times with an integration time of 5 s, and the values were averaged. Scanning was done at 1 nm steps, and the slit width was 1 nm. Liposome/pDNA complexes were prepared at 3/1 +/− charge ratio at 50 μg/mL pDNA concentration. Sample values were subtracted from those obtained for dextrose solution (for pDNA) or corresponding liposome solution in dextrose (for liposome/pDNA complexes) as blank.

Transmission Electron Microscopy. Liposome/pDNA complexes prepared at 3/1 (+/−) charge ratio were visualized by transmission electron microscopy (TEM) using 300-mesh carbon coated copper (Cu) grids, at 60 kV and magnifications ranging from 40 000× to 300 000×. Complexes were prepared at 3/1 charge ratio and 0.01 μg/μL pDNA concentration. Ten microliter complexes were loaded on the Cu grid for 2 min followed by blotting of the excess liquid and incubation for another 2 min with 1% uranyl acetate followed by blotting. The grid was air-dried for another 2 min and visualized under the electron microscope. Digital images were captured using the instrument software.

Statistical Analysis. Statistical significane of differences between samples was evaluated using one-way and two-way ANOVA using SPSS software (SPSS Inc., Chicago, IL) with Tukey's posthoc analysis. Significance level used for all studies was $p = 0.05$.

RESULTS

Syntheses and Purification. Tertiary amine (DADP, III), quaternary amine (HE-DADP, IV), and mesylate (EMS-DADP, V) derivatives of a two-chain fatty acid containing cationic lipid with only one positively charged headgroup were synthesized using dimethylaminoglycerol (I) as a template (Figure 1). Oleoyl chloride (II) was conjugated to the free hydroxyls of the glycerol backbone by esterification in chloroform followed by extraction in a series of aqueous solutions to give DADP. DADP was converted to HE-DADP by reaction with 2-bromoethanol in *N*,*N*-dimethylformamide, followed by mesylation in methylene chloride to yield EMS-DADP. Purity of the isolated compounds was assessed by both NMR and electrospray ionization-mass spectroscopy (ESI-MS). The mesylate compound was further converted to polyamine derivative (VI) using polyethyleneimine of low molecular weight (PEI 423) by simple heating.

NMR results of DADP are as follows (Figure 2a). ^{1}H NMR (300 MHz, chloroform-d_1): δ ~0.8−1.0 ppm (H of terminal (CH_3) of lipid chains (a)); δ ~1.2−1.7 (H of (CH_2) methylenes of lipid chains except the ones neighboring the double bond and the carbonyl (b)); δ ~2.0−2.1 (H of (CH_2) methylenes of lipid chains neighboring the double bond (c)); δ ~5.3−5.5 (H of (CH=) groups of lipid chains participating in double bond (d)); δ ~2.1−2.3 (H of (CH_3) methyl groups attached to the tertiary nitrogen (e)); δ ~4.3−4.4 (H of (CH_2) methylene of glycerol backbone (f)); δ ~2.2−2.4 (H of (CH_2) methylenes next to carbonyl on the lipid chains (g)); δ ~4.1 (H of (CH) from the glycerol backbone (h)); δ ~2.3−2.5 (H of (CH_2) methylene of the glycerol backbone connecting the amine (i)).

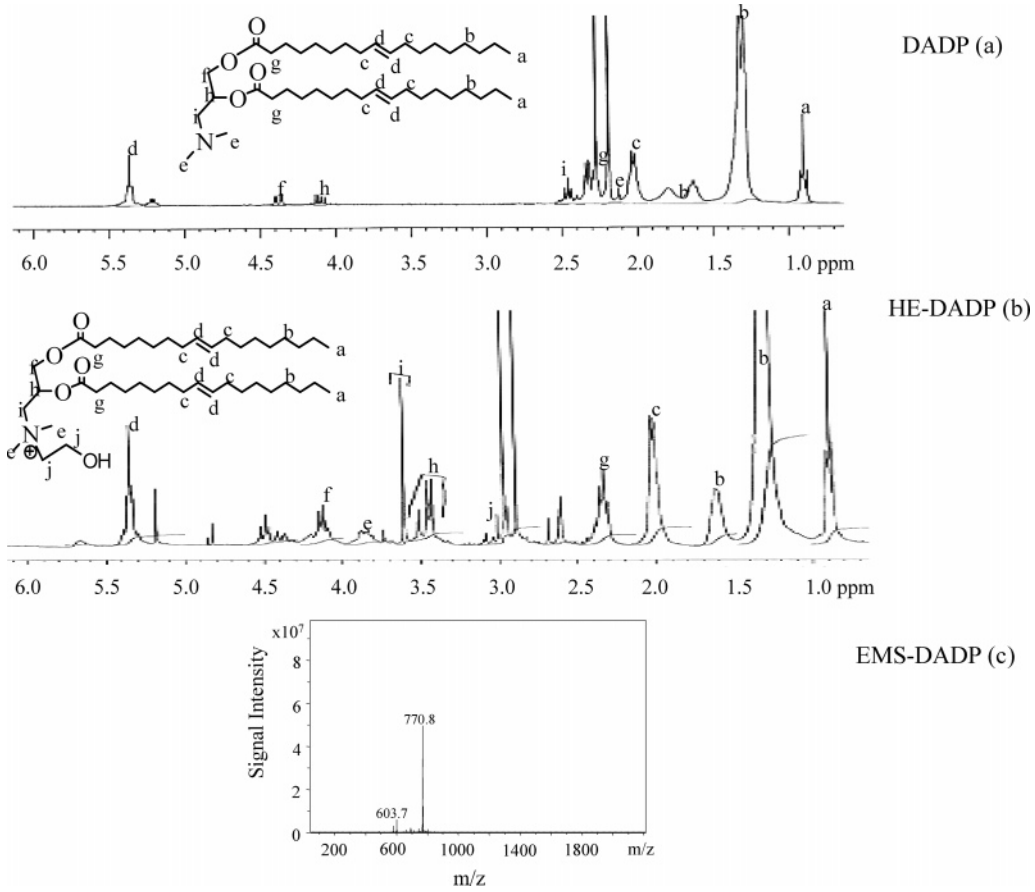

Figure 2. Proton nuclear magnetic resonance (NMR) spectra for 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP, III) and 3-(*N*-2-hydroxyethyl dimethylamino)-1,2-dioleoyl propanediol (HE-DADP, IV) and electrospray ionization (ESI) mass spectrum (MS) for 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP, V).

NMR results of HE-DADP are as follows (Figure 2b). ^{1}H NMR (300 MHz, chloroform-d_1): δ ~0.9 ppm (H of terminal (CH_3) of lipid chains (a)); δ ~1.2−1.7 (H of (CH_2) methylenes of lipid chains except the ones neighboring the double bond and the carbonyl (b)); δ ~2.1 (H of (CH_2) methylenes of lipid chains neighboring the double bond (c)); δ ~5.4 (H of (CH═) groups of lipid chains participating in double bond (d)); δ ~3.8−3.9 (H of (CH_3) methyl groups attached to the tertiary nitrogen (e)); δ ~4.1−4.2 (H of (CH_2) methylene of glycerol backbone (f)); δ ~2.2−2.4 (H of (CH_2) methylenes next to carbonyl on the lipid chains (g)); δ ~3.3−3.5 (H of (CH) from the glycerol backbone (h)); δ ~3.6 (H of (CH_2) methylene of the glycerol backbone connecting the amine (i)); δ ~2.9−3.1 (H of (CH_2) methylenes of hydroxy ethanol attached to the quaternary nitrogen (j).

EMS-DADP was also analyzed by NMR; however, the spectrum was too complicated to clearly distinguish product formation (data not shown). Hence, product formation and purity of EMS-DADP were assessed by the ESI-MS appearance of the molecular ion peak (770.8 corresponding to M. W. of 770, Figure 2c).

DNA Condensation. To determine the effect of cationic lipid headgroup structures, cationic lipid-to-colipid ratio, cationic lipid to pDNA charge ratio, and pH on DNA condensation, pDNA complexes were prepared by adjusting the stoichiometry of plasmid and cationic liposomes, using liposomes prepared from a cationic lipid (DADP, HE-DADP, or EMS-DADP) and a colipid (cholesterol or DOPE) at various molar ratios. Effective DNA condensation was achieved with cationic liposomes prepared using HE-DADP or EMS-DADP at charge ratios 3/1 and above, as no mobility of pDNA was observed at these charge ratios (data not shown). In contrast, DADP was relatively inefficient at DNA condensation, as some free DNA was seen even at 5/1 (+/−) charge ratio (data not shown). At a given charge ratio, cationic lipid to colipid molar ratios had little influence on DNA condensation (data not shown).

