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Physicochemical Characteristics Associated with Transfection of Cationic Cholesterol-based Gene Delivery Vectors in the Presence of DOPE

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Abstract

The physicochemical properties of a novel series of cholesterol-based cationic lipids in the presence of DOPE were studied by various techniques in an effort to correlate cationic lipid structure with transfection efficacy. It was found that while DOPE improves the β -gal activity of the active AC and MC derivatives, the overall zeta potential of the particles, pDNA complexation and condensation is not improved. This is in stark contrast with the tertiary amine derivative DC whose dispersion properties were improved and its monolayer surface potential is restored at high molecular surface density in the presence of DOPE. Overall the transfection activity mediated by DC and the quaternary ammonium TC derivative was greatly improved in the presence of DOPE and is attributed to decreased cytotoxicity, improved fusogenicity and cellular association.

Keywords

DC-Chol; DOPE; cationic lipids; lipoplex; gene therapy; monolayer; surface potential; zeta potential

1. Introduction

It is well established that a direct relationship exists between the efficacy of gene delivery and the structure of the cationic lipid complex to which the nucleic acid cargo is associated during the delivery process. Fundamental contributions to the success of that relationship are interactions with DNA facilitated by the cationic nature of the constituent lipids. It is accepted that in order for cationic surfactants to successfully deliver genetic material to the cell's interior, they must first electrostatically complex with and mediate internalization of the DNA (Ciani et al., 2004, Kennedy et al., 2000). Once inside the cell however, it is the dissociation of the DNA from the lipoplex that confers the initiation of a translational event. Such an event has been attributed to the fusion of the cationic lipid moiety with membrane components facilitated by the acquisition of a change in lipid phase state upon their interaction (Xu and Szoka, 1996; Zelphati and Szoka, 1996).

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Oftentimes, effective delivery is achieved by the inclusion of DOPE in cationic liposome formulations to overcome toxicity and to efficiently complex with and condense plasmid DNA at charge ratios that produce net positively charged structures that preferentially bind to cell membranes (Chesnoy et al., 2000; Koltover et al., 1999; Zuidam and Barenholz, 1997; Felgner et al., 1987). Examples of enhanced pDNA transfer mediated by the zwitterionic helper lipid DOPE on cholesterol-based cationic lipids include formulations composed of DC-Chol (herein termed DC) and cholesterol based Gemini lipids (Gao and Huang, 1991; Bajaj et al., 2007; Bajaj et al., 2008). While the inclusion of DOPE alone has led to increased transfection levels for many non cholesterol based vectors, its use in conjunction with cholesterol has demonstrated enhanced transfection activity by cationic acyl chain derivatives (Mukherjee et al., 2005). The role of endogenous cholesterol in biological membranes is thought to control fluidity by interactions with phospholipids to promote the formation of liquid ordered phases (El-Sayed et al., 1986; Almeida et al., 1992; Silvius, 2003).

Recently, cationic lipids based on the structure of DC-Chol (DC) were synthesized in this laboratory and evaluated for their contribution to the biophysical relationship that exists between transfection efficiency and endosomal escape. The study suggested that the particular cholesterol-based derivatives lacking highly substituted head groups interact with membrane mimetic vesicles to promote transfection in the absence of helper lipids (Kearns et al., 2007). Here we examine the physicochemical contribution of DOPE in improving transfection when used in conjunction with cholesterol-based amphiphiles.

2. Experimental

2.1 Materials and Apparatus

3β -[N-(N'-methylaminoethane)-carbamoyl]-cholesterol (MC), 3β -[N-(aminoethane)-carbamoyl]-cholesterol (AC), (3β -[N-(N',N'-dimethylaminoethane)-carbamoyl]-cholesterol (DC), and 3β -[N-(N',N',N'-trimethylaminoethane)-carbamoyl]-cholesterol iodide (TC) were synthesized to mass purity > 98.5 % as described elsewhere (Kearns et al., 2007; Scheme 1). 1,2-Dioleoyl-3-Trimethylammonium-Propane hydrochloride (DOTAP) and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The fluorescein labeling reagent together with the G50 microspin columns (*Label IT*[®] Fluorescein Labeling Kit) were purchased from Mirus Bio Corporation (Madison, WI). 2-(*N*-morpholine)-ethane sulphonic acid (MES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *o*-nitrophenyl- β -D-galactopyranoside (ONPG) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse melanoma cells (B16F0), human cervical carcinoma cells (HeLa), Dulbecco's modified Eagle's medium (DMEM) and Eagle's Minimum Essential Medium with Earle's Balanced salt solution (EMEM) were obtained from American Type Culture Collection (Manassas, VA). Fetal calf serum, penicillin (5,000 units)-streptomycin (5,000 μ g) solution and ethidium bromide solution (10 mg mL⁻¹) were obtained from Invitrogen Corporation (Grand Island, NY). All salts, acids and bases used in buffer preparation were of molecular biology grade and were obtained from Fisher Scientific (Suwanee, GA) and Spectrum Chemical Manufacturing Company (New Brunswick, NJ). Solvents from commercial suppliers were of reagent grade and were used without further purification.

