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Physicochemical and biological characterization of 1,2-dialkoylamidopropane-based lipoplexes for gene delivery

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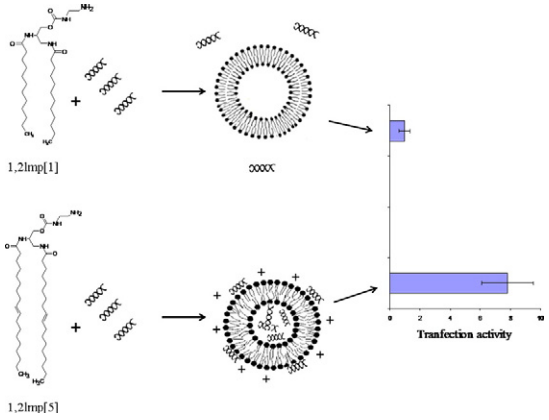
HIGHLIGHTS

- We investigated the molecular requirements for improved lipofection activity of ionizable cationic lipids.
- Only cationic lipids with sufficient bilayer fluidity and extent of ionization promoted significant lipofection.
- The activity of these lipids was boosted by inclusion of the helper lipid DOPE in the formulation.
- The improved lipofection is attributed to the efficient association and encapsulation of plasmid DNA by such formulations.

GRAPHICAL ABSTRACT



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ABSTRACT

Elucidation of the molecular and formulation requirements for efficient lipofection is a prerequisite to enhance the biological activity of cationic lipid-mediated gene delivery systems. To this end, the in vitro lipofection activity of the ionizable asymmetric 1,2-dialkoylamidopropane-based derivatives bearing a single primary amine group as the cationic head group was evaluated. The electrostatic interactions of these cationic lipids with plasmid DNA in serum-free medium were investigated by means of gel electrophoresis retardation and Eth-Br quenching assays. The effect of the inclusion of the helper lipid DOPE in the formulation on these interactions was also considered. The physicochemical properties of these lipids in terms of bilayer fluidity and extent of ionization were investigated using fluorescence anisotropy and surface potential techniques, respectively. The results showed that only the active lipid, 1,2lmp[5], existed in a liquid crystalline state at physiological temperature. Moreover, the extent of ionization of this lipid in assemblies was significantly higher that it's saturated analogues. Inclusion of the helper lipid DOPE improved the encapsulation and association between 1,2lmp[5] and plasmid DNA, which was reflected by the significant boost of lipofection activity of the 1,2lmp[5]/DOPE formulation as

Abbreviations: Eth-Br, ethidium bromide; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane hydrochloride; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; lmp, lipid monovalent primary; MmA, mean molecular area; N/P, nitrogen to phosphate; MLV, multilamellar vesicles.

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compared to the lipid alone. In conclusion, membrane fluidity and sufficient protonation of ionizable cationic lipid are required for efficient association and encapsulation of plasmid DNA and elicit of improved in vitro lipofection activity.

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1. Introduction

Lipoplexes, the colloidal complexes of cationic lipids with nucleic acids, have been the most thoroughly investigated and clinically experimented alternative of viral vectors for the delivery of nucleic acid therapeutics [11,25,29]. This is attributed to their obvious advantages of low immunogenicity, manufacturability to industrial standards, targeting flexibility, and ease of handling. However, their progress into a therapeutically feasible gene medicine is impeded by two major hurdles. These hurdles are their colloidal instability and low transfection activity compared to viral vectors [26,28].

To overcome these limitations, different structures of cationic lipids were synthesized and evaluated for lipofection effectiveness in the past two decades [6,7,18,20,23,42,43]. Moreover, the physicochemical properties of these lipids and their complexes with nucleic acids (DNA, oligonucleotides, and siRNA) were investigated in an attempt to derive a structure activity relationship [4,27,38]. Substantial improvement of the efficiency of cationic lipid-based vectors was achieved through the discovery and synthesis of new and more effective cationic lipids [19]. Nevertheless, transfection activity can also be improved through the optimization of formulation aspects of these vectors. Lipoplexes are formed spontaneously through the electrostatic interaction between the positively charged cationic lipids and the negatively charged nucleic acid backbone [21]. That association is followed by collapse of the nucleic acid structure as well as aggregation, rupture and fusion of lipid vesicles to condense and entrap the nucleic acid [14,41]. Several processing and formulation factors are expected to affect the efficiency with which these events can take place and subsequently, their biological activity and the colloidal characteristics of the resultant particles. Examples of such factors include (1) lipid composition [15,24] (2) charge ratio [31,32,44] (3) type and ratio of helper lipid [8,13,34] (4) type of the hydrophobic anchor of PEG-lipids in PEGylated lipoplexes [17] and (5) complexing medium [12,22].

However, these lipoplex-formulation issues would not be optimized without considering the physicochemical properties of the cationic lipid itself. Only lipids that possess sufficient molecular fluidity [1,3,35,40], interfacial elasticity [2,31,32], and hydration [5] were found to interact, complex and mediate efficient plasmid DNA transfection. With regard to charge ratio, the extent of ionization becomes a determining factor when ionizable cationic lipids are used [30]. Therefore, detailed characterization and comprehensive understanding of all of the aforementioned issues is undoubtedly a prerequisite for the development of pharmaceutically sound and efficient lipoplexes.