Formulation Factors Influencing Transfection Efficiency. The effect of various physicochemical parameters on in vitro transfection into CT-26 colon carcinoma cells was examined as a function of cationic lipid headgroup structure, colipid type, cationic lipid/colipid molar ratio, cationic lipid/DNA charge ratio, and liposome size.

The headgroup of most effective cationic lipids is either a polyamine or a quaternary amine, with the exception of Lipofectamine, which is a combination of both the motifs. Lipofectamine is composed of the cationic lipid DOSPA with colipid DOPE at 3/1 weight ratio. Since Lipofectamine gives one of the best transfection efficiencies and was used in our previous studies on gene delivery to human pancreatic islets (*16*, *17*), we compared the transfection efficiency of this commercially available reagent with our newly synthesized DADP, HE-DADP, and EMS-DADP lipid-based formulations. Transfection efficiency is reported as % relative light units (RLU) per mg of total protein content, expressed as a percentage of the transfection activity observed with Lipofectamine. Each bar in Figure 3 represents an average of six samples, with standard deviation being $\leq$10%.

The molar ratio of cationic lipid to colipid influences the surface chemistry of lipid/plasmid complexes and phase transition temperature of the liposomes. This may affect the plasmid uptake, opsonization, and endosomal

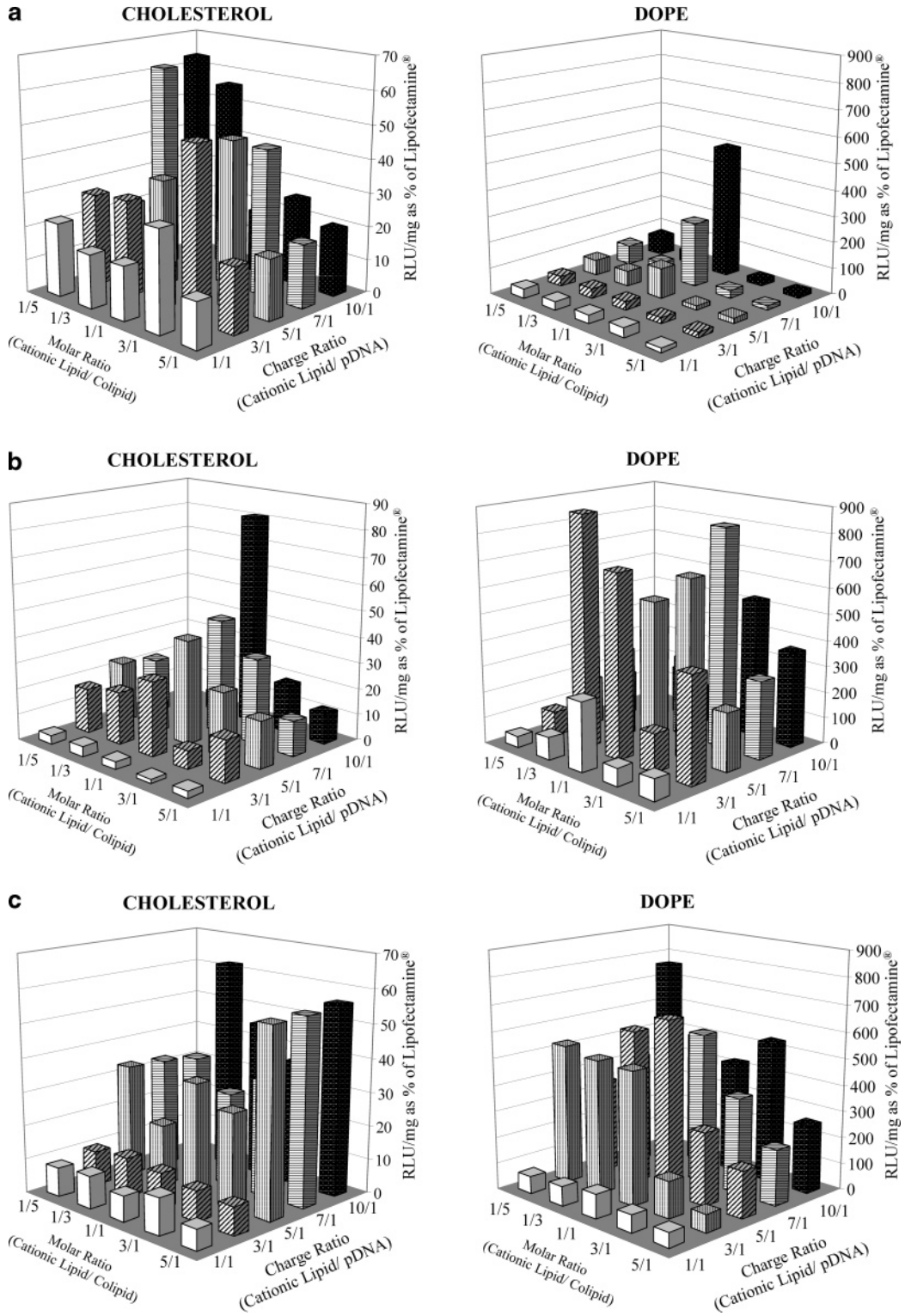

Figure 3. Transfection efficiency of liposomes prepared with 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP) (a), 3-(*N*-2-hydroxyethyldimethylamino)-1,2-dioleoylpropanediol (HE-DADP) (b), or 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP) (c) lipid derivatives prepared with cholesterol or DOPE as a colipid, as % relative light units (RLU) measured by luciferase assay, per mg of protein for $n = 6$. Transfection efficiency of Lipofectamine was considered 100% for comparison purpose.

release thus affecting the efficiency of gene expression. Most of the cationic liposome formulations incorporate colipids, but the colipid amount does not normally exceed 50% because of the need for high cationic lipid content to complex with and neutralize the large number of negative charges of DNA.

As shown in Figure 3, transfection efficiency was strongly influenced by the relative proportions of cationic lipid, colipid, and plasmid DNA. In general, at a fixed DNA concentration, the cationic lipid dose−response curves were bell-shaped due to the toxicity of cationic lipid at higher dose or charge ratios. The transfection efficiency of these cationic lipids was in the following order: DADP < HE-DADP < EMS-DADP. The use of cholesterol as a colipid showed poor transfection, while DOPE as a colipid gave higher transfection.

Transfection efficiency of DADP was higher for the 3/1 molar ratio of cationic lipid/colipid while decreasing on

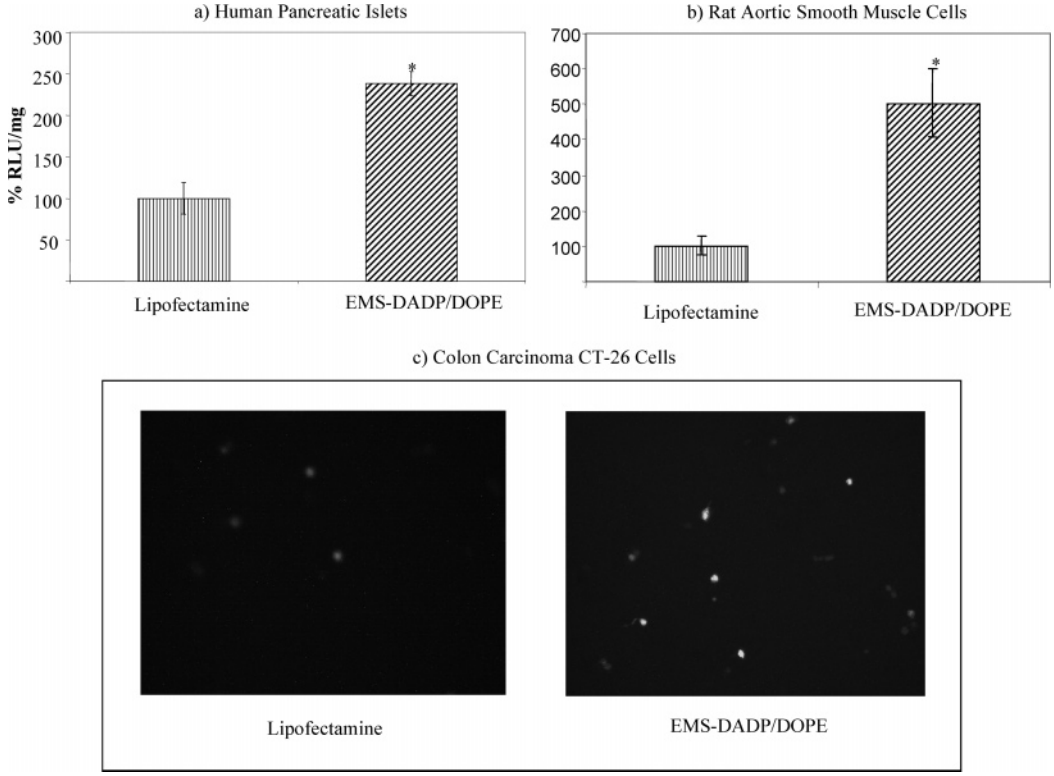

Figure 4. Transfection efficiency of pcDNA3-Luc/EMS-DADP (3/1, +/−) complexes in human pancreatic islets (a) and in rat aortic smooth muscle cells (b), by luciferase assay, and of pCMS-EGFP//EMS-DADP (3/1, +/−) complexes in colon carcinoma CT-26 cells (c), by fluorescence microscopy. (*) indicates statistically significantly ($p < 0.05$) differences in transfection efficiency by two-way ANOVA analysis.

either side, except for the 10/1 (+/−) cationic lipid/pDNA charge ratio for transfection. With respect to the influence of charge ratio, in liposomes with higher cholesterol content (1/5 and 1/3 molar ratios) transfection efficiency was higher with increasing charge ratios while liposomes with equal or less colipid content (1/1, 3/1, or 5/1 molar ratios) showed higher transfection efficiencies at intermediate charge ratios of 3/1 and 5/1.