2.2 Preparation of Liposomal Dispersions

Anhydrous lipid films of cationic cholesterol lipid derivatives with and without DOPE were formed by dissolution in chloroform followed by evaporation. Subsequent hydration in appropriate volumes of 40 mM tris pH 7.2 allowed for self assembly of the cationic amphiphilic molecules.

2.3 Lipofection and Cytotoxicity

B16F0 and HeLa cell lines (50,000 cells per well; 48-well plate) were transfected with dispersions of cationic lipid/DOPE mixture at molar ratios of 8:2 and 6:4 complexed with β -gal encoded pUC19 plasmids. Lipoplexes were prepared in serum free media 20 to 30 minutes prior to transfection by incubating aliquots of each lipid dispersion with plasmid DNA at positive to negative charge ratios of 1:1, 2:1, 4:1. Negative and positive controls were naked pDNA and DOTAP:pDNA (+/- charge ratio of 2:1), respectively, while the total amount of pDNA was kept constant at 1 μ g per well. Treated cells were incubated at 37 °C with 5% CO₂ for 4 hours during which time lipofection was allowed to occur. Approximately 48 hours post transfection, cells were lysed and transfection activity was quantified by a microplate calorimetric assay utilizing the β -galactosidase substrate ONPG. Quantification of the percent of viable cells was done using an MTT assay (Kearns et al., 2007).

2.4 Fluorescence Microscopy of Transfected B16F0 Cells

To determine the location of lipoplexes and/or plasmid DNA, B16F0 cell lines were transfected with fluorescently labeled pDNA. The fluorescein nucleic acid labeling reagents used were designed to covalently attach fluorescein-isothiocyanate (FITC) to the plasmid DNA with an efficiency of one label for every 20-60 base pairs. Cell transfections were performed as described above by modifying the ratios of labeled to unlabeled plasmid until optimal fluorescence intensity was achieved. After a 4 hour treatment time images of transfected cells were obtained using the Zeiss fluorescent inverted microscope (Axiovert 200M) equipped with a FITC fluorescence filter.

2.5 Agarose Gel Electrophoresis

The molar charge ratio at which complexation occurs between the cationic lipids formulated with DOPE and plasmid DNA was analyzed by agarose gel (0.8 %) electrophoresis visualized with the aid of ethidium bromide (0.5 μ g mL⁻¹). Complexation efficiency was measured by the ability of cationic lipids to exclude EtBr from diffusing into lipoplexes and intercalating into the pDNA double helix. Cationic lipid/DOPE lipoplexes were formulated at +/-charge ratios of 0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1. Samples were prepared in centrifuge tubes by the addition of 0.2 μ g of pDNA followed by cationic lipid to achieve each desired charge ratio. Appropriate volumes of 40 mM tris buffer pH 7.2 and serum free media (SFM) were added to achieve the final desired volumes.

2.6 Ethidium Bromide Displacement

Ethidium bromide (EtBr) displacement from plasmid DNA was utilized in this study as a measurement of the lipids' ability to condense plasmid DNA. Experiments were carried out in 40 mM tris buffer, pH 7.2 as well as in SFM and were conducted on a Cary Eclipse Fluorescence spectrophotometer (Varian Inc.) at excitation and emission wavelengths of 515 nm and 605 nm, respectively, as described elsewhere (Savva et al., 2005).

2.7 Photon Correlation Spectroscopy (PCS) and Laser Doppler Velocimetry

Particle size and zeta potential of lipid dispersions were analyzed using the Malvern Zetasizer Nano system (Malvern Instruments, Inc., Southborough, MA) in 40 mM tris buffer, pH 7.2 and SFM. Lipid dispersions (~120 nmol) were added to cuvettes followed by the addition of 40 mM Tris buffer pH 7.2, to a final volume of 1.2 mL. Plasmid DNA was subsequently added in ratios equivalent to those used in the transfection experiments. The resulting mixtures were incubated while stirring for 10 minutes after which time, the scattered light intensity of each sample at a 90° angle was measured at 23 °C. The reported hydrodynamic radii distributions were derived using the Zetasizer Nano system's self-analyzing autocorrelation function. The polydispersity indices corresponding to the widths of each size distribution correlation curve

was a parameter derived from the cumulants analysis method embedded within the software. After measuring the particle distribution within each sample, 0.75 mL (0.1 μ mol) was withdrawn using a 1 mL syringe and placed in the Zetasizer's folded capillary cell for ζ -potential measurement.

2.8 Interfacial Studies

Cationic lipid – DOPE monolayers were studied by their deposition at an air/water interface using a PC controlled analytical film balance (KSV Instruments Ltd, Helsinki, Finland). Surface pressure-Area (Π -A) isotherms of equimolar amounts of cationic lipid and/or DOPE monolayer films were obtained via symmetric isothermal (23 °C) compression of monolayers spread at an air/water interface facilitated by the addition of 150 mL of a 40 mM tris buffer, pH 7.2 subphase. To avoid dust contamination of surface-active substances, all glassware was cleaned and rinsed with purified water and subphase buffer was prepared using HPLC grade water and stored under a hood. Lipid stock solutions from which the monolayers were spread were prepared with HPLC grade chloroform.