The present study aims to continue the characterization of dialkylamidopropane-based cationic lipids that we developed in order to identify the molecular and formulation requirements for improved transfection activity [2,3,31,32,33,36–38]. Herein, the in vitro lipofection activity of the asymmetric 1,2-dialkoylamidopropane-based derivatives bearing single primary amine group as the cationic head group was evaluated. The electrostatic interactions of these cationic lipids with plasmid DNA in serum-free medium were investigated with several techniques. In addition, the effects of the presence of the helper lipid on these interactions are described. The physicochemical properties of these lipids in terms of bilayer fluidity and extent of ionization in the absence of the helper lipid were investigated and correlated with their in vitro lipofection activity.

2. Materials and methods

2.1. Materials

Cholesterol (>99%), 1,6-diphenyl-1,3,5-hexatriene (DPH), and o-nitrophenyl β-D-galactopyranoside were purchased from Sigma-Aldrich (St. Louis, MO). Tris (hydroxymethyl) aminomethane (99.8 + %) and ammonium acetate were from Fisher Scientific (Pittsburgh, PA). DOPE and DPPC were from AVANTI Polar Lipids Inc. (Alabaster, AL). Agarose, ethidium bromide solution (10 mg/ml), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, combined penicillin–streptomycin aqueous solution (10,000 U/ml and 10,000 μg/ml, respectively), sodium pyruvate and trypsin–EDTA 1× (10 mg/ml) were from Invitrogen Life Technologies (Carlsbad, CA). Water for buffer preparation was obtained from a Barnstead NANOpure ultrapure water system (Barnstead, Dubuque, IA). The resistivity and surface tension of the ultrapure water were 16–18 × 10^6 Ω cm and 72.5 mN/m, respectively.

2.2. Plasmid DNA preparation

pEGFP-N1 plasmid DNA vector containing the green fluorescent protein (GFP) reporter gene was amplified in *Escherichia coli* DH5α competent cells and extracted, purified, and quantified as described earlier [3]. High quality plasmids free from protein and salt contamination were obtained as verified by gel electrophoresis and A260/A280 ratio of 1.89.

2.3. Preparation of liposomes

In vitro lipofection, gel electrophoresis retardation and Eth-Br fluorescence quenching assays were performed with liposomal dispersions prepared at 0.6 mM cationic lipid concentration in Tris buffer. First, solutions of 1,2lmp derivatives (Fig. 1) in a 4:1 v/v chloroform/methanol solvent mixture were prepared as a weight per volume concentration. Aliquots of these solutions along with DOPE at cationic lipid/DOPE molar ratio of 6:4 were transferred to 12 × 75 mm borosilicate glass tubes (VWR). Films of the lipids were deposited on the walls of the tubes by evaporating the bulk solvent with a stream of dry nitrogen gas. To remove any residual solvents, the tubes were vacuum desiccated for an additional 4 h. MLV were prepared by hydrating the dry lipid films with 40 mM tris buffer pH 7.2 at 90 °C for 1 h with occasional vortexing. The tubes were carefully sealed to prevent any loss of water during this process. The prepared MLV were finally downsized by sonicating the tubes for 5 min.

2.4. In vitro lipofection

Cationic lipids formulated in the presence and absence of the helper lipids were screened for activity against human cervical carcinoma (HeLa) cells (ATCC, CCl-2) at the following cationic lipid/DNA N/P molar ratios: 1/1, 2/1 and 4/1. All calculations were based on the nucleotide average molecular weight taken to be 330. HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin.

Approximately 50,000 cells were seeded 12 h before transfection in 1 mm wells (48-well plate). Lipoplexes were prepared 30 min prior to transfection by mixing appropriate amounts of the cationic lipid aqueous dispersions with plasmid DNA in serum-free media. The cells

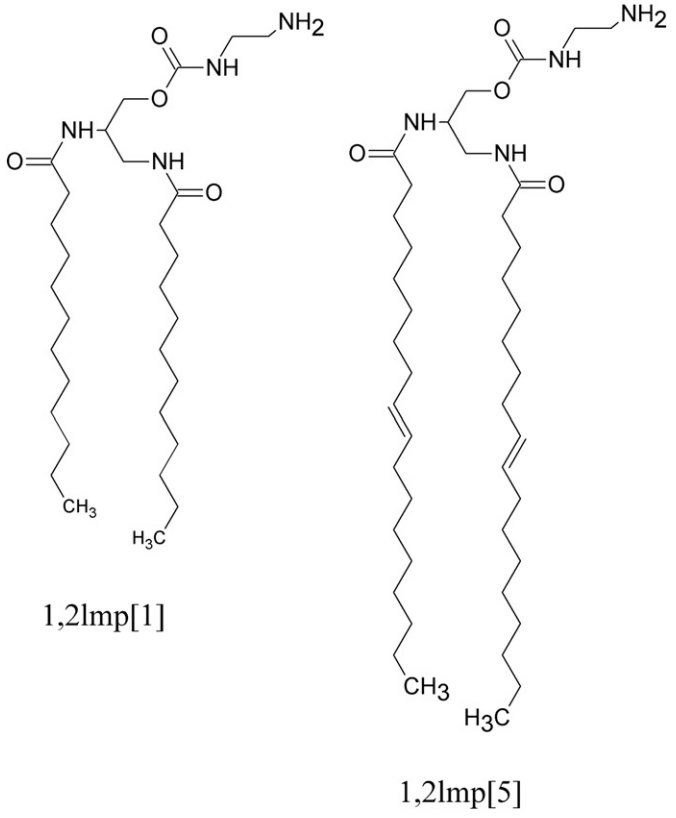

Fig. 1. Chemical structure of the lauroyl and oleoyl derivatives of 1,2-dialkoylamidopropane-based cationic lipids with a single primary amine head group.

were transfected with 250 μl of lipoplex dispersions and incubated at 37 °C for 4 h. The lipoplexes were then aspirated and fresh DMEM medium containing 10% FBS was added to each well. Cells transfected with plasmid DNA containing the β-Gal reporter gene was assayed for enzymatic activity 48 h after transfection, using a 96-well microplate colorimetric assay that employed the substrate o-nitrophenyl β-D-galactopyranoside as described elsewhere [3].