HE-DADP showed a distinct trend of higher transfection activity at 1/1 molar ratio and decreasing trend with an increase in either the cationic or the colipid component of liposomes. However, the transfection efficiency of DADP, HE-DADP, and EMS-DADP was less when cholesterol was used as a colipid compared to Lipofectamine, with none of them exceeding 80% of the efficiency observed with Lipofectamine (Figure 3a). In contrast, transfection efficiency was signficantly higher for HE-DADP and EMS-DADP when DOPE was used as a colipid for liposome preparation. There was, furthermore, a clear trend of the transfection being dependent on the molar ratio of cationic lipid/colipid with 1/1 ratio giving higher activity than increasing the content of either component. HE-DADP and EMS-DADP/DOPE liposome/pDNA complexes, however, presented much higher transfection efficiency than DADP (∼2−5 times), as well as the commercially available reagent Lipofectamine (∼5 times) (Figure 3b). In the case of EMS-DADP/DOPE liposomes, the transfection activity was higher at higher molar ratios of DOPE.

We have previously reported that transfection efficiency of Lipofectamine/pCAGGS-hVEGF complexes into human islets is low and dependent on the incubation period of islets with the complexes (*16*, *17*). There are also reports of low transfection of Lipofectin/pDNA, Lipofectamine/pDNA, DOSPA:DOPE liposome/pDNA, and polyethylenimine (PEI)/pDNA complexes into intact mouse, rat, and porcine islets (*26*). Unlike the 3:1 w/w ratio Lipofectamine/pDNA used in the present study, Benhamou et al. (*24*) used relatively large amount of cationic lipid (DOTAP, complexes were formulated at 12/1 DOTAP/pDNA weight ratio). These authors also suggested that islet transfection is predominantly in the peripheral cells due to the relative inaccessibility of the central core cells to the vector. Low transfection efficiency in islets was also reported by Saldeen et al. (*25*) To compensate for low transfection efficiency, some workers transfect the dispersed islet cells, as opposed to intact islets, which gives higher transfection due to greater number of cells becoming exposed to the transfection agent after dispersion (*24*, *26*). This, however, often compromises the glucose response function of β-cells and will not be suitable for transplantation (*25*). In the present study, transfection efficiency was observed to be ∼2.5 times higher than Lipofectamine in the human pancreatic islets, and ∼5 times in RASM cells (Figure 4 a,b). In an attempt to further enhance transfection efficiency in human islets, we are in process of testing our lead formulations in conjunction with fusogenic peptides.

We also analyzed GFP positive CT-26 cells by flow cytometry after transfection with Lipofectamine and the lead formulation EMS-DADP/DOPE (1/1) complexed with pDNA at 3/1 (+/−) charge ratio. The results indicated that the transfection efficiency for EMS-DADP/DOPE liposomes was ∼1.5 times higher than Lipofectamine. Concurrent fluorescence microscopic observations of the same samples indicated higher levels of and difference in GFP expression. This could be because flow cytometric analysis would only capture cells that are relatively brightly fluorescent.

We and others (*3*, *11*, *27*) have shown that the transfection efficiency of larger size liposomes is higher than small size liposomes. To test the validity of this observa-

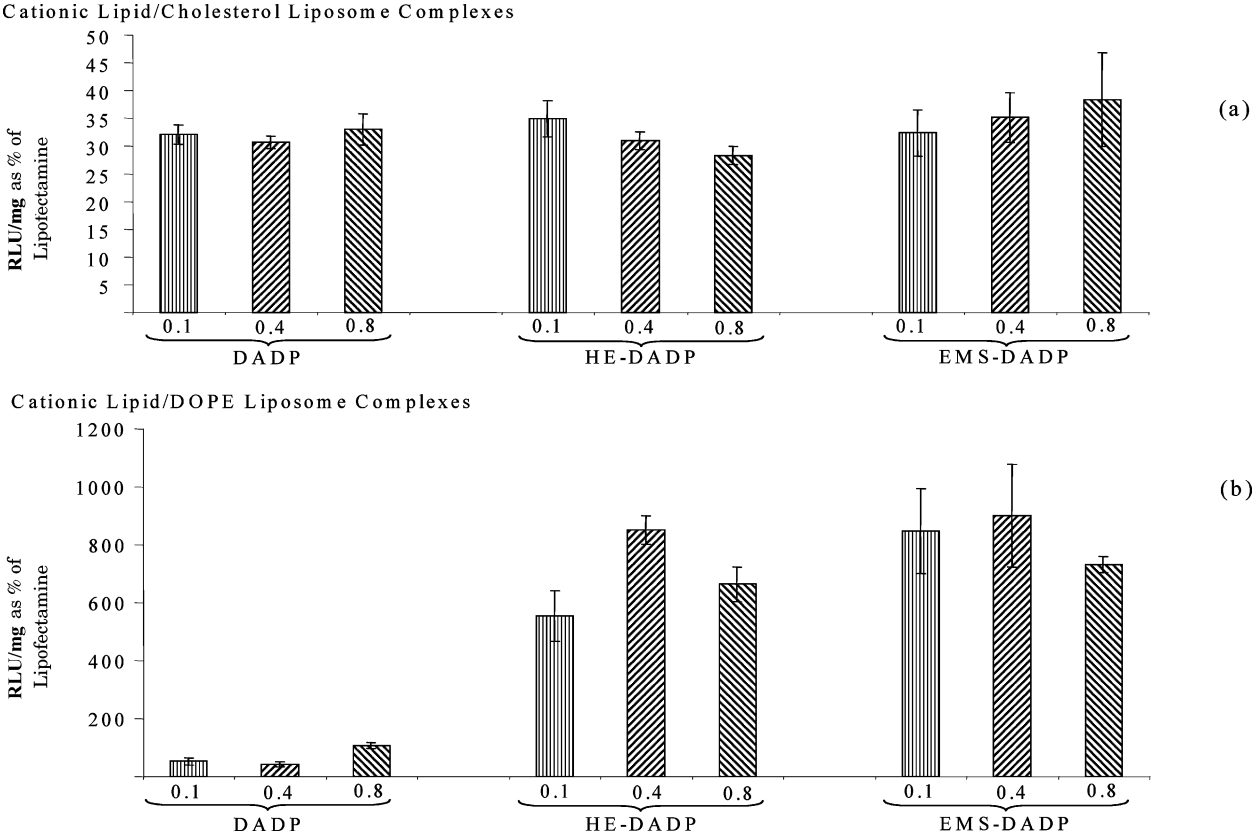

Figure 5. Effect of liposome size on transfection efficiency of pDNA/liposome complexes using DADP, HE-DADP, and EMS-DADP liposomes, extruded through 0.1, 0.4, and 0.8 μm polycarbonate membranes, using (a) cholesterol or (b) DOPE as a colipid. Transfection activity is expressed as % relative light units (RLU) measured by luciferase assay per mg of protein for $n = 6$. Transfection efficiency of Lipofectamine was considered 100% for comparison. There was no significant difference ($p = 0.05$) in transfection efficiency between different liposome size samples of the same lipid-based formulations.

tion with our novel lipids, we examined the effect of liposome size on transfection efficiency by extrusion of liposomes through 100, 400, and 800 nm pore size polycarbonate membranes and complex formation with pcDNA3-Luc at 3/1 (+/−) charge ratio (Figure 5). In the present study, liposome size did not show any significant effect on transfection efficiency for DOPE and cholesterol-based liposome/pDNA complex formulations, since 100, 400, and 800 nm extruded liposomes showed similar %RLU/mg total protein (Figure 5).