To obtain complementary information about the orientation of these molecules, the change in the monolayer surface potential, ΔV , upon monolayer compression was measured using the KSV SPOT1 surface potential meter that operates based on the contactless and nondestructive vibrating capacitor method. Briefly, a 140 Hz audiofrequency current is created by a sound card and drives a magnet creating electrode vibration that causes a variation in the capacity across the air gap setting up an alternate current in the second circuit, the magnitude of which depends on the voltage across the gap (5 mm). The potentiometer that is adjusted to minimize the current is attached on the metal plate placed in the subphase (counter electrode). Calibration of the measuring head when connected to the counter electrode was done to assure a plate resonance frequency of 140 Hz with an amplitude of 50. Prior to the application of the lipid monolayer, the potential of the surface between the vibrating measuring head and the air-buffer subphase interface was compensated by zeroing the measuring head in order to correct for the surface potential of the buffer.

3. Results and Discussion

3.1 Lipofection and Cytotoxicity

Fig. 1A compares the overall transfection activity for all four cholesterol-based cationic lipids in the absence and presence of DOPE. In the presence of DOPE, there is an overall increase in activity for all four derivatives compared with their performance in its absence. AC and MC however, consistently exhibit higher transfection activities at all charge ratios in melanoma cells compared with DC and TC with and without the incorporation of DOPE. Transfection activity was also determined for HeLa cells where the presence of DOPE also mediated higher levels of transfection, albeit at less efficient levels than results obtained in B16F0 cells (Fig. 1B). In almost all cases, incorporation of 40 mole percent DOPE was optimal compared with that of 20 mole percent (not shown) with the most efficient level of transfection observed at charge ratios of 1:1 and 2:1.

To rule out cytotoxic effects, viability studies were performed on both cells lines in concert with the transfection studies (Fig. 2A and B). The detection of formazan crystals at 630 nm facilitated by mitochondrial dehydrogenase confirmed that the viability of transfected cells was improved for DC and TC in the presence of DOPE compared with transfections carried out with the cationic derivatives alone in both cell lines.

In support of the β -gal activity, representative images of cell associated and internalized FITC labeled plasmid DNA are presented in Fig. 3. In the absence of DOPE fluorescence due to

FITC-labeled DNA is observed at all three charge ratios for AC and MC but not for DC and TC cationic lipids. In the presence of DOPE, cellular association of all cationic lipids, especially for DC and TC was dramatically improved. It can therefore be concluded that the increased transfection activity mediated by all four cationic lipids in the presence of DOPE could be due to the enhanced cellular association and in the case of DC and TC due to increased cell viability, as well.

3.2 Agarose Gel Electrophoresis

Given the increased cell membrane association of the lipoplexes in the presence of DOPE, the physicochemical evaluation of each lipoplex suspension began with their ability to complex with pDNA. Complexation studies with pDNA in the absence and presence of DOPE in 40 mM tris buffer, pH 7.2 suggests that the charge ratio at which complete complexation occurs is relatively similar for all four lipids, with complete retardation occurring at a charge ratio of 4:1 (Fig. 4A).

When exposed to high ionic strength conditions however, complexation seems to improve only with the DC derivative in the presence of DOPE while complexation of TC with pDNA was essentially unaffected (Fig. 4B). Thus, in agreement with the pDNA complexation data consisting of partial to complete interactions between charge ratios 2:1 and 4:1, lipoplex formulations containing DOPE displayed enhanced and optimum biological activity at charge ratio 2:1.

3.3 Ethidium Bromide Displacement

In the presence of 40 mole % DOPE and when suspended in 40 mM tris buffer pH 7.2, lipoplexes composed of AC and MC condense significantly more pDNA than DC and TC (Fig. 5A). These percentages were slightly higher in comparison with exclusion data obtained for the AC and MC cholesterol derivatives without the inclusion of DOPE in the formulations (Kearns et al., 2007). The higher methylated derivatives, DC and TC, mediated 20 to 30 % condensation regardless of the inclusion of DOPE in the mixtures. No significant condensation of pDNA was mediated by any of the cationic lipids in the presence of DOPE under high ionic strength conditions.

3.4 Particle Size Distribution and Zeta Potential

In the absence of DNA, all lipid dispersions with and without DOPE were of small size in 40 mM tris, pH 7.2. In the physiological ionic strength SFM, particle size drastically increased for AC and MC, whereas for DC and TC particle size was practically unaffected. All lipid dispersions exhibited a positive zeta potential in both media but they were significantly reduced in SFM. DOPE had no significant effect on the zeta potential of lipid dispersions (Table 1).