2.5. Fluorescence anisotropy

For determination of phase transition temperatures of 1,2lmp bilayers, liposomes of 1,2lmp derivatives as well as DPPC were prepared at 0.5 mM concentration and at lipid:DPH ratio of 200:1. Steady state fluorescence anisotropy measurements (r) were recorded with a carry eclipse fluorescence spectrophotometer (Varian Inc., CA.) equipped with automated polarizer and peltier thermostated single cell holder with temperature accuracy of ± 0.1 °C. Samples were maintained in the cuvette for 2 min per 1 °C before taking measurement. All samples were excited at 364 nm wavelength and emission was collected at 427 nm. Anisotropy (r) values were calculated by the eclipse software using the equation

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities of the emitted light parallel and perpendicular to the polarization plane of the excitation light, respectively. G is a factor that accounts for the polarization bias of the instrument.

The experimental data were fitted with PSIplot software (Poly Software International, version 3, New York, USA) using the following equation:

$$r = \mathrm{A} - \frac{\mathrm{B}}{1 + \mathrm{e}^{-(\mathrm{C})\ (\mathrm{T}-\mathrm{D})}} \quad (2)$$

where: A, B, C and D are constant parameters to be determined. The parameter D represents the gel to liquid crystalline phase transition temperature, T_m. Nonlinear fitting of the experimental data was done in an iterative fashion where initial parameters for A, B were r_{max} and $(r_{max} - r_{min})$, respectively. r_{max} is the maximum anisotropy or average of the upper third and r_{min} represents the minimum anisotropy value or the average of the lower third of the anisotropy values of data points. The initial value of D (or T_m) is the average of the temperature values of the cross-over area. Goodness of fit parameters were assessed within a 95% confidence interval.

2.6. Surface potential measurements

A computer controlled KSV minitrough film balance (KSV instruments LTD, Helsinki, Finland) was used to construct the ΔV/A isotherms of monolayers of the different 1,2lmp cationic derivatives in isolation at the air/water interface. This film balance was equipped with two hydrophilic polyacetal made barriers, a platinum Wilhelmy plate, a KSV SPOT1 surface potential meter based on vibrating plate method, and a metal plate as a counter electrode. The trough used in this study was made of solid PTFE with dimensions of 1364 × 75 mm. As a subphase, 140 ml of 40 mM tris buffer, pH 7.2, was used. Measurements were carried out at constant subphase temperatures of 23 °C maintained by the aid of an external water bath circulator.

1,2lmp lipid solutions at 0.75 μM concentration were prepared in chloroform/methanol (4:1) solvent mixture, stored at −20 °C and used within two days of preparation. Preliminary analysis of ΔV/A isotherms revealed that variation of the number of 1,2lmp molecules spread did not affect the reproducibility of the isotherms. Therefore, 30 μl aliquots of 1,2lmp lipid-spreading solutions were constantly applied drop by drop to the surface of the aqueous subphase with the aid of a Hamilton glass micro syringe. After an initial delay period of 10 min, to ensure chloroform evaporation, the barriers were closed at a constant speed of 9.99 mm/min. Each experiment was repeated 3–4 times in an open-air vibration-free environment to ensure isotherms reproducibility. After each experiment, the trough and glasswares were exhaustively washed with purified water. Surface potential at monolayer collapse (ΔV_{max}) and phase transition areas were identified using the first derivative of surface potential with respect to mean molecular area. All transitions and collapse areas reported here are onset parameters [16].

2.7. Gel electrophoresis retardation assay

The electrophoretic equipment (casting apparatus and power source) utilized in this assay was obtained from BioRad® (Hercules, CA). The gel mold provided with the apparatus was filled to an approximate thickness of 5.0 mm with a standard Low-EEO agarose in a 16 mM TAE buffer pH 7.2. The concentration of the gel used in this retardation assay was 0.8%. Ethidium bromide was added to the gel solution at a concentration of 0.5 μg/ml for visualization of any migrated DNA.

Lipoplexes at N/P molar ratios of 0.5, 1, 2, 4, 6 and 8 were prepared in microfuge tubes by mixing 0.2 μg of DNA with the appropriate amount of the cationic lipid aqueous dispersions. One μl of a 0.25% bromophenol solution (30.0% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was added to each tube and the total volume was brought to 11 μl with serum-free DMEM medium. Lipoplexes were briefly centrifuged at a speed of 14,000 rpm, and then allowed to incubate for 30 min. Naked DNA was loaded on the outermost lanes as a negative control. All samples were electrophoresed by applying a voltage of 5 V per centimeter length of casted gel for 35 min. The migration of lipoplexes was visualized using a KODAK Gel Logic 200 gel Imaging System (Eastman Kodak Co., Rochester, NY) equipped with a 590 nm filter to eliminate light other than 590 nm wavelength produced by ethidium bromide.