Particle Size and Zeta Potential. Particle diameter of liposome/pDNA complexes (Figure 6a) was ~500 nm for DADP and HE-DADP with both colipids, while EMS-DADP had a larger particle size (~1.0 μm with cholesterol as a colipid and ~1.8 μm with DOPE as a colipid). The particle size of complexes of all liposomes was larger than that of Lipofectamine and was closer to the membrane size used for extrusion of liposomes i.e., 400 nm (Figure 6a).

Zeta potential of liposome/pDNA complexes was ~ +40mV for both HE-DADP and EMS-DADP liposomes with either cholesterol or DOPE as colipids (Figure 6b). These were comparable to the zeta potential observed for Lipofectamine, ~ +45mV. The ξ-potential for complexes with DADP liposomes was, however, on the negative side (~−15mV). This was indicative of insufficient positive charge on the tertiary nitrogen to cause effective DNA condensation.

Circular Dichroism. Circular dichroism spectra were recorded for naked plasmid and lipid/plasmid complexes in 5% dextrose solution. Since dextrose is a chiral molecule, CD spectra of dextrose alone was subtracted from that of pDNA in dextrose solution to obtain the profile for pDNA. Similarly, the spectra of complexes were recorded with background normalization to the profiles of corresponding liposomes alone in dextrose solution. The spectra were recorded from 220 nm to 360 nm to observe changes in the conformation of pDNA arising from its interactions with the cationic lipids. The charge ratio of cationic lipids to pDNA as well as the molar ratio of cationic lipids to colipids was kept constant in order to delineate the differences arising from different structures of cationic lipids. The CD spectra of naked DNA and lipid/plasmid complexes are shown in Figure 7. The spectrum of naked DNA presented B-type DNA curve. The CD spectra of liposome/pDNA complexes indicated a significant change in DNA conformation being brought about by the interaction with cationic lipids. CD spectra of Lipofectamine/plasmid complexes were also recorded as a positive control. These data indicated that the difference in transfection efficiency obtained with these different liposome formulations could not be directly correlated to the change in DNA conforamation/winding of the duplex brought about by the cationic lipids.

Transmission Electron Microscopy. Morphology of lipid/plasmid complexes was visualized at magnifications of 40000 to 300000 under the transmission electron microscope after negative staining of DNA using uranyl acetate. The complexes formed with cationic lipids were compared with those formed with Lipofectamine (Figure 8a). The complexes with EMS-DADP had the tendency to be elongated and have more surface area than those formed with Lipofectamine, which were relatively compact. Furthermore, some incompletely condensed pDNA molecules could be visualized in these samples (Figure 8b), which indicated that a significantly higher amount

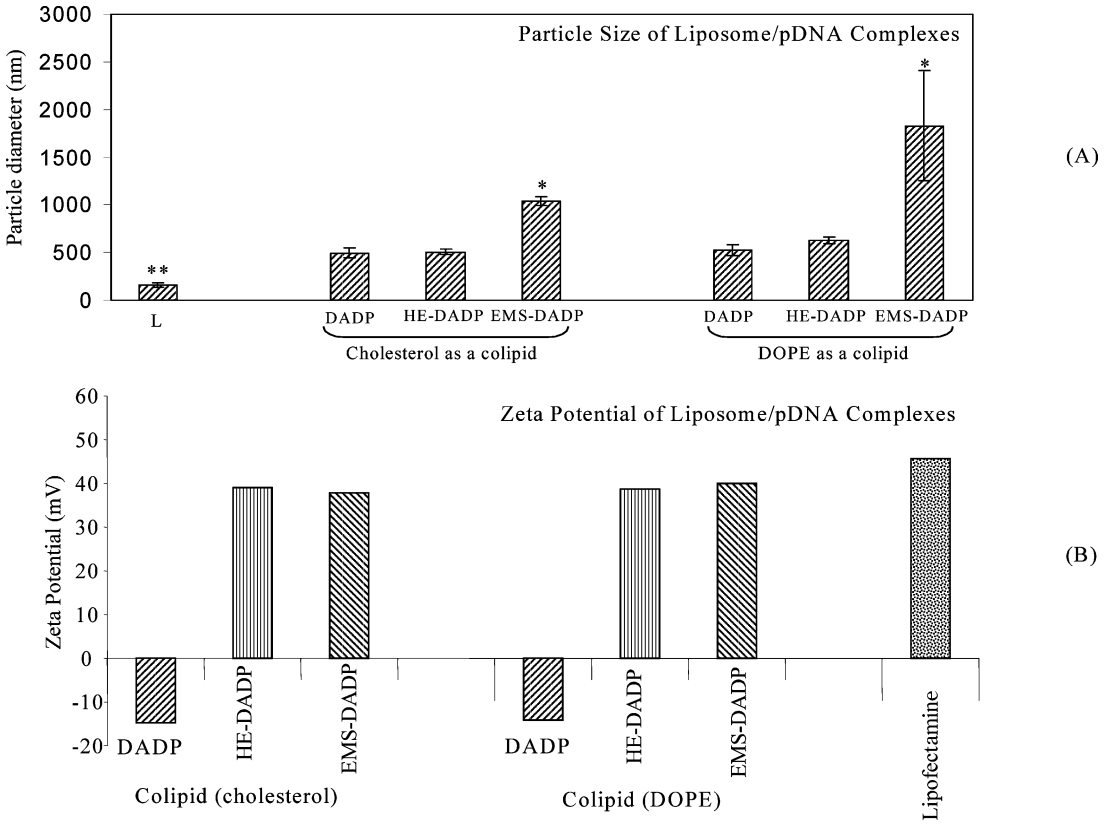

Figure 6. Particle size (a) and zeta potential (b) of liposome/pDNA complexes prepared at 3/1 (+/−) charge ratio using either 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP), 3-(*N*-2-hydroxyethyldimethylamino)-1,2-dioleoylpropanediol (HE-DADP), or 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP) as a cationic lipid and cholesterol or DOPE as a colipid at 1/1 molar ratio. Lipofectamine/pDNA complexes (3/1 molar ratio) were used as a control. (*) indicates statistically significant difference of these EMS-DADP samples from DADP, HE-DADP, and Lipofectamine. Lipofectamine (**) sample was significantly different from all other samples. Two-way ANOVA was used to assess statistical signficance of differences at $p < 0.05$ level. Significance tests could not be applied to the zeta potential data since it was based on only one sample readings.

of the lipids were attached to the DNA molecules in the case of EMS-DADP than Lipofectamine.

DISCUSSION

Cationic lipid structure, colipid type, lipid/DNA ratio, cationic lipid/colipid ratio, and liposome size have been shown to influence gene transfer efficiency both in vitro and in vivo (*2*, *3*, *11*). Here we report the effect of these parameters on the transfection efficiency of our newly synthesized cationic lipids in vitro. These lipid derivatives have two C_{18} oleoyl lipid chains attached to the glycerol backbone through an ester linkage. To the C-3 carbon of the glycerol backbone is attached either a tertiary or a quaternary amine as a hydrophilic headgroup. A review of research (*2*−*4*, *28*−*35*) and review (*36*−*42*) papers, and patents (US 5550289, 4897355, 5736392, 5527928, 5744625, 5892071, 5869715, 5171678, 5824812, 6251433, 6376248, 6051429, 6087325, 6143716, 6339069, 5736392, 5334761, and 6346516), revealed various design features and structure−activity relationships of cationic lipids. On the basis of this information, it was concluded that a two chain symmetrical C_{18} mono-unsaturated oleoyl ester chain based lipid conjugate would be the most suitable with a glycerol backbone connecting it to the cationic headgroup.

Significant differences were observed in the transfection efficiency of the three lipid derivatives prepared viz. DADP, HE-DADP, and EMS-DADP (Figure 3). The charge ratio of cationic lipid to pDNA, type of colipid, and molar ratio of cationic lipid to colipid affected the transfection efficiency of liposome/plasmid complexes. One possible reason for different transfection efficiency is the difference in their DNA condensing ability. Unlike most quaternary lipids currently being used for transfection, HE-DADP has an additional hydroxyl functionality separated from the quaternary amine by a two-carbon chain alkyl moiety that confers to it a distance of $\sim$ 5 Å. The hydroxyl group can participate in hydrogen bond formation as both a hydrogen bond donor and/or acceptor. The distance of the hydroxyl group from the quaternary amine correlates with the molecular distances within the DNA bases, indicating a possibility of concurrent hydrogen bond interactions along with electrostatic interactions, thus leading to better DNA condensation.