Lipoplexes exhibited a very different trend and were more heterogeneous than lipid dispersions in the absence of pDNA (Table 2). More specifically, lipoplexes composed of AC, MC and DC exhibited a similar trend, i.e., in 40 mM tris, pH 7.2, at +/- charge ratio of 1:1 and 2:1 particle size was relatively small without DOPE and it significantly increase at +/- ratio of 4:1. In SFM lipoplexes composed of these three cationic derivatives were significantly larger at all charge ratios. At DOPE molar ratio of 0.4, the small size measured at charge ratios of 1 and 2 for AC and MC lipoplexes, drastically increased at charge ratio 4. In the presence of DOPE, DC-lipoplex size in 40 mM tris remained below 300 nm at all charge ratios. Similarly to the other CL, this improved stability of DC/DOPE lipoplexes was limited in SFM and big aggregates were observed at DOPE mole ratio of 0.4 and charge ratio of 2 to 4. A similar trend to the other cationic lipids was observed for TC in the absence of DOPE in tris buffer, pH 7.2 and SFM. However, in sharp contrast to the other cationic lipids, the presence of DOPE in lipoplex formulations composed of TC ameliorated the aggregation in SFM observed with

corresponding lipoplexes in the absence of DOPE. Regarding the zeta potential in tris buffer, the behavior of lipoplexes composed of AC, MC and DC were similar, with the values being negative at low charge ratios and reaching positive values of zeta potential at +/- charge ratio of 4 with and without DOPE. Lipoplexes composed of DC/DOPE exhibited negative zeta potential even at the highest charge ratio, in contrast to the results of EtBr exclusion assay. Lipoplexes composed of TC were quite unique in their interaction with DNA exhibiting a positive zeta potential even at +/- charge ratio of 2:1, although in the presence of DOPE, positive zeta potential was exhibited only at charge ratio of 4. Lipoplexes composed of AC, MC and DC in SFM in the presence and absence of DOPE exhibited negative zeta potential at all charge ratios, while lipoplexes composed of TC exhibited positive zeta potential values only at the highest charge ratio with and without DOPE.

3.5 Interfacial Studies

Isothermal compression of monolayers of each cationic cholesterol lipid in the presence of 40 mole percent DOPE at the air-water interface enabled the comparison of compression parameters with corresponding self assembly properties of each insoluble, ionizable layer (Fig. 6 A-H). Monolayers of each formulation were spread keeping the number of molecules constant so that analysis of the measured surface tension would reflect spatial effects due to inter-lipid association.

As each monolayer was laterally and isothermally compressed, the mean molecular area corresponding to monolayer collapse increased, as expected, in the presence of DOPE compared with the cationic lipids in isolation (Table 3). This increased area confirmed the incorporation of DOPE with the lipids due to its higher molecular weight. It is interesting to note that the collapse pressures of mixed monolayers do not differ significantly from collapse pressures of monolayers composed of pure cationic lipids; primarily because at DOPE mole fraction of 0.4 the magnitude of monolayer collapse pressure is very similar to that of the cationic cholesterol derivatives and of cholesterol (Savva et al., 2009). Increased fluidity or elasticity is observed both qualitatively and quantitatively in the presence of DOPE; the slopes corresponding to the monolayer isotherms of all four lipids decreased in the presence of DOPE, a trend that is more pronounced for DC (Table 3). This would help to explain the increased interactions with the cell membranes observed in the pDNA localization studies and the improvement in transfection (Fig. 2 and 4).

The effective dipole moment densities of single-component and mixed monolayers was studied by surface potential measurements. From the fundamental Helmholtz equation used to calculate potential difference in parallel-plate capacitors (eq 1),

$$\Delta V = \frac{4 \cdot \pi \cdot \sigma \cdot d}{\epsilon} \quad (1)$$

where σ is the enclosed charge density equal to charge Q over plate surface area, d is the distance between the parallel plates and ϵ the dielectric constant of the medium between the plates that varies between 1 to 80 for gas and water, respectively, one could derive the simplest model relating the surface potential drop and molecular dipole moment μ (eq 2), with the assumption that charge separation due to the presence of dipole moment simulates a parallel plate condenser,

$$\Delta V = \frac{\Gamma \cdot \mu_{\perp}}{\varepsilon \cdot \varepsilon_o} \quad (2)$$

where Γ is the molecular surface density in units of molecules/ $\text{\AA}^2$ and ε_o is the permittivity of empty space equal to $8.854 \cdot 10^{-12} \text{ C}^2/\text{N m}^2$.