2.8. Eth-Br fluorescence quenching assay

Fluorescence measurements were collected with a Cary Eclipse fluorescence spectrophotometer (Varian Inc., CA) at an excitation wavelength of 515 nm (slit width 5 nm) and an emission wavelength of 595–605 nm (slit width 2.5 nm). Briefly, 22.5 μg of plasmid DNA and 0.8 μg of ethidium bromide (DNA:Eth-Br complex ratio 34:1) were added to a quartz cuvette and diluted to 3 ml with serum-free DMEM medium. Cationic lipids were added in aliquots under continuous stirring at 23 °C and fluorescence was monitored continuously until the reading became stable. A sample without Eth-Br was run side by side to the original experiment to correct for light scattering. To provide the relative Eth-Br displacement from plasmid DNA, the fluorescence signal of the Eth-Br blank solution was subtracted from all measurements and the fluorescence intensity obtained from the plasmid DNA solution containing Eth-Br in the absence of lipid (F_o) was assigned a value of 100. The resulting fluorescence intensity after addition of the cationic lipids ($F_{complex}$) was normalized as follows:

$$\%\,\text{Displacement} = \frac{F_{complex}}{F_o} \times 100 \quad (3)$$

3. Results and discussion

3.1. In vitro lipofection

Lipofection levels of the reporter gene that were elicited by the various 1,2lmp cationic lipids in HeLa cells are summarized in Fig. 2. Only the dioleoyl derivative 1,2lmp[5] could induce considerable lipofection levels on its own. Inclusion of the helper lipid DOPE in 1,2lmp[5] lipoplex formulation at a cationic lipid/DOPE molar ratio of 6:4 resulted in a significant improvement of its lipofection efficiency. As Fig. 2 shows, cells that were treated with 1,2lmp[5]/DOPE lipoplex formulation at 1:1 N/P molar ratio expressed similar levels of the reporter gene as that induced by the commercial cationic lipid DOTAP. On the other hand, the other four saturated derivatives were completely ineffective in inducing any significant lipofection as compared to the naked plasmid DNA control (data not shown). This is in agreement with their previously reported poor efficacy in B16-F0 mouse melanoma cells [3]. Upon inclusion of DOPE in the lipoplex formulation of these derivatives, modest signs of improvement in the lipofection efficiency could be seen with the shortest saturated derivative 1,2lmp[1] only. Similar to the general activity trend presented in Fig. 2, the maximum lipofection efficiency was induced at 1:1 N/P molar ratio and it declined as the N/P molar ratio is increased.

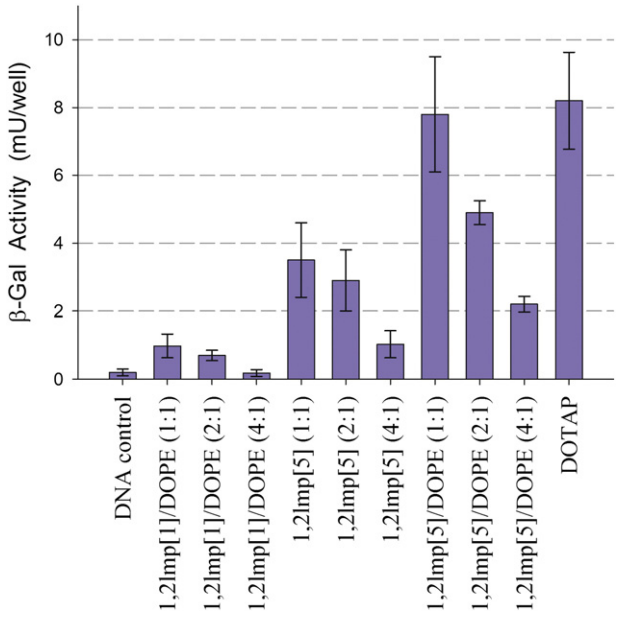

Fig. 2. Lipofection efficiencies of cationic lipoplexes in HeLa cells. The data shown present the average of three wells treated at the same day. β-Galactosidase levels are expressed as mU/well, where each well is equivalent to 150 μl of cell lysate. Numbers in parenthesis denote N/P molar ratios.

3.2. Fluorescence anisotropy

The depolarization of DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescent probe was used to monitor phase transitions of 1,2lmp cationic liposomes at pH 7.2. Fig. 3 shows the calculated mean anisotropy of DPH in liposomal dispersion of the five 1,2lmp derivatives as well as DPPC as a function of temperature. The onset, offset, and mean phase transition temperatures of the various I,2lmp liposomal dispersions were determined from the derivative profile of the anisotropy temperature plot (Fig. 4) and their values are summarized in Table 1. Noticeably, only the lipofection active 1,2lmp[5] derivative underwent its phase transition below physiological temperature with a T_m of 21.28 °C. Conversely, the phase transition of all the other saturated derivatives took place at considerably higher temperatures starting at a T_m of 59.14 °C for the short lauroyl derivative. Interestingly, a linear relationship could be seen when the phase transition temperatures of the saturated derivatives were plotted against the length of the hydrocarbon chain of these lipids (Fig. 5). On average, increasing the acyl chain length of saturated 1,2lmp derivatives by two carbons was accompanied by 8.14 °C increase in T_{onset}, T_{offset}, or T_m.

Previously, the fluorescence anisotropy-based phase transitions of liposomal dispersions of N,N\-diacyl-1,2-diaminopropyl-3-carbamoyl[bis-(2-dimethylaminoethane)] cationic lipids (referred to as 1,2lb lipids) were reported [31]. These divalent analogues of the monovalent 1,2lmp lipids exhibited wide onset–offset temperature range of the phase transitions. Moreover, the broad phase transitions were characterized by more than one inflection in their fluorescence anisotropy profile. In contrary, all five 1,2lmp cationic lipids exhibited one inflection in their fluorescence anisotropy profile representing the gel-to-liquid crystalline phase transition. The phase transitions of the saturated derivatives were sharp and represented by small temperature ranges, indicating the very high cooperative nature of the phase transition of these derivatives. For the unsaturated derivative, 1,2lmp[5], a wider temperature range of the phase transition was found. This less cooperative behavior of 1,2lmp[5] phase transition is expected considering the increased disorder offered by the presence of a cis double