On the basis of the high transfection obtained with lipid/plasmid complexes prepared at 3/1 (+/−) charge ratio and 1/1 molar ratio of cationic lipid/colipid, we selected formulations of DADP, HE-DADP, and EMS-DADP with both cholesterol and DOPE as colipids for further studies. Plasmid DNA solution in water was used as a control. We hypothesize that the higher transfection efficiency of HE-DADP and EMS-DADP is due to stronger interactions between cationic lipid and plasmid DNA that originate in the presence of the hydroxyl functionality in HE-DADP and the methylsulfonate group in the case of EMS-DADP. The hydroxyl group has the potential for hydrogen bonding interactions both as a hydrogen bond donor as well as an acceptor. The position of the hydroxyl group on HE-DADP is such that it is only two carbons away from the positively charged nitrogen. Given the interaction of the positive nitrogen with the negative phosphate on the DNA backbone, which would bring the

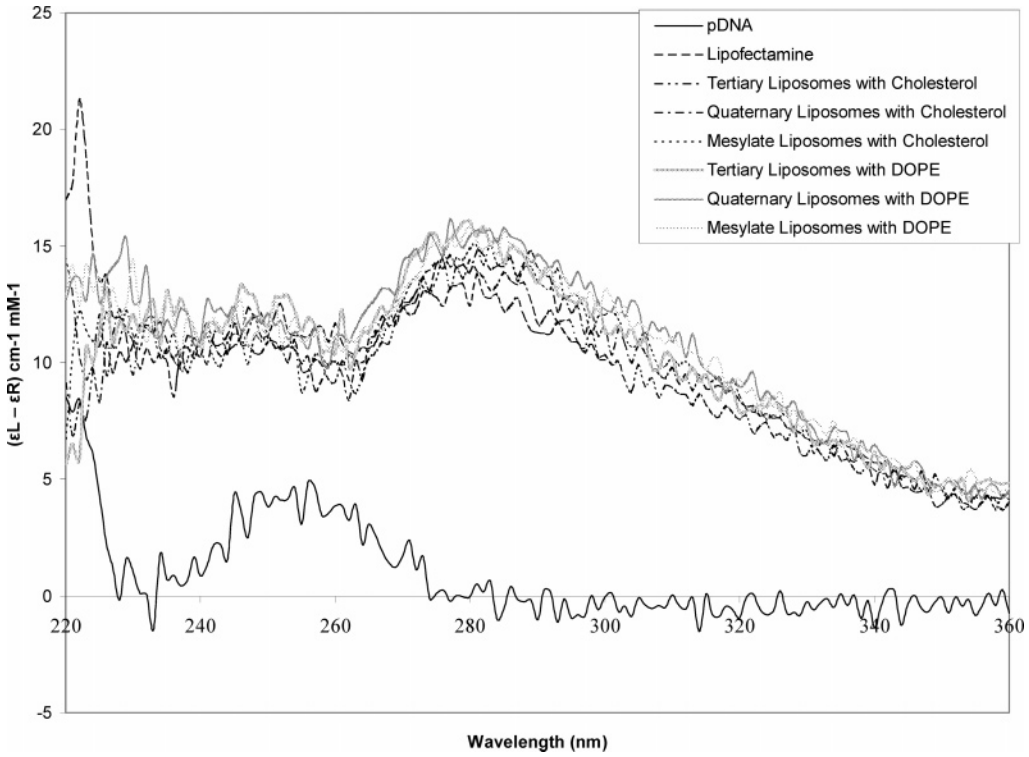

Figure 7. Circular dichroism (CD) spectra of lipid/plasmid complexes. Liposomes were prepared at 1/1 molar ratio of either 3-(dimethylamino)-1,2-dioleoylpropanediol (DADP), 3-(*N*-2-hydroxyethyldimethylamino)-1,2-dioleoylpropanediol (HE-DADP), or 3-(*N*-ethyl-2-methanesulfonyldimethylamino)-1,2-dioleoylpropanediol (EMS-DADP) as a cationic lipid and cholesterol or DOPE as a colipid. Complexes were prepared at 3/1 charge ratio of cationic lipid/colipid, and CD was recorded at a pDNA concentration of 50 μg/mL. The CD spectra has been compared to the profile for pDNA alone. Spectral profiles of liposomes with dextrose or of dextrose alone were subtracted as blank.

headgroup of the cationic lipid in close proximity to the DNA, the hydroxyl group possibly engages in hydrogen bonding interactions, either directly or through water bridge(s), with the neighboring polar or charged groups on the surface of DNA duplex. Multiple hydrogen bonding interaction of the cationic lipids with surface charged groups on the DNA double helix would be sufficient to significantly increase the overall strength of interaction between the lipid and the plasmid DNA.

In the case of EMS-DADP, the presence of the uncharged, bulky methanesulfonyl group above the quaternary amine headgroup is expected to hinder the electrostatic interactions between the positively charged nitrogen with the negatively charged phosphates on the DNA backbone. However, the transfection efficiency of EMS-DADP was not reduced compared to the quaternary compound, HE-DADP. Mesylates have been shown to alkylate DNA (*43*−*47*) and can react with amine functionalities in aqueous conditions at room temperature (*48*). Furthermore, the steric positioning of the mesylate group is expected to be close to the neighboring phosphates on the surface of double stranded DNA molecule, when the quaternary nitrogen forms electrostatic bonds with the anionic phosphates. The mesylate group, therefore, possibly forms a covalent bond or engages in hydrogen bonding interactions through the water bridges.

We have earlier evaluated in vivo transfection efficiency of cationic liposomes in mice after tail vein injection of lipid/plasmid complexes. Human growth hormone (hGH) gene expression level in the serum was about 6-fold higher when 400 nm extruded liposomes were used compared to 100 nm extruded liposomes (*11*). Ross et al. (1999) also observed a linear increase in lipoplex association, cellular uptake, and gene expression with increase in liposome size, composed of DOTAP as the cationic lipid and DOPE as the colipid (*49*). Also, Zelphati et al. (1998) have shown the transfection activity to be almost double for an increase in particle size from 100 nm to 400 nm (*50*). Felgner et al. (1994) have evaluated different particle size formulations of the cationic lipid DORIE, which is very similar to our lipid HE-DADP, and observed a 2-fold difference in the transfection efficiency of larger multilamellar vesicles and smaller unilamellar vesicles of these liposomes (*3*). In contrast, we did not observe a signficant (p 0.05, ANOVA) difference in transfection efficiency with increase in liposome size for all cationic lipids (Figure 5).

Zeta potential of the complexes has an important role in transfection efficiency by promoting adhesion of the complexes to the negatively charged cell membrane. The ξ-potential of lipid/plasmid complexes prepared using HE-DADP and EMS-DADP was ∼(+)40 mV, which was comparable to that for Lipofectamine/plasmid complexes (∼ +45 mV) indicating that higher ξ-potential was not the reason for higher transfection. The ξ-potential for complexes prepared with the liposomes of DADP was ∼ −15 mV. The low transfection efficiency of DADP could be correlated to negative zeta potential.

We have earlier shown that interaction with cationic lipopolymers significantly affects the conformation of pDNA, as inferred by CD, and that this is a function of +/− charge ratio used for the preparation of complexes (*14*). Therefore, in the present study, we have scanned pDNA and liposome/pDNA complexes over a range of 220−360 nm at a fixed charge ratio of 3/1 (+/−), to assess the comparative degree of change in conformational state of plasmid DNA with liposomes of different cationic lipids. The CD profile obtained for a given pDNA molecule depends on its sequence of base pairs, %AT content of bases, and the interactions among bases (*51*, *52*).