Simultaneous analysis of the surface potential isotherms of the monolayers enabled the detection of intermolecular interactions prior to and during compression. This included two comparisons; firstly, mean molecular areas at collapse pressures Π_c versus maximum surface potential $\Delta V_{\max}$ and secondly, $\Delta V_{\max}$ corresponding to the mean molecular areas in the presence and absence of DOPE (Fig. 6). Reflective of a reduced hydrophobicity among the four cationic lipids AC exhibited the lowest ΔV at molecular areas much larger than the lift-off areas. Evidently, at mean molecular areas around $200 \text{ \AA}^2/\text{molecule}$ the AC monolayer is in a gas phase with its ring structure, polar head and linker lying flat on the interface. Upon compression, the increase in ΔV is monotonic in accordance with a gradual upright close alignment of molecules on the interface. Furthermore, unlike the primary, secondary and quaternary ammonium derivatives, comparison of Π -A and ΔV -A isotherms of the DC monolayer is most striking. The reduction observed in $\Delta V_{\max}$ of mixed monolayers as compared to the pure AC, MC and TC monolayers is expected considering that the $\Delta V_{\max}$ of pure DOPE monolayers is very similar to that of DC monolayers, around 450 mV (Bordi et al., 2006). Examination of the DC isotherms reveal that the maximum change in surface potential occurs at the point of monolayer lift-off in the absence of DOPE (470 mV at $76 \text{ \AA}^2/\text{molecule}$) versus at the point of monolayer collapse in the presence of DOPE (470 mV at $72 \text{ \AA}^2/\text{molecule}$). In the absence of DOPE, $\Delta V_{\max}$ occurred at $76 \text{ \AA}^2/\text{molecule}$ but the monolayer collapse as determined by the collapse pressure occurred at $32 \text{ \AA}^2/\text{molecule}$. These areas indicate that the DC molecules in isolation collapse long after (approximately $44 \text{ \AA}^2$) the highest levels of ionization per mean molecular area is achieved. At low compression pressures and in the liquid-expanded state, spatial restrictions are not imposed on the charges on the head groups. As compression proceeds in the absence of DOPE, the headgroups continue to rearrange laterally, gain proximity and effectively compromising charge separation and the overall basicity of the assembled monolayer, although molecular realignment at the interface is no longer uniform. Contrary to that, the presence of DOPE facilitates increased fluidity in the monolayer. This suggests that DOPE not only plays a role in stabilizing the monolayer but bridges the gap between individual DC molecules thereby allowing the loose interjection of water molecules to serve as spacer molecules leading to a more stable packing conformation that promotes better hydration and efficient cell membrane association. This type of “spatial stability” allows for the maintenance of a uniform charge at the surface of liposomes formed by DC and DOPE, one that would enable efficient interactions with pDNA as well.

Taken together, the monolayer experiments help to characterize sensitive physical forces of molecular distribution associated with the chemical makeup of each cationic cholesterol moiety in the presence of DOPE. We have observed that all the amphiphiles self assemble, not only upon compression, but spontaneously in a manner that reflects the degree of methylation on their hydrophilic headgroups.

4. Conclusion

The four planar cholesterol-based lipids employed in this study vary with increasing degrees of methylation on their headgroup and as a result we continue to observe remarkable differences in their performances as cationic delivery vectors. In the absence of the helper lipid DOPE, the secondary and tertiary derivatives, DC and TC, were inactive, toxic, inefficiently condensed

pDNA, poorly associated with cell membranes and failed to mediate calcein leakage from membrane mimetic vesicles (Kearns et al., 2007). In that same study, the primary and secondary derivatives, AC and MC, demonstrated high levels of biological efficacy and imparted contrary physicochemical characteristics in the absence of DOPE. While the biological studies performed herein demonstrate elevated transfection levels in the presence of DOPE, corresponding physical studies point to varying parameters that help to explain the supportive role that DOPE imparts on each lipid.

Formulations composed of DC and TC that include DOPE at near equimolar ratios increased transfection, increased cell surface interactions with FITC labeled pDNA and reduced toxicity. The physical studies performed indicate that these events are attributed to the degree of available ionized surface charge together with improved spatial characteristics of the molecules. Such characteristics are a direct reflection of the polar head groups. Monolayer studies attest to the latter where we observe that DOPE significantly imparts fluidity to the cholesterol components, a trend that is described by the slopes of each isotherm and is pronounced as methylation increases in the presence of DOPE. This is most evident with DC. In the absence of DOPE the increased lipophilicity of the DC molecules causes a drop to the maximum surface potential prior to monolayer collapse. Upon the addition of DOPE monolayer collapse identified by surface pressure measurements coincided with that determined by surface potential measurements and suggests that DOPE promotes charge separation at the polar region. This enables better grafting to the water at the air/water interface and suggests an increased hydration of the liposomal layers.

In the presence of 40 mole % DOPE, formulations composed of already active AC and MC derivatives mediated enhanced β -gal levels. This was not due to a significant decrease in toxicity compared with studies performed with the lipids in isolation. With 40 mole % DOPE, pDNA complexation is restored under high ionic strength conditions, however, it does not improve the condensation nor the cell viability. Cell associations however, are improved and occur at lower charge ratios. It follows that while DOPE may not increase the amount of pDNA that gets incorporated into the liposome, it protects what electrostatically binds with the lipid, and efficiently delivers the resulting lipoplex in SFM to the surface of the cell. The monolayer studies support this conclusion and reveal that with monolayers composed of AC and MC, a charge separation is spontaneously achieved and maintained signifying decreased lipophilicity and hydration regardless of the inclusion of DOPE.