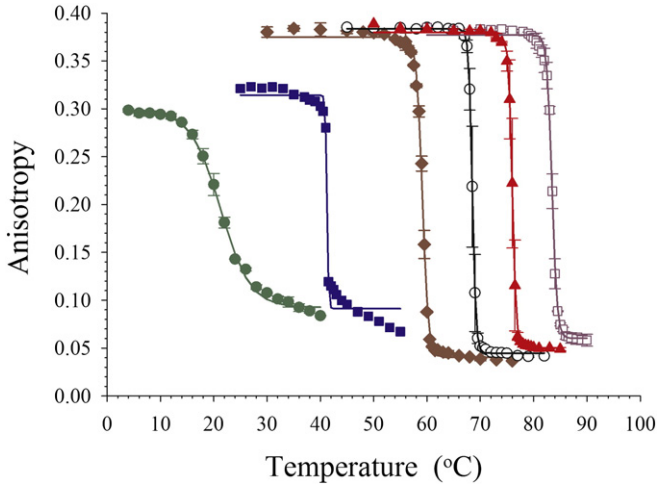

Fig. 3. Fluorescence anisotropy (r) against temperature plots for cationic lipid dispersions at pH 7.2. continuous lines are the curve fits of the experimental data (symbols): 1,2lmp[1] (♦), 1,2 lmp[2] (○), 1,2lmp[3] (▲), 1,2lmp[4] (□), 1,2lmp[5] (●) and DPPC (■).

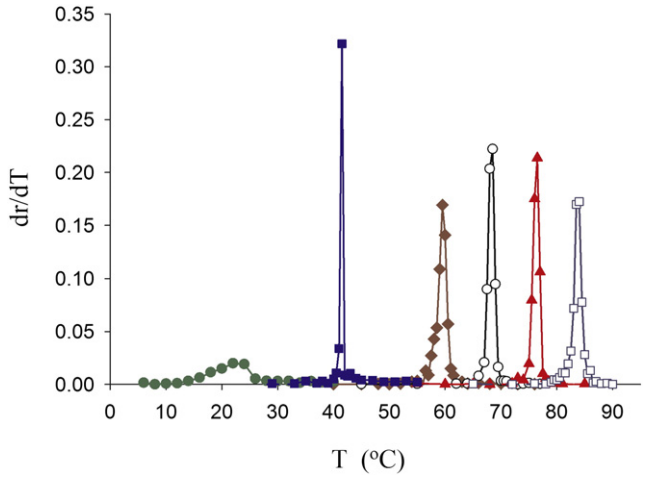

Fig. 4. Derivative data of fluorescence anisotropy (r) against temperature plots for cationic lipid dispersions at pH 7.2. 1,2lmp[1] (♦), 1,2 lmp[2] (○), 1,2lmp[3] (▲), 1,2lmp[4] (□), 1,2lmp[5] (●) and DPPC (■).

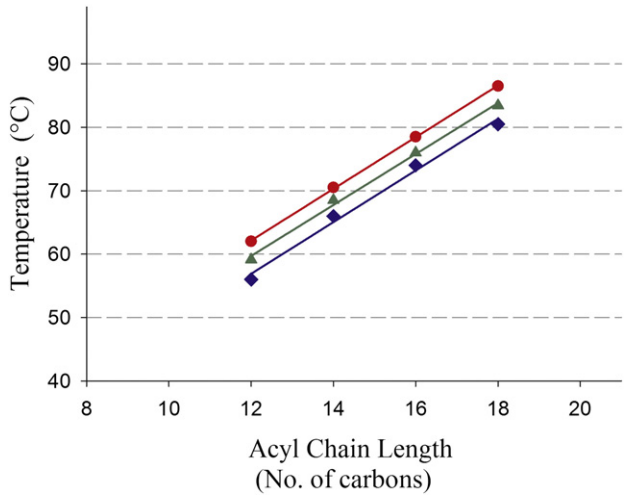

Fig. 5. Main phase transition temperatures [T_{onset} (♦),T_{offset} (●), and T_m (▲)] of saturated 1,2lmp derivatives in 40 mM tris buffer, pH 7.2, as a function of chain length. T_{onset} and T_{offset} for cationic lipid dispersions were obtained from the derivative data of fluorescence anisotropy (r) as a function of temperature, and T_m was obtained from fitting the results of fluorescence anisotropy (r) as a function of temperature to Eq. (2).

bond in each of its hydrocarbon chains. In addition, the low T_m is a direct consequence of the presence of these cis double bonds in the structure [39].

3.3. Surface potential measurements

To obtain complementary information about the polarity and ionization of 1,2lmp derivatives upon assembly into complexed structures, the change in the monolayer surface potential, ΔV, upon monolayer compression was measured and compared for the five lipids (Fig. 6). Since 1,2lmp derivatives form charged monolayers at the air–water interface, the measured surface potential is a sum of two terms, a dipolar term (μ) and an electrostatic one (ψ_o). For the first term, the surface potential is correlated with the dipole moments of the monolayer-forming molecules through the Helmholtz equation:

$$\Delta V = \frac{\mu_{\perp}}{\varepsilon \cdot \varepsilon_o \cdot A} \tag{4}$$

where $\mu_{\perp}$ is the effective dipole moment, ε is the dielectric constant of the medium involving this dipole, ε_o is the vacuum permittivity, and A is the area occupied by a molecule. However, calculation of $\mu_{\perp}$ from surface potential measurements is compromised by the inherent complexity and variability of ε at the monolayer/water interface. The varying contributions from the reorientation of water molecules due to the presence of the monolayer, from the polar head group, and from hydrophobic chain tails make any quantitative assessments of $\mu_{\perp}$ very difficult to make.