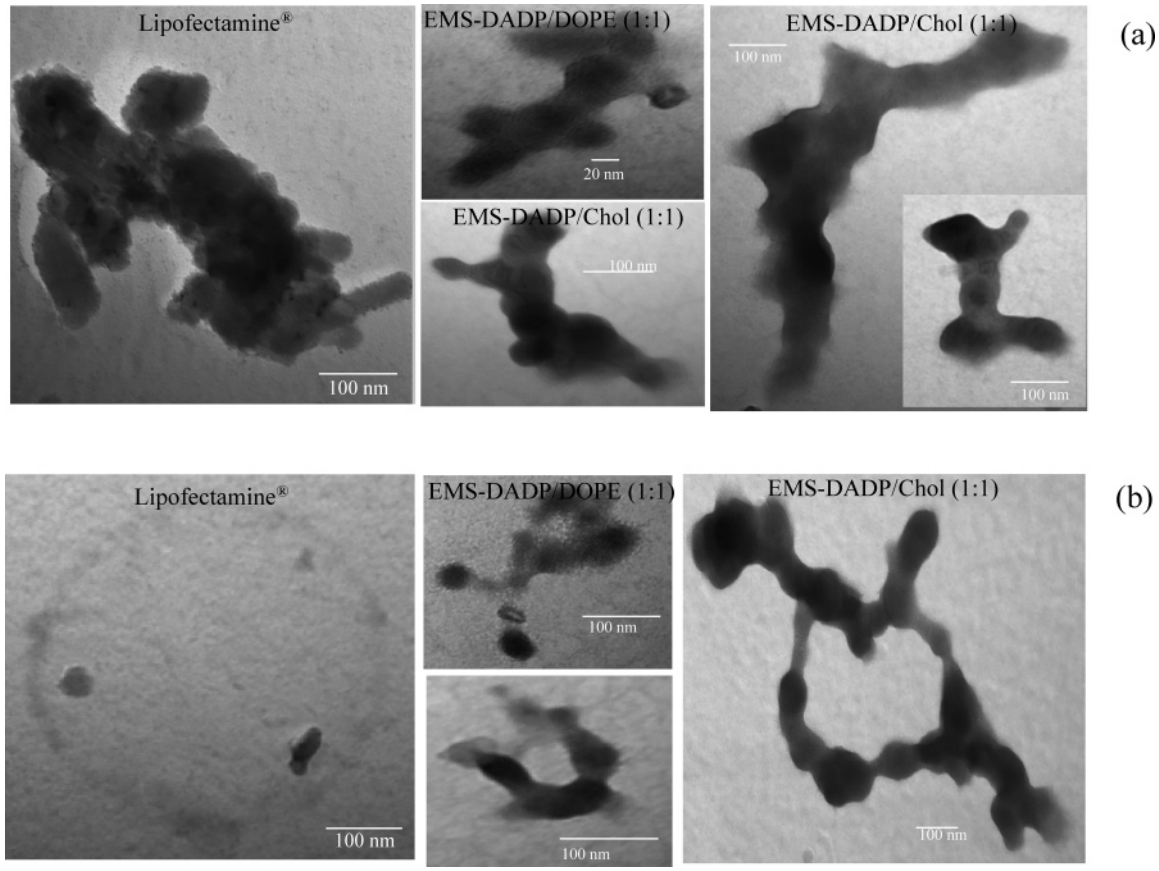

Figure 8. Transmission electron microscopy (TEM) images of cationic liposome/pDNA complexes and occasional uncondensed plasmid DNA when complexes were prepared at 3/1 (+/−) charge ratio using cationic liposomes prepared with 1/1 molar ratio of the cationic lipid with the colipid. Samples were prepared at the pDNA concentration of 0.01 μg/μL and negatively stained on carbon-coated copper grids using uranyl acetate aqueous solution. Complexes of the mesylate lipid with either DOPE or cholesterol as a colipid in liposome formulations have been compared with Lipofectamine.

Hence, the CD spectra of pDNA obtained in our current study was somewhat different than that obtained earlier (*14*) and seems closer to the poly [d(A-C-C). d(G-G-T)] two repeating trinucleotide polymer at 67% GC content shown by Gray et al. (*51*). The goal of our study here was to examine the relative difference in changes in conformation induced by different cationic lipids. The results (Figure 7) indicated a significant change in the conformation of pDNA upon complexation with the cationic lipids in the appearance of a significant positive peak ~ 280 nm, where there was a positive peak at ~ 255 nm in the naked pDNA and the disappearance of the slight negative peak at ~235 nm. However, the relative difference in the pDNA profile with different cationic lipids, including Lipofectamine, was insignificant. This indicated that the degree of conformational change/winding-up of the helix caused by the cationic lipids was similar in all cases examined.

The particle size data (Figure 6) as well as the results of transfection studies (Figure 3) implied the possibility that the higher in vitro transfection could be due to the aggregation/agglomeration of complexes. Therefore, we sought to visualize the complexes using transmission electron microscopy (TEM) to confirm the observations of particle size data and any possible aggregation of the complexes. The size, agglomeration, and structural geometry of the complexes has been shown to depend greatly upon the +/− charge ratio of the cationic polymer/liposomes to pDNA. Lower charge ratios of 1/1.2 to 1/1.3 were shown to form large-sized complexes and aggregates (*53*). Pitaard et al. (1997) had shown the progressive decrease in diameter and increase in compactness of complexes formed at (+/−) charge ratios of 0.3/1, through 1.65/1 to 6/1 (*54*). Rackstraw et al. (2002) had shown the increase in density and size of complexes formed at 1/1 to 5/1 charge ratio (*55*). The complexes were seen to be compact and spheroidal in all cases. Aggregate formation was also visible in some cases, especially at lower charge ratios. An interesting observation was reported by Thomas et al. (2003), with the deposition of a lot of lipid on the circular pDNA at an extremely high charge ratio of 150/1 (*56*). We have seen similar phenomena in the case of pDNA/EMS-DADP:DOPE liposome complexes but at a lower charge ratio of 3/1 (+/−).

The TEM of the pDNA complexes of Lipofectamine liposomes was also visualized as a control. Both complexes (Figure 8a) as well as occasional uncondensed pDNA (Figure 8b) could be seen. The observation of uncondensed DNA could be due to the relatively lower amount of liposomes used for pDNA condensation. The results indicated the size of most of the complexes to be in the range of the particle size data obtained using light scattering technique (Figure 6a). Furthermore, two significant differences were observed between Lipofectamine/plasmid and EMS-DADP:DOPE liposome/plasmid complexes. While the former appeared more compact in nature, the latter were more 'spread-out' and elongated, with higher surface area (Figure 8). The observation of elongated complexes indicated that the higher particle size obtained with the light scattering technique for EMS-DADP complexes could be due to the varying orientation of the elongated structures. This would also explain the significantly higher variability seen with the EMS-DADP/DOPE complexes in Figure 6a. The amount of lipid

attached to the plasmid DNA was seen to be significantly higher for EMS-DADP than for Lipofectamine complexes. This observation is in line with our hypothesis that the conformational placement of the hydroxyl or the mesylate group allows them to form additional bonding interactions with the DNA double helix, leading to a greater degree of attraction between the lipid molecules and the plasmid DNA. This results in more amount of lipid 'sticking' to the pDNA. This also explains the relatively elongated or spread-out nature of the complexes formed with EMS-DADP as being due to the packing of an excessive amount of material on the DNA duplex with a limited surface area.

The observation of uncondensed DNA could be due to the relatively lower amount of liposomes used for pDNA condensation. Although electrophoretic mobility of plasmid DNA was seen to be prevented at charge ratios of 3/1 and higher, effective charge neutralization might not result in formation of compact lipid/DNA complexes for all 100% molecules at 3/1 (+/−) charge ratio. Hence, some incompletely compacted pDNA molecules, which did have the lipid attached, were observed under the electron microscope at this charge ratio, although they were very few.

Bennett et al. (1997) have evaluated in vivo transfection efficiency and lipid hydration of a series of cationic lipids with variations in headgroup structures (*57*). Among other lipids, their lipid structure RI, which is the same as HE-DADP, was compared with its methoxy derivative (MEP) to evalute the difference in hydration and the role of hydrogen bonding interactions. They observed an increase in lipid hydration with increasing bulk of the polar headgroup with methoxy modification, which was attributed to reduced interactions with the colipid, DOPE. Thus, greater exclusion of interstitial water in the case of hydroxyl containing lipids due to intermolecular hydrogen bonding, would lead to reduced lipid hydration with increased tendency of liposomes to form nonbilayer structures. Such lipids were observed to be more active than the non-hydroxyl-containing lipids. The same lipid, HE-DADP, was also studied by Felgner et al. (1994) as DORI in their evaluation of a series of cationic lipid structures differing in headgroup as well as lipid domain structures (*3*). Addition of a hydroxyalkyl moiety on the quaternary amine in DORI was seen to give ~2-fold higher transfection than the non-hydroxyl group-containing lipid, DOTMA. Presence of polar hydroxyl functionality was postulated to affect hydrogen bonding associations with DOPE to form a heterodimer with different net charge dependent on the medium pH. The authors proposed that ion pair heterodimer formation with DOPE would help maintain the integrity of bilayer structure with changing pH, resulting in different aggregation and fusion properties of vesicles that impact intracellular trafficking. Hence, the hydrogen bonding potential of the hydroxyl functionality in the headgroup has been postulated to improve transfection efficiency either by destabilization of liposomes due to reduced hydration or maintaining the integrity of bilayer structure with changing pH.

Irrespective of the hypothesis regarding vesicle properties that influence transfection activity, cationic lipid interactions with the colipid DOPE were deemed important to the higher transfection efficiency of hydroxyl-containing cationic lipids by both Bennet et al. (*57*) and Felgner et al. (*3*). Felgner et al. also showed that an alternate colipid, dioleoyl phosphatidylcholine (DOPC), which had a permanently positively charged quaternary amine in the headgroup, which would display pH independent charge of the cationic lipid/colipid heterodimer. Lower transfection efficiency was seen with DOPC as a colipid in liposome formulations as compared to DOPE. Hence, the role of colipid remains very important to transfection efficiency because of its potential interactions with the cationic lipid. We expect that this should be true also for cholesterol versus DOPE as a colipid in our formulations.