While DOPE enhances transfection for all four derivatives in this series, the underlying reasons depend on the chemical structure of the cationic lipid itself. It appears that the cationic cholesterol derivatives are able to achieve the common goal of improved interactions with membrane lipids which promote improved delivery. This feat stems from varying levels of spatial configurations achieved between each cationic lipid and DOPE as a function of their polar methylation.

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References

- Almeida PFF, Vaz WLC, Thompson TE. Lateral Diffusion in the Liquid Phases of Dimyristoylphosphatidylcholine/Cholesterol Lipid Bilayers: A Free Volume Analysis. *Biochemistry* 1992;31:6739–6747. [PubMed: 1637810]

- Bajaj A, Kondiah P, Bhattacharya S. Design, Synthesis, and in Vitro Gene Delivery Efficacies of Novel Cholesterol-Based Gemini Cationic Lipids and Their Serum Compatibility: A Structure – Activity Investigation. *J Med Chem* 2007;50(10):2432–2442. [PubMed: 17444625]
- Bajaj A, Kondiah P, Bhattacharya S. Effect of the Nature of the Spacer on Gene Transfer Efficacies of Novel Thiocholesterol Derived Gemini Lipids in Different Cell Lines: A Structure-Activity Investigation. *J Med Chem* 2008;51:2533–2540. [PubMed: 18376817]
- Bordi F, Cametti C, Sennato S, Paoli B, Marianecchi C. Charge renormalization in planar and spherical charged lipidic aqueous interfaces. *J Phys Chem B* 2006 Mar 16;110(10):4808–14. [PubMed: 16526718]
- Chesnoy S, Huang L. Structure and Function of Lipid-DNA Complexes for Gene Delivery. *Annu Rev Biophys Biomol Struct* 2000;29:27–47. [PubMed: 10940242]
- Ciani L, Ristori S, Salvati A, Calamai L, Martini G. DOTAP/DOPE and DC-Chol/DOPE Lipoplexes for Gene Delivery: Zeta Potential Measurements and Electron Spin Resonance Spectra. *Biochim Biophys Acta* 2004;1664:70–79. [PubMed: 15238260]
- El-Sayed MY, Guion TA, Fayer MD. Effect of Cholesterol on Viscoelastic Properties of Dipalmitoylphosphatidylcholine Multibilayers as Measured by a Laser-Induced Ultrasonic Probe. *Biochemistry* 1986;25:4825–4832. [PubMed: 3768316]
- Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* 1995;1235(2):289–295. [PubMed: 7756337]
- Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielson M. Lipofection: A highly Efficient, Lipid-mediated DNA-transfection Procedure. *Proc Natl Acad Sci USA* 1987;84:7413–7417. [PubMed: 2823261]
- Gao X, Huang L. A Novel Cationic Liposome reagent for Efficient Transfection of Mammalian Cells. *Biochem Biophys Res Commun* 1991;179(1):280–285. [PubMed: 1883357]
- Kearns MD, Donkor AM, Savva M. Structure-transfection activity studies of novel cationic cholesterol-based amphiphiles. *Mol Pharm* 2007;5(1):128–139. [PubMed: 18159927]
- Kennedy MT, Pozharski EV, Rakhmanova VA, MacDonald RC. Factors Governing the Assembly of Cationic Phospholipid-DNA Complexes. *Biophys J* 2000;78:1620–1633. [PubMed: 10692346]
- Koltover I, Salditt T, Safinya CR. Phase Diagram, Stability, and Overcharging of Lamellar Cationic Lipid-DNA Self-Assembled Complexes. *Biophys J* 1999;77:915–924. [PubMed: 10423436]
- Mukherjee K, Sen J, Chaudhuri A. Common Co-lipids, in Synergy, Impart High Gene Transfer Properties to Transfection-incompetent Cationic Lipids. *FEBS Letters* 2005;579:1291–1300. [PubMed: 15710428]
- Savva M, Acheampong S. The interaction energies of cholesterol and 1,2-dioleoyl-phosphoethanolamine in spread mixed monolayers at the air-water interface. *J Phys Chem B* 2009;113:9811–9820. [PubMed: 19569618]
- Savva M, Chen P, Aljaberi A, Selvi B, Spelios M. In Vitro Lipofection with Novel Asymmetric Series of 1,2-Dialkoylamidopropane-Based Cytotoxins Containing Single Symmetric Bis-(2-dimethylaminoethane) Polar Headgroups. *Bioconjugate Chem* 2005;16:1411–1422.
- Silvius JR. Role of Cholesterol in Lipid Raft Formation: Lessons from Lipid Model Systems. *Biochim Biophys Acta* 2003;1610:174–183. [PubMed: 12648772]
- Xu YH, Szoka FC. Mechanism of DNA Release from Cationic Liposome/DNA Complexes used in Cell Transfection. *Biochemistry* 1996;35:5616–5623. [PubMed: 8639519]
- Zelphati O, Szoka FC. Mechanisms of Oligonucleotide Release from Cationic Liposomes. *Proc Natl Acad Sci U S A* 1996;93:11493–11498. [PubMed: 8876163]
- Zhi D, Zhang S, Zhao Y, Yang B, Yu S. Transfection efficiency of cationic lipids with different hydrophobic domains in gene delivery. *Bioconjugate Chem* 2010;21:563–577.
- Zuidam NJ, Barenholz Y. Electrostatic Parameters of Cationic Liposomes Commonly used for Gene Delivery as Determined by 4-heptadecyl-7-hydroxycoumarin. *Biochim Biophys Acta* 1997;1329:211–222. [PubMed: 9371413]

