Ionization and transfection activity of n-methyl-substituted carbamoyl-cholesterol derivatives

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ABSTRACT

Five novel cationic lipids, the polar head group of which was attached to the cholesterol backbone via a tertiary carbamate linker, were synthesized and their physicochemical properties were compared to their transfection efficiencies. Transfection activity of the primary amine analog was highest among the series, while the quaternary ammonium iodide salt was essentially transfection incompetent. Contrary to DC-Chol, methyl and ethyl carbamoyl derivatives of DC-Chol mediated high levels of transfection in the absence of DOPE. Ionization of the cationic assemblies in 40 mM Tris buffer pH 7.2 exactly correlated with the competitive nature of the inductive and steric effects of the methyl groups on the aliphatic nitrogen of the lipids' polar moiety. Interestingly, the pH interaction zone of all lipid dispersions at 25˚C was extended by ±2 pH units from the pKa, while the pKa of the cationic lipids determined in mixed vesicles composed of 90% DOPC and cholesterol was approximately 1.3 to 1.5 times higher than that of pure cationic assemblies. The interaction of cationic lipids with plasmid DNA was correlated with pKa, but not the transfection activity.

Keywords: Cationic Lipids; DC-Chol; Transfection; Pka; Plasmid DNA; Monolayer; Surface Pressure

1. INTRODUCTION

Cholesterol-based cationic lipids are one of the most promising series of reagents for *in vitro* and *in vivo* gene delivery [1-4]. Over the years, these structures were systematically varied in an effort to generate structure-activity relationships to rationalize their design and effectively optimize their transfection efficiency [5-8]. Most of the approaches focus on attaching a hydrophilic basic moiety capable of binding to the negatively char-

ged nucleic acid, to the membrane compatible cholesterol moiety via a chemical linker. Quite frequently, the polar head group of these lipids is composed of a primary, secondary, tertiary or quaternary amine and almost always these lipids require the presence of the helper lipid DOPE in order to mediate transfection. DOPE is a zwitterionic amphiphil that forms reverse hexagonal phases (H_{II}) at physiological pH and is widely believed to promote fusion with cell membranes, facilitate endosomal escape, reduce the toxicity and increase the transfection activity of lipoplexes [9-13].

In our recent report, the physicochemical properties of cholesterol-based cationic lipids carrying a secondary carbamate linker were correlated with their transfection activity in the absence of helper lipids. The study found that the tertiary and quaternary amine derivatives, DC-Chol and TC-Chol (herein termed DC and TC), failed to mediate transfection and further concluded that the high transfection activity mediated by the primary and secondary analogs AC and MC in the absence of DOPE, was due to their increased association with plasma membrane and endosomal escape [14]. The aim of this investigation was to examine how methylation on the carbamoyl nitrogen affects the lipids' ionization, interaction with plasmid DNA and transfection activity.

2. MATERIAL AND METHODS

N-methylethylenediamine 95%, N,N'-dimethylethylenediamine 99%, N,N,N'-trimethylethylene-diamine 97%, N,N-dimethyl-N'-ethylethylenediamine 98%, Cholesteryl chloroformate 97%, iodomethane 99.5%, 2-hydroxyethylmercaptan, 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). Tris-(hydroxymethyl)-aminomethane (ultrapure grade) and edetate disodium dihydrate (EDTA) were purchased from Spectrum Chemical Manufacturing Company (New Brunswick, NJ). Murine melanoma cells B16F0 and Dulbecco's modified
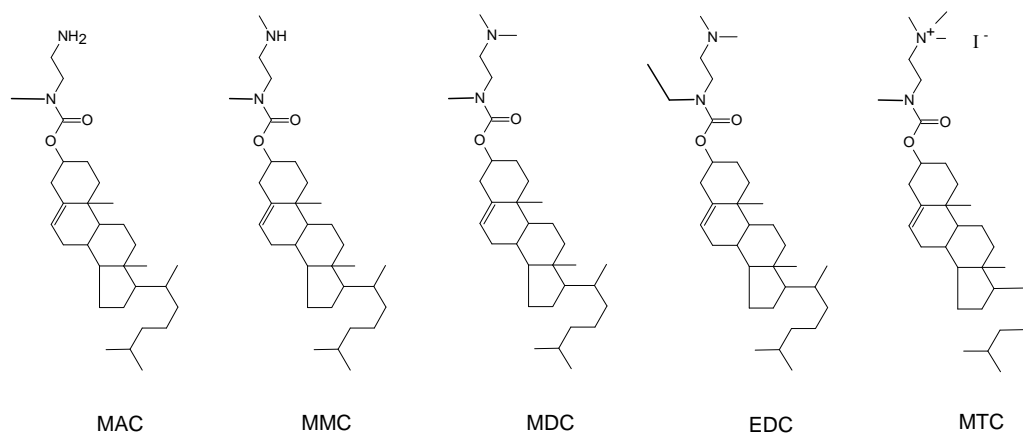
Scheme 1. Cholesterol-based cationic lipids.