Indeed, the transfection efficiency of EMS-DADP with DOPE-based liposomes is much higher than that with cholesterol based liposomes (Figure 3). The observation of higher amount of lipid attachment to plasmid DNA with mesylate lipid-based liposomes, however, is consistent with both types of colipids (Figure 8). This indicates that more lipid attachment is not the only mechanism involved. Literature also suggests that membrane fusion, promoted by lipid attachment to DNA, may not be the predominant mechanism for cell penetration (*58*). The predominant mechanism for membrane penetration is endocytosis rather than fusion. Membrane fusion and disruption, however, is a critical requirement for the next stage of endosomal escape. Higher affinity of the mesylate headgroup for DNA would also promote higher colipid attachment to pDNA because of the proximity of both lipids in the liposome formulation. Also, the attachment of DOPE as a colipid may be more than cholesterol because of zwitterionic nature of DOPE and its possible interactions with EMS-DADP as well as plasmid DNA. The fusogenic colipid DOPE is known to have significantly higher impact on endosomal disruption than cholesterol. Hence, we hypothesize that while higher lipid attachment may be protective for plasmid DNA and slightly enhancing cell penetration in the case of either colipid, higher transfection efficiencies are seen only with DOPE as a colipid because of its role in membrane fusion and disruption at the stage of endosomal escape. This is indicated by the significantly higher transfection efficiency of EMS-DADP liposomes having a higher molar content of DOPE in the liposomes (1/5, 1/3, and 1/1 EMS-DADP/DOPE molar ratios consistently give higher transfection than 3/1 and 5/1 molar ratios irrespective of the charge ratio used, Figure 3b). No such significant effect of colipid content was seen with cholesterol-based liposomes (Figure 3a). Hence, these cationic lipid formulations may be acting predominantly at a stage of endosomal disruption and prevention of lysosomal degradation instead of cellular internalization. These hypotheses will be the subject of our further investigations in this area.

The incomplete condensation observed at 3/1 (+/−) charge ratio may adversely impact in vivo gene expression of lipid/plasmid complexes. However, there is a need to maintain a delicate balance between transfection efficiency and toxicity. High charge ratios are known to efficiently condense DNA and increase transfection efficiency, but also increase cytotoxicity. Moreover, high charge ratios of cationic lipid to pDNA in complexes is known to cause complement activation after systemic administration (*59*). To minimize cytotoxicity, we will use 3/1 (+/−) charge ratio complexes for further evaluation of our novel lipids.

While our compounds with both polyamine and the quaternary amine were water-soluble and had lower transfection efficiencies than the commercially available Lipofectamine (data not shown), the HE-DADP and EMS-DADP showed unexpectedly higher transfection efficiencies. The cytotoxicity observed with these compounds, however, was not significantly higher than Lipofectamine, which was used as a positive control. We conclude that the possible reason for the higher transfection efficiency

observed with EMS-DADP lipsomes is due to increased lipid affinity for and attachment to the plasmid DNA.

ACKNOWLEDGMENT

This work was supported by the University of Tennessee New Faculty Start-up Fund and the UT Vascular Biology Center of Excellence.

LITERATURE CITED

(1) Mahato, R. I., Rolland, A., and Tomlinson, E. (1997) Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm. Res. 14*, 853−859.

(2) Lee, E. R., Marshall, J., Siegel, C. S., Jiang, C., Yew, N. S., Nichols, M. R., Nietupski, J. B., Ziegler, R. J., Lane, M. B., Wang, K. X., Wan, N. C., Scheule, R. K., Harris, D. J., Smith, A. E., and Cheng, S. H. (1996) Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther. 7*, 1701−1717.

(3) Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem. 269*, 2550−2561.

(4) Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A. 84*, 7413−7417.

(5) Gao, X., and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun. 179*, 280−285.

(6) Behr, J. P., Demeneix, B., Loeffler, J. P., and Perez-Mutul, J. (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. U.S.A. 86*, 6982−6986.

(7) Edelstein, M. L., Abedi, M. R., Wixon, J., and Edelstein, R. M. (2004) Gene therapy clinical trials worldwide 1989−2004-an overview. *J. Gene. Med. 6*, 597−602.

(8) Hui, K. M., Ang, P. T., Huang, L., and Tay, S. K. (1997) Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA-liposome complexes. *Gene Ther. 4*, 783−790.

(9) Galanis, E., Hersh, E. M., Stopeck, A. T., Gonzalez, R., Burch, P., Spier, C., Akporiaye, E. T., Rinehart, J. J., Edmonson, J., Sobol, R. E., Forscher, C., Sondak, V. K., Lewis, B. D., Unger, E. C., O'driscoll, M., Selk, L., and Rubin, J. (1999) Immunotherapy of advanced malignancy by direct gene transfer of an interleukin-2 DNA/DMRIE/DOPE lipid complex: phase I/II experience. *J. Clin. Oncol. 17*, 3313−3323.

(10) Ruiz, F. E., Clancy, J. P., Perricone, M. A., Bebok, Z., Hong, J. S., Cheng, S. H., Meeker, D. P., Young, K. R., Schoumacher, R. A., Weatherly, M. R., Wing, L., Morris, J. E., Sindel, L., Rosenberg, M., Van Ginkel, F. W., Mcghee, J. R., Kelly, D., Lyrene, R. K., and Sorscher, E. J. (2001) A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. *Hum. Gene Ther. 12*, 751−761.

(11) Mahato, R. I., Anwer, K., Tagliaferri, F., Meaney, C., Leonard, P., Wadhwa, M. S., Logan, M., French, M., and Rolland, A. (1998) Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. *Hum. Gene Ther. 9*, 2083−2099.

(12) Nomura, T., Nakajima, S., Kawabata, K., Yamashita, F., Takakura, Y., and Hashida, M. (1997) Intratumoral pharmacokinetics and in vivo gene expression of naked plasmid DNA and its cationic liposome complexes after direct gene transfer. *Cancer Res. 57*, 2681−2686.

(13) Nomura, T., Yasuda, K., Yamada, T., Okamoto, S., Mahato, R. I., Watanabe, Y., Takakura, Y., and Hashida, M. (1999) Gene expression and antitumor effects following direct interferon (IFN)-gamma gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice. *Gene Ther. 6*, 121−129.

(14) Wang, D. A., Narang, A. S., Kotb, M., Gaber, A. O., Miller, D. D., Kim, S. W., and Mahato, R. I. (2002) Novel branched poly(ethylenimine)-cholesterol water-soluble lipopolymers for gene delivery. *Biomacromolecules 3*, 1197−1207.

(15) Han, S., Mahato, R. I., Sung, Y. K., and Kim, S. W. (2000) Development of biomaterials for gene therapy. *Mol. Ther. 2*, 302−317.

(16) Mahato, R. I., Henry, J., Narang, A. S., Sabek, O., Fraga, D., Kotb, M., and Gaber, A. O. (2003) Cationic lipid and polymer-based gene delivery to human pancreatic islets. *Mol. Ther. 7*, 89−100.

(17) Narang, A. S., Cheng, K., Henry, J., Zhang, C., Sabek, O., Fraga, D., Kotb, M., Gaber, A. O., and Mahato, R. I. (2004) Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. *Pharm. Res. 21*, 15−25.

(18) Gaber, A. O., Fraga, D. W., Callicutt, C. S., Gerling, I. C., Sabek, O. M., and Kotb, M. Y. (2001) Improved in vivo pancreatic islet function after prolonged in vitro islet culture. *Transplantation 72*, 1730−1736.

(19) Ferrari, M. E., Rusalov, D., Enas, J., and Wheeler, C. J. (2002) Synergy between cationic lipid and co-lipid determines the macroscopic structure and transfection activity of lipoplexes. *Nucleic Acids Res. 30*, 1808−1816.

(20) Matulis, D., Rouzina, I., and Bloomfield, V. A. (2002) Thermodynamics of cationic lipid binding to DNA and DNA condensation: roles of electrostatics and hydrophobicity. *J. Am. Chem. Soc. 124*, 7331−7342.

(21) Floch, V., Le Bolc'h, G., Audrezet, M. P., Yaouanc, J. J., Clement, J. C., Des Abbayes, H., Mercier, B., Abgrall, J. F., and Ferec, C. (1997) Cationic phosphonolipids as non viral vectors for DNA transfection in hematopoietic cell lines and CD34+ cells. *Blood Cells Mol. Dis. 23*, 69−87.

(22) Mahato, R. I., Kawabata, K., Nomura, T., Takakura, Y., and Hashida, M. (1995) Physicochemical and pharmacokinetic characteristics of plasmid DNA/cationic liposome complexes. *J. Pharm. Sci. 84*, 1267−1271.