On the other hand, the ψ_o component of the surface potential can be estimated using the Gouy–Chapman double layer theory

$$\Psi_o = \frac{2kT}{e} \sinh^{-1}\left[\frac{e\alpha}{A \cdot (5.88 \times 10^{-7} \cdot c \cdot \varepsilon \cdot T)^{1/2}}\right] \tag{5}$$

where k is the Boltzmann constant, T is temperature, e is the proton charge, α is the degree of ionization of the head group, c is the ionic strength of the subphase, A is the area per molecule and ε is the dielectric constant in the double-layer region.

1,2lmp lipids share identical head groups (Fig. 1) and all the conditions during monolayers compression, e.g., temperature and subphase composition were kept constant. Therefore, changes in ΔV can be attributed to (1) changes in $\mu_{\perp}$ due to varying contribution from the various 1,2lmp acyl chains and (2) different degrees of ionization of the head group. As expected, it can be seen from Table 2 that decreasing the chain length of the saturated derivatives from 18 to 12 carbons was

Table 1
Phase transition temperatures of 1,2lmp derivatives as determined by fluorescence anisotropy.

Lipid	T_m (°C)	Goodness of fit statistics[a]		T_{onset} (°C)[d]	T_{offset} (°C)[d]	$T_{offset - onset}$
		C O D[b]	Corrl[c]			
1,2lmp[1]	59.14	0.9981	0.9990	56	62	6
1,2lmp[2]	68.51	0.9998	0.9999	66	70.5	4.5
1,2lmp[3]	76.0	0.9991	0.9996	74	78.5	4.5
1,2lmp[4]	83.45	0.9984	0.9991	80.5	86.5	6.5
1,2lmp[5]	21.28	0.9982	0.9991	12	28	16
DPPC	41.24	0.9884	0.9942	40	42	2

[a] Goodness of fit statistics for T_m were assessed within a 95% confidence interval.
[b] C O D: coefficient of determination.
[c] Corrl: correlation.
[d] Onset and offset transition temperature values were determined from the first derivative analysis presented in Fig. 4.

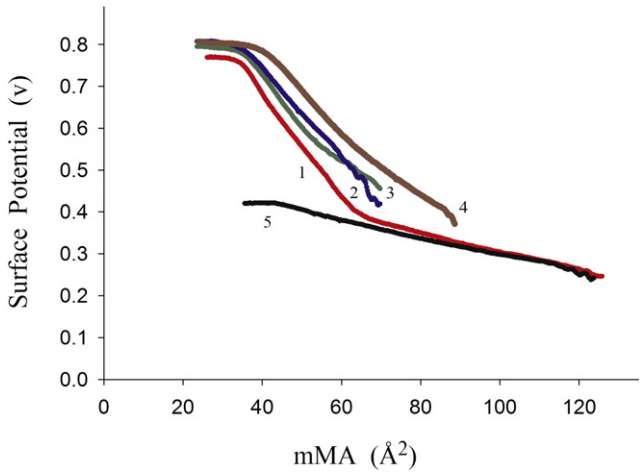

Fig. 6. Representative *surface potential–area* isotherms of 1,2lmp cationic derivatives at the air/water interface at 23 °C. Tris buffer (40 mM, pH 7.2) was used as the subphase. (1) 1,2lmp[1]; (2) 1,2lmp[2]; (3) 1,2lmp[3]; (4) 1,2lmp[4]; and (5) 1,2lmp[5].

accompanied by a gradual decrease in ΔV_{max} from 796.00 ± 8.76 to 755.33 ± 12.50 mV, indicating increased polarity of the molecules. Likewise, the polarity of 1,2lmp[5] will also increase due to increased polarity of the unsaturated acyl chains.

Nevertheless, that increase in molecular polarity of 1,2lmp[5] is not the main cause for the substantial drop in 1,2lmp[5] ΔV_{max} to 424.33 ± 2.89 mV. In fact, it may be attributed to the larger degree of ionization of 1,2lmp[5] as compared to its saturated counterparts. Insight on this increased ionization can be obtained by considering the packing of the lipid molecule when assembled into monolayers and eventually into bilayers. 1,2lmp[5] molecules occupied significantly larger mean molecular areas of 48.04 ± 2.79 Å^2 at ΔV_{max} as compared to the ~34.5 Å^2 for the saturated derivatives. This is in agreement with the previously reported mean molecular surface areas at monolayer collapse (51 versus 38 Å^2) obtained from corresponding π/A isotherms. The kinks in the acyl chain of 1,2lmp[5] introduced by the cis double bond prevent tight packing of lipid molecules which allow improved hydration and higher degree of ionization of its primary amine head group.

3.4. Gel electrophoresis retardation assay

In general, the first step of the lipofection process is considered to be the electrostatic interaction of the cationic lipids with DNA and the subsequent formation of the lipoplexes. It is of utmost importance to characterize different processes that take place during this step such as, DNA encapsulation, condensation, dehydration, as well as the influence of various formulation components on the efficiency of these processes. The quality of the resultant lipoplexes in terms of their colloidal physicochemical properties will determine their biological activity, and both will dramatically vary by slight modification of the formulation components such as helper lipid, molar ratio, ionic strength, etc.