Eagle's medium (DMEM) were obtained from American Type Culture Collection (Manassas, VA). Fetal calf serum, penicillin (5 000 units)/streptomycin (5,000 μg) and ethidium bromide solution (10 mg·mL^{-1}) were obtained from Invitrogen Corporation (Grand Island, NY). 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

2.1. Synthesis

The cholesterol-based analogues MAC, MMC, MDC and EDC (Scheme 1) were synthesized from N-methyle-thylenediamine, N,N'-dimethylethylenediamine, N,N,N'-trimethylethylene-diamine and N,N-dimethyl-N'-ethyle-thylenediamine, respectively, at yields > 75 %, as described elsewhere [14]. The quaternary ammonium derivative MTC was synthesized by refluxing MDC in the presence of an equimolar concentration of methyl iodide for 2 h.

3β-[2-(aminoethyl)-methylcarbamoyl]-cholesterol (MAC)

Anal. Calcd for $C_{31}H_{54}N_2O_2$ (MW 486.42): C, 76.42; H, 11.09; N, 5.75, Found: C, 75.44; H, 11.09; N, 5.68, MS (Positive/Negative ES) m/z 487.43 [M+H]$^+$; ^{1}H NMR (400 MHz, CdCl$_3$, 20˚C, TMS) δ 0.68 (s, 3H, H'18), 0.85 - 0.88 (d, 6H, H'26 - H'27), 0.90 - 0.92 (d, 3H, H'21), 1.02 (s, 3H, H'19), 1.04 - 1.59 (m, 21H, H'1, H'9, H'11 - H'12, H'14 - H'17, H'20, H'22 - H'25), 1.81 - 2.02 (m, 5H, H'2, H'7, H'8), 2.30 - 2.35 (m, 2H, H'4), 2.83 - 2.94 (m, 5H, H$_2$NCH$_2$, OCON(CH$_3$)CH$_2$), 3.31 - 3.32 (m, 2H, OCON(CH$_3$)CH$_2$), 4.53 (m, 1H, H´3), 5.37 - 5.38 (d, 1H, *J* = 5, H'6).

3β-[[2-(methylamino)ethyl]-methylcarbamoyl]-cholesterol (MMC)

Anal. Calcd for $C_{32}H_{56}N_2O_2$ (MW 500.4): C, 76.68; H, 11.18; N, 5.59, Found: C, 75.34; H, 11.04; N, 5.49, MS (Positive/Negative ES) m/z 501.3 [M + H]$^+$; ^{1}H NMR (400 MHz, CdCl$_3$, 20˚C, TMS) δ 0.67 (s, 3H, H´18), 0.85 - 0.88 (d, 6H, H'26-H'27), 0.90 - 0.92 (d, 3H, H'21), 1.02 (s, 3H, H'19), 1.04 - 1.63 (m, 21H, H'1, H'9, H'11 - H'12, H'14 - H'17, H'20, H'22 - H'25), 1.78 - 2.02 (m, 5H, H'2, H'7, H'8), 2.26 - 2.38 (m, 2H, H'4), 2.46 (s, 3H, NH(CH$_3$)), 2.74 (m, 2H, (CH$_3$)HNCH$_2$), 2.93 (s, 3H, OCON(CH$_3$)CH$_2$), 3.38 (m, 2H, OCON(CH$_3$)CH$_2$), 4.51 (m, 1H, H'3), 5.37 - 5.38 (d, 1H, *J* = 5, H'6).

3β-[[2-(dimethylamino)ethyl]-methylcarbamoyl]-cholesterol (MDC)

Anal. Calcd for $C_{33}H_{58}N_2O_2$ (MW 514.4): C, 76.92; H, 11.27; N, 5.44, Found: C, 76.93; H, 11.64; N, 5.33, MS (Positive/Negative ES) m/z 515.3 [M + H]$^+$; ^{1}H NMR (400 MHz, CdCl$_3$, 20˚C, TMS) δ 0.63 (s, 3H, H'18), 0.82 - 0.84 (d, 6H, H'26 - H'27), 0.87 - 0.90 (d, 3H, H'21), 0.97 (s, 3H, H'19), 1.00-1.60 (m, 21H, H'1, H'9, H'11-H'12, H'14 - H'17, H'20, H'22 - H'25), 1.75 - 1.98 (m, 5H, H'2, H'7, H'8), 2.22 (s, 6H, N(CH$_3$)$_2$), 2.36 - 2.40 (m, 4H, H'4, (CH$_3$)$_2$NCH$_2$), 2.87 (s, 3H, OCON(CH$_3$) CH$_2$), 3.20 - 3.36 (m, 2H, OCON(CH$_3$)CH$_2$), 4.48 - 4.52 (m, 1H, H´3), 5.36 - 5.38 (d, 1H, *J* = 5, H'6).

3β-[[2-(dimethylamino)ethyl]-ethylcarbamoyl]-cholesterol (EDC)

Anal. Calcd for $C_{34}H_{60}N_2O_2$ (MW 528.5): C, 77.15; H, 11.34; N, 5.29, Found: C, 76.05; H, 11.38; N, 4.79, MS (Positive/Negative ES) m/z 529.5 [M + H]$^+$; ^{1}H NMR (400 MHz, CdCl$_3$, 20˚C, TMS) δ 0.63 (s, 3H, H´18), 0.81 - 0.83 (d, 6H, H'26 - H'27), 0.86 - 0.88 (d, 3H, H'21), 0.97 (s, 3H, H´19), 1.08 - 1.58 (m, 21H, H'1, H'9, H'11 - H'12, H'14 - H'17, H'20, H'22 - H'25), 1.76 - 2.00 (m, 5H, H'2, H'7, H'8), 2.20 - 2.36 (m, 2H, H'4), 2.42 (s, 6H, N(CH$_3$)$_2$), 2.70 (m, 2H, (CH$_3$)$_2$NCH$_2$), 2.91 (bs, 5H, OCON(C$_2$H$_5$)CH$_2$), 3.38 (m, 2H, OCON(C$_2$H$_5$)CH$_2$), 4.48 - 4.52 (m, 1H, H´3), 5.36 - 5.38 (d, 1H, *J* = 5, H´6).

3β-[[2-(trimethylamino)ethyl]-methylcarbamoyl]-cholesterol iodide (MTC)

Anal. Calcd for $C_{34}H_{61}N_2O_2$ I (MW 656.4): C, 62.16; H, 9.45; N, 4.27, Found: C, 58.19; H, 9.37; N, 4.05, MS (Positive/Negative ES) m/z 529.4 [cation of salt], ^{1}H NMR (400 MHz, CdCl$_3$, 20°C, TMS) δ 0.68 (s, 3H, H'18), 0.85 - 1.74 (m, 33H, H'1, H'9, H'11 - H'12, H'14 - H'17, H'19 – H'27), 1.85 - 1.97 (m, 5H, H'2, H'7, H'8), 2.35 (m, 2H, H'4), 3.08 (bs, 3H, OCON(CH$_3$)CH$_2$), 3.50 (s, 9H, N(CH$_3$)$_3$), 3.80 - 4.04 (m, 4H, NCH$_2$CH$_2$N), 4.45 (m, 1H, H'3), 5.40 (d, 1H, J = 5, H'6).

2.2. Transfection and Cytotoxicity

The studies were conducted as described elsewhere [14]. Briefly, the plasmid DNA pUC19-β-gal was propagated in DH5α-competent cells using standard protocols [15] and purified by gel permeation chromatography using Sepharose 4B as the stationary phase and 2.5 M ammonium acetate as the mobile phase. Fractions containing plasmid DNA were pooled and precipitated with isopropyl alcohol. The precipitate was resuspended in a few mL of 40 mM TE buffer pH 8, dialyzed for 2 - 4 h at room temperature against 40 mM TE buffer to eliminate traces of ammonium acetate and quantified by its absorption at 