Abbreviations

CL	cationic lipid
AC-Chol	3 β -[N-(aminoethane)-carbamoyl]-cholesterol
MC-Chol	3 β -[N-(N'-methylaminoethane)-carbamoyl]-cholesterol
DC-Chol	(3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]-cholesterol
TC-Chol	3 β -[N-(N',N',N'-trimethylaminoethane)-carbamoyl]-cholesterol iodide
DOTAP	1,2-Dioleoyl-3-Trimethylammonium-Propane hydrochloride
DOPE	1,2-Dioleoyl- <i>sn</i> -Glycero-3-Phosphoethanolamine
MES	2-(<i>N</i> -morpholine)-ethane sulphonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ONPG	<i>o</i> -nitrophenyl- β -D-galactopyranoside
B16F0	mouse melanoma cells
HeLa	human cervical carcinoma cells
DMEM	Dulbecco's modified Eagle's medium
EMEM	Eagle's Minimum Essential Medium with Earle's Balanced salt solution

Figure 1.

Lipofection of cationic lipids (solid bars) and CL/DOPE 6/4 mol/mol (white bars) in murine melanoma cells and human cervical cancer cells. The positive control included in each assay is DOTAP/pDNA at +/- ratio 2:1 (stripped bars). The negative control in each transfection assay was naked plasmid DNA (dotted bars). Ratios on the abscissa indicate cationic lipid to pDNA +/- charge ratios. Standard deviations are representative of experiments performed in triplicate.

Figure 2.

Cell viability of cationic lipids (solid bars) compared with CL/DOPE 6/4 (white bars) in B16F0 cells and in HeLa cells. All experiments were performed in triplicate of which standard deviations are indicated by crossbars on each column. Ratios next to each lipid formulation on the abscissa composition indicate CL:pDNA ratios.

PANEL A

AC

1:1

2:1

4:1

AC/DOPE 6/4

1:1

2:1

4:1

PANEL B

MC

1:1

2:1

4:1

MC/DOPE 6/4

1:1

2:1

4:1

Figure 3. Cellular localization of FITC labeled pDNA at +/- ratios 1:1, 2:1, and 4:1 mediated by (A) AC, (B) MC, (C) DC and (D), in isolation and in the presence of 40 mole % DOPE as indicated in each panel of images.

Figure 4.

Gel electrophoresis performed in 40 mM tris pH 7.2 (A) and SFM (B) with indicated lipid formulations. The ionic strength of SFM (0.150 M) varies with +/- charge ratio as follows: pDNA control, 0.5, 1, 2, 4, 6 and 8 are 0.130 M, 0.123 M, 0.116 M, 0.103 M, 0.080 M, 0.089 M and 0.082 M, respectively. The heading over each lane represents +/- charge ratio of cationic lipid to pDNA.

Figure 5. Charge ratio (+/-) plotted as a function of the percent of maximum ethidium bromide displaced. Graphs represent CL/DOPE 6/4 (mol/mol) in 40 mM tris buffer pH 7.2 (A), and SFM (B). Symbols are the average values calculated from three independent experiments.

Figure 6.

Monolayer compression isotherms consisting of surface pressure-area and surface potential-area curves for AC (A), AC/DOPE (B), MC (C), MC/DOPE (D), DC (E), DC/DOPE (F), TC (G), and TC/DOPE (H). Graphs B, D, F and H depict isotherms obtained with cationic lipid and DOPE performed at a molar ratio of 6:4. Isotherms in bold type represent surface pressure-area curves while those in light type correspond to the surface potential-area (secondary axis) curves.

Scheme 1.

Chemical structures of the four cholesterol-based cationic lipids. AC, MC and DC were synthesized and used as a free base whereas TC was synthesized and used as the iodide salt.

Hydrodynamic characterization experiments of lipid dispersions (in the absence of DNA) were carried out in 40 mM tris buffer (pH 7.2) and serum free media (SFM) at room temperature. Standard uncertainties were calculated from average values measured in three separate experiments.

Table 1

Composition (mol/mol)	Hydrodynamic Radius (nm)	Polydispersity index (PDI)		Zeta Potential (mV)
	40 mM Tris	SFM	40 mM Tris	SFM
AC	204	2322	0.24	1.0
AC/DOPE (6:4)	219	571	0.17	0.21
MC	169	1418	0.21	0.79
MC/DOPE (6:4)	631	889	0.35	0.56
DC	210	182	0.29	0.31
DC/DOPE (6:4)	137	220	0.45	0.67
TC	141	203	0.23	0.24
TC/DOPE (6:4)	115	97	0.43	0.25

Table 2

Hydrodynamic characterization of lipoplexes determined at various charge ratios carried out in 40 mM tris buffer pH 7.2 and serum free media (SFM) at room temperature. Standard uncertainties were calculated from average values measured in three independent experiments.