The gel electrophoresis retardation assay is a useful mean to examine the effectiveness with which cationic lipid formulations can complex plasmid DNA. Retardation of the electrophoretic migration of DNA in the gel is indicative of the association between plasmid DNA and the cationic liposomes and the subsequent complexation and lipoplex formation. The ability of 1,2lmp cationic lipids to complex DNA in the absence and presence of DOPE in DMEM is illustrated in Fig. 7. In the absence of DOPE, the saturated derivatives did not induce efficient complexation of DNA as evident by the similar electrophoretic migration of plasmid DNA to the reference naked DNA control. The dioleoyl derivative on the other hand exhibited improved association and complexation with plasmid DNA and produced complete retardation at N/P molar ratio of 6. Incorporation of DOPE in the lipoplex formulation of 1,2lmp[5] did not cause any further improvement in its DNA retardation efficiency. However, it has a profound effect on the lipoplexes prepared with the lauroyl derivative 1,2lmp[1]. Their electrophoretic migration profile was found to mimic that of 1,2lmp[5]. Apparently, the helper lipid improved the efficiency of DNA complexation of 1,2lmp[1] which might explains the slight activity observed for this formulation.

Table 2
Summary of surface potential data of 1,2lmp amphiphilic series[a].

	MmA (Å^2) at max ΔV	Max ΔV (mV)
1,2lmp1	34.02 ± 2.40	755.33 ± 12.50
	69.15 ± 3.14[b]	380.33 ± 3.79
1,2lmp2	33.87 ± 1.71	788.50 ± 2.12
1,2lmp3	34.76 ± 0.71	785.00 ± 8.49
1,2lmp4	35.25 ± 0.49	796.00 ± 8.76
1,3lmp5	48.04 ± 2.79	424.33 ± 2.89

MmA denotes mean molecular area expressed in Å^2 per molecule.
[a] Measured in 40 mM tris buffer, pH 7.2, 23 °C.
[b] Phase transition denoted by a maximum in the dV/dA versus area plot.

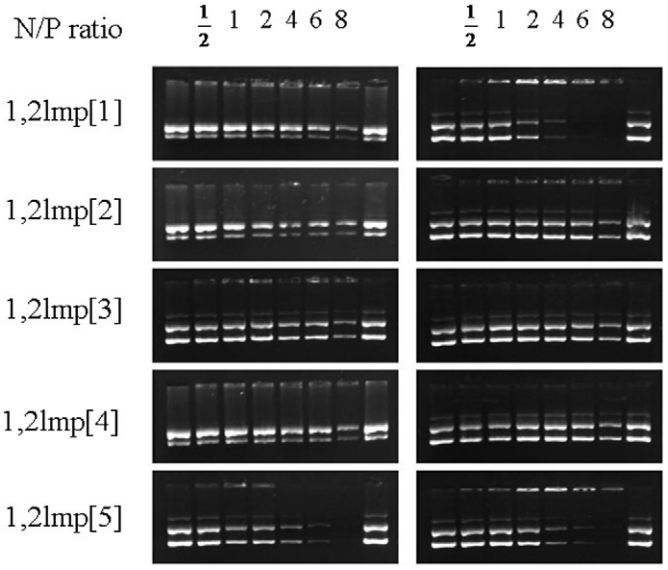

Fig. 7. Representative gel-electrophoresis pictures of lipoplexes prepared with various 1,2lmp formulations in serum-free media. Outermost left and right lanes are naked DNA. Left panel: Lipoplexes prepared with cationic lipids alone. Right panel: Lipoplexes prepared with cationic lipid/DOPE (6:4 molar ratios).

3.5. Eth-Br fluorescence quenching assay

The electrostatic interactions involved during the formation of 1,2lmp lipoplexes were further investigated by the Eth-Br fluorescence quenching assay. The reduction of the fluorescence signal of the intercalated Eth-Br probe upon titration with cationic dispersions allows accurate quantification of the cationic lipid-induced compaction of plasmid DNA. Interestingly, the Eth-Br fluorescence quenching profiles of various 1,2lmp formulations presented in Fig. 8 confirm the findings of the agarose gel electrophoresis retardation assay described above. The lipofection efficient 1,2lmp[5] derivative induced 67% condensation of plasmid DNA at N/P molar ratio of 2.2. That percentage slightly

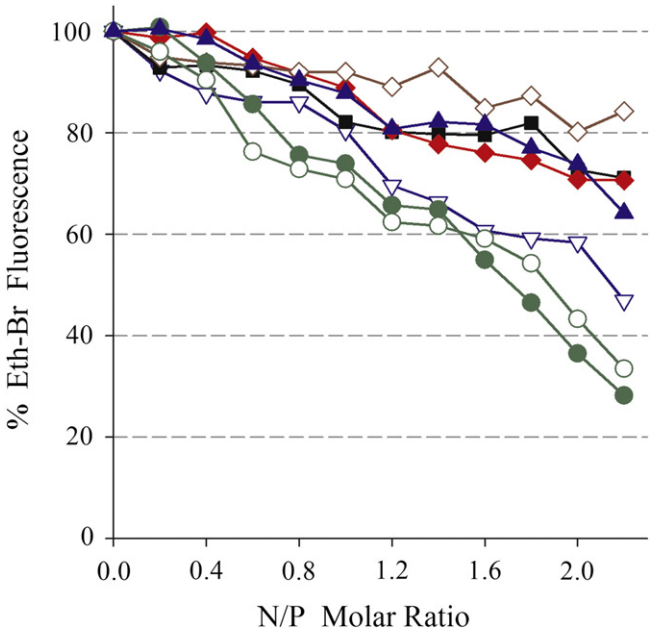

Fig. 8. Cationic lipid induced DNA condensation in serum-free DMEM medium monitored by the Eth-Br fluorescence quenching assay: 1,2lmp[1] (▲),1,2lmp[1]/Dope (▽), 1,2 lmp[2] (■), 1,2lmp[3] (◆), 1,2lmp[4] (◊), 1,2lmp[5] (●) and 1,2lmp[5]/Dope (○).

improved upon the inclusion of DOPE (72%). Much lower condensation of plasmid DNA was observed with the saturated lipids in absence of DOPE that ranged from 38% for 1,2lmp[1] to 20% for 1,2lmp[4]. Inclusion of DOPE did improve the plasmid DNA condensation efficiency of 1,2lmp[2–4] (data not shown). However, it increased the efficiency of 1,2lmp[1] to 55% at N/P molar ratio of 2.2. Finally, no representative data could be collected beyond 2.2 N/P molar ratio because of the severe aggregation and erroneous light scattering that took place.