(23) Mahato, R. I., Lee, M., Han, S., Maheshwari, A., and Kim, S. W. (2001) Intratumoral delivery of p2CMVmIL-12 using water-soluble lipopolymers. *Mol. Ther. 4*, 130−138.

(24) Benhamou, P. Y., Moriscot, C., Prevost, P., Rolland, E., Halimi, S., and Chroboczek, J. (1997) Standardization of procedure for efficient ex vivo gene transfer into porcine pancreatic islets with cationic liposomes. *Transplantation 63*, 1798−1803.

(25) Saldeen, J., Sandler, S., Bendtzen, K., and Welsh, N. (2000) Liposome-mediated transfer of IL-1 receptor antagonist gene to dispersed islet cells does not prevent recurrence of disease in syngeneically transplanted NOD mice. *Cytokine 12*, 405−408.

(26) Saldeen, J., Curiel, D. T., Eizirik, D. L., Andersson, A., Strandell, E., Buschard, K., and Welsh, N. (1996) Efficient gene transfer to dispersed human pancreatic islet cells in vitro using adenovirus-polylysine/DNA complexes or polycationic liposomes. *Diabetes 45*, 1197−1203.

(27) Liu, Y., Mounkes, L. C., Liggitt, H. D., Brown, C. S., Solodin, I., Heath, T. D., and Debs, R. J. (1997) Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol. 15*, 167−173.

(28) Byk, G., Dubertret, C., Escriou, V., Frederic, M., Jaslin, G., Rangara, R., Pitard, B., Crouzet, J., Wils, P., Schwartz, B., and Scherman, D. (1998) Synthesis, activity, and structure−activity relationship studies of novel cationic lipids for DNA transfer. *J. Med. Chem. 41*, 229−235.

(29) Banerjee, R., Mahidhar, Y. V., Chaudhuri, A., Gopal, V., and Rao, N. M. (2001) Design, synthesis, and transfection biology of novel cationic glycolipids for use in liposomal gene delivery. *J. Med. Chem. 44*, 4176−4185.

(30) Regelin, A. E., Fankhaenel, S., Gurtesch, L., Prinz, C., Von Kiedrowski, G., and Massing, U. (2000) Biophysical and lipofection studies of DOTAP analogues. *Biochim. Biophys. Acta 1464*, 151−164.

(31) Venkata Srilakshmi, G., Sen, J., Chaudhuri, A., Ramadas, Y., and Madhusudhana Rao, N. (2002) Anchor-dependent lipofection with nonglycerol based cytofectins containing

single 2-hydroxyethyl headgroups. *Biochim. Biophys. Acta 1559*, 87−95.
(32) Heyes, J. A., Niculescu-Duvaz, D., Cooper, R. G., and Springer, C. J. (2002) Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer. *J. Med. Chem. 45*, 99−114.
(33) Vigneron, J. P., Oudrhiri, N., Fauquet, M., Vergely, L., Bradley, J. C., Basseville, M., Lehn, P., and Lehn, J. M. (1996) Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. *Proc. Natl. Acad. Sci. U.S.A. 93*, 9682−9686.
(34) Ren, T., Song, Y. K., Zhang, G., and Liu, D. (2000) Structural basis of DOTMA for its high intravenous transfection activity in mouse. *Gene Ther. 7*, 764−768.
(35) Gaucheron, J., Wong, T., Wong, K. F., Maurer, N., and Cullis, P. R. (2002) Synthesis and properties of novel tetraalkyl cationic lipids. *Bioconjugate Chem. 13*, 671−675.
(36) Miller, A. D. (1998) Cationic Liposomes for Gene Therapy. *Angew Chem., Int. Ed. 37*, 1768−1765.
(37) Niculescu-Duvaz, D., Heyes, J., and Springer, C. J. (2003) Structure−activity relationship in cationic lipid mediated gene transfection. *Curr. Med. Chem. 10*, 1233−1261.
(38) Liu, D., Ren, T., and Gao, X. (2003) Cationic Transfection Lipids. *Curr. Med. Chem. 10*, 1005−1013.
(39) Nicolazzi, C., Garinot, M., Mignet, N., Scherman, D., and Bessodes, M. (2003) Cationic lipids for transfection. *Curr. Med. Chem. 10*, 1263−1277.
(40) Kumar, V. V., Singh, R. S., and Chaudhuri, A. (2003) Cationic transfection lipids in gene therapy: successes, setbacks, challenges and promises. *Curr. Med. Chem. 10*, 1297−1306.
(41) Miller, A. D. (2003) The problem with cationic liposome/micelle-based nonviral vector systems for gene therapy. *Curr. Med. Chem. 10*, 1195−1211.
(42) Behr, J. P. (1994) Gene transfer with synthetic cationic amphiphiles: prospects for gene therapy. *Bioconjugate Chem. 5*, 382−389.
(43) Bartsch, H., Terracini, B., Malaveille, C., Tomatis, L., Wahrendorf, J., Brun, G., and Dodet, B. (1983) Quantitative comparison of carcinogenicity, mutagenicity and electrophilicity of 10 direct-acting alkylating agents and of the initial O6: 7-alkylguanine ratio in DNA with carcinogenic potency in rodents. *Mutat. Res. 110*, 181−219.
(44) Eder, E., Kutt, W., and Deininger, C. (2001) On the role of alkylating mechanisms, O-alkylation and DNA-repair in genotoxicity and mutagenicity of alkylating methanesulfonates of widely varying structures in bacterial systems. *Chem. Biol. Interact. 137*, 89−99.
(45) Peterson, A. R. (1980) DNA synthesis, mutagenesis, DNA damage, and cytotoxicity in cultured mammalian cells treated with alkylating agents. *Cancer Res. 40*, 682−688.
(46) Platzek, T., and Bochert, G. (2000) DNA alkylation studies of combined treatment with methylnitrosourea and ethylmethanesulfonate in mice. *Teratog., Carcinog., Mutagen 20*, 27−34.
(47) Snyder, R. D., and Bhatt, S. (1993) Alterations in repair of alkylating agent-induced DNA damage in polyamine-depleted human cells. *Cancer Lett 72*, 83−90.
(48) Hudson, R. F., and Withey, R. J. (1964) Nucleophilic reactivity. V. The reaction between amines and ethyl methanesulfonate. *J. Chem. Soc.* 3513−3517.
(49) Ross, P. C., and Hui, S. W. (1999) Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther. 6*, 651−659.
(50) Zelphati, O., Nguyen, C., Ferrari, M., Felgner, J., Tsai, Y., and Felgner, P. L. (1998) Stable and monodisperse lipoplex formulations for gene delivery. *Gene Ther. 5*, 1272−1282.
(51) Gray, D. M., Johnson, K. H., Vaughan, M. R., Morris, P. A., Sutherland, J. C., and Ratliff, R. L. (1990) The vacuum UV CD bands of repeating DNA sequences are dependent on sequence and conformation. *Biopolymers 29*, 317−323.
(52) Allen, F. S., Gray, D. M., and Ratliff, R. L. (1984) On the first-neighbor analysis of nucleic acid CD spectra: the definitive T matrix and considerations of various methods. *Biopolymers 23*, 2639−2659.
(53) Gustafsson, J., Arvidson, G., Karlsson, G., and Almgren, M. (1995) Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim. Biophys. Acta 1235*, 305−312.
(54) Pitard, B., Aguerre, O., Airiau, M., Lachages, A. M., Boukhnikachvili, T., Byk, G., Dubertret, C., Herviou, C., Scherman, D., Mayaux, J. F., and Crouzet, J. (1997) Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer. *Proc. Natl. Acad. Sci. U.S.A. 94*, 14412−14417.
(55) Rackstraw, B. J., Stolnik, S., Davis, S. S., Bignotti, F., and Garnett, M. C. (2002) Development of multicomponent DNA delivery systems based upon poly(amidoamine)-PEG copolymers. *Biochim. Biophys. Acta 1576*, 269−286.
(56) Thomas, M., and Klibanov, A. M. (2003) Conjugation to gold nanoparticles enhances polyethylenimine's transfer of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. U.S.A. 100*, 9138−9143.
(57) Bennett, M. J., Aberle, A. M., Balasubramaniam, R. P., Malone, J. G., Malone, R. W., and Nantz, M. H. (1997) Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. *J. Med. Chem. 40*, 4069−4078.
(58) Bloomfield, V. A. (1996) DNA condensation. *Curr. Opin. Struct. Biol. 6*, 334−341.
(59) Plank, C., Mechtler, K., Szoka, F. C., Jr., and Wagner, E. (1996) Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther. 7*, 1437−1446.

BC049818Q