Composition (mol/mol)	+/- charge ratio	Hydrodynamic Radius (nm)		PDI	Zeta Potential (mV)	
		40 mM Tris ^a	SFM ^b	40 mM Tris	40 mM TB	SFM
AC	1/1	285	1620	0.25	-34 ± 7	-29 ± 57
AC	2/1	298	1331	0.27	-33 ± 8	-30 ± 56
AC	4/1	1248	891	0.33	+38 ± 5	-33 ± 53
AC/DOPE (6:4)	1/1	235	448	0.21	-8.5 ± 9.4	-41 ± 26
AC/DOPE (6:4)	2/1	283	509	0.22	-35 ± 12	-34 ± 18
AC/DOPE (6:4)	4/1	5721	3634	0.57	+22 ± 9	-40 ± 20
MC	1/1	185	862	0.24	-34 ± 10	-31 ± 35
MC	2/1	258	886	0.24	-33 ± 6	-31 ± 46
MC	4/1	782	877	0.19	+41 ± 6	-30 ± 23
MC/DOPE (6:4)	1/1	567	1356	0.41	-55 ± 8	-37 ± 45
MC/DOPE (6:4)	2/1	491	2034	0.33	-56 ± 8.5	-38 ± 36
MC/DOPE (6:4)	4/1	1232	1258	0.30	+7 ± 9.4	-20 ± 41
DC	1/1	236	377	0.36	-36 ± 12	-32 ± 15
DC	2/1	250	417	0.25	-36 ± 11	-30 ± 11
DC	4/1	1359	987	0.80	+28 ± 4	-24 ± 12
DC/DOPE (6:4)	1/1	132	484	0.62	-47 ± 11	-36 ± 18
DC/DOPE (6:4)	2/1	151	837	0.46	-41 ± 5.8	-35 ± 25
DC/DOPE (6:4)	4/1	302	4129	0.40	-26 ± 6.1	-11 ± 30
TC	1/1	160	316	0.21	-34 ± 7.6	-29 ± 16
TC	2/1	324	1467	0.29	+15 ± 5.6	-5 ± 17
TC	4/1	1496	4446	0.99	+19 ± 4.6	+14 ± 14
TC/DOPE (6:4)	1/1	310	181	0.34	-56 ± 5	-34 ± 21
TC/DOPE (6:4)	2/1	6089	633	0.32	-35 ± 28	-12 ± 26
TC/DOPE (6:4)	4/1	2192	530	1.0	+43 ± 4.5	+18 ± 13

Table 3

Monolayer values obtained from compression isotherms (23 °C) of lipids in isolation and with the inclusion of 40 % DOPE. The number of molecules sprayed onto the interface was kept constant for all formulations. Collapse areas and pressures of monolayers in isolation were repeated to obtain simultaneous surface potential values and are being included in this manuscript for comparison (Kearns et al., 2007). Standard deviations represent errors calculated for 3 or 4 consecutive runs.

Composition (mol/mol)	Mma ($\text{\AA}^2$) at collapse Π	Mma ($\text{\AA}^2$) at max ΔV	Π (mN/m)	ΔV (mV)	d Π /dA
AC	46 $\pm$ 0.03	48 $\pm$ 0.03	33.1 $\pm$ 3.3	600 $\pm$ 0.86	2.8 $\pm$ 0.7
AC/DOPE (6:4)	78 $\pm$ 0.01	75 $\pm$ 0.4	33.9 $\pm$ 8.1	560 $\pm$ 0.75	2.1 $\pm$ 0.6
MC	37 $\pm$ 0.06	44 $\pm$ 0.02	32.0 $\pm$ 0.7	645 $\pm$ 0.94	3.3 $\pm$ 0.3
MC/DOPE (6:4)	72 $\pm$ 0.02	70 $\pm$ 0.3	36.5 $\pm$ 3.7	510 $\pm$ 0.84	1.5 $\pm$ 0.3
DC	37 $\pm$ 0.05	76 $\pm$ 0.001	35.8 $\pm$ 0.7	470 $\pm$ 0.63	2.3 $\pm$ 0.05
DC/DOPE (6:4)	72 $\pm$ 0.04	72 $\pm$ 0.3	38.4 $\pm$ 1.0	470 $\pm$ 0.57	0.8 $\pm$ 0.02
TC	42 $\pm$ 4.3	34 $\pm$ 0.005	39.1 $\pm$ 0.5	672 $\pm$ 0.01	3.5 $\pm$ 0.7
TC/DOPE (6:4)	104 $\pm$ 17	70 $\pm$ 0.3	34.3 $\pm$ 2.6	650 $\pm$ 0.005	1.6 $\pm$ 0.2