4. Discussion

Improving the lipofection efficiency of cationic lipoplexes is necessary for the development of this type of colloidal dispersions to become a clinically-effective gene medicine. Toward that end, the physicochemical properties of cationic lipids in isolation that are associated with efficient lipofection functionality of these lipids must be identified. In the present work, the N,N′-diacyl-1,2-diaminopropyl-3-carbamoyl-(aminoethane) derivatives were used as model cationic lipids to investigate the correlation between cationic lipid's physicochemical properties and their lipofection activity. Furthermore, the electrostatic interactions involved in the formation of lipoplexes with these lipids were evaluated. In these experiments, a particular interest was toward evaluating these interactions in serum-free medium as the complexing medium. In addition, the effect of including the helper lipid DOPE in the lipoplex formulation on the aforementioned interactions was investigated.

Only the active lipid formulations of 1,2lmp[1]/DOPE, 1,2lmp[5], and 1,2lmp[5]/DOPE were found to be able to complex and condense plasmid DNA in DMEM serum-free medium. Insight on the plasmid DNA-induced complexation and compaction and the subsequent lipofection activity can be obtained from the fundamental understanding of the factors that influence these processes. Among these factors, the membrane fluidity of cationic liposomes prepared from the different 1,2lmp derivatives and their ionization state were characterized thoroughly.

The significance of membrane fluidity arises from the fact that several processes during the lipofection process will not efficiently proceed unless the temperature is above the phase transition temperature of the lipid bilayer. Examples of these processes include homogeneous incorporation of helper lipid during the preparation of cationic liposomes, formation of lipoplexes and escape from endosomal degradation. Rigidity of the cationic lipid will result in phase separation of the different lipid components of the liposomal formulation upon storage. As mentioned earlier, it has been demonstrated that significant restructuring of cationic lipid and fusion of liposomes occurs during the interaction of cationic liposomes with plasmid DNA. Therefore, only cationic lipids with phase transition temperatures below the experimental temperature will promote efficient complexation and condensation of plasmid DNA during the lipoplex formation process. In addition, interaction and fusion of lipoplexes with biological membrane, such as endosomal membrane, and the subsequent translocation of DNA cargo into the cytoplasm, are essential for efficient in vitro transfection. Rigidity of the lipid bilayer is believed to suppress the fusogenic events involved during this process thereby, resulting in degradation of lipoplexes in the endosome and failure to induce significant in vitro transfection.

In the present work, fluorescence anisotropy measurements indicated that only 1,2lmp[5] lipoplexes are expected to exist in a liquid crystalline state at physiological temperature. Moreover, the extent of ionization of this lipid in assemblies is significantly higher that it's saturated analogues as suggested by the surface potential measurements. All the currently used cationic lipids, with a few exceptions, require the presence of the neutral phospholipid DOPE for high transfection activity [9,10]. DOPE is known to facilitate fusogenic events that take place during the association and encapsulation of DNA by cationic lipids as well as promoting the destabilization of endosomal membrane, thereby facilitating the release of DNA into the cytoplasm and improving marker gene expression levels. These roles of DOPE may partly explain the efficiency of plasmid DNA complexation and condensation promoted by 1,2lmp[1]/DOPE formulation. Although that efficiency was not translated into significant lipofection activity, it is still an interesting phenomenon. This efficiency has to be promoted by significant ionization of 1,2lmp[1] in 1,2lmp[1]/DOPE liposomes as compared to other saturated derivatives. All the saturated derivatives exhibited similar ΔV as seen in Table 2. Apparently, the highly rigid saturated derivatives 1,2lmp[2–4] could not incorporate DOPE in their cationic dispersions and thus failed to induce any association with plasmid DNA. On the other hand, effective incorporation of DOPE was possible with shortest derivative 1,2lmp[1]. Incorporation of DOPE at 40% molar ratio is expected to exert sufficient dilution effect to prevent tight packing of 1,2lmp[1] molecules. This will allow the head group of 1,2lmp[1] to become protonated to a higher extent in the presence of DOPE. In spite of the improved ionization of 1,2lmp[1] and it's association with plasmid DNA, the lack of sufficient membrane fluidity prohibited efficient lipofection with this lipid.

5. Conclusion

In summary, the current findings suggest that efficient association and encapsulation of plasmid DNA by cationic lipids are required for improved in vitro transfection activity. These processes can be only promoted by cationic lipids that are characterized by the following: (1) acyl chain fluidity indicated by a main phase transition temperatures of bilayers below 37 °C, (2) in the case of ionizable lipids, sufficient protonation indicated by low surface potential. In addition, DNA complexation and condensation studies indicated that inclusion of the helper lipid DOPE in the formulation resulted in improved association between cationic lipids and plasmid DNA. The improved association was translated into a significant boost of the lipofection activity of the cationic lipids/DOPE formulations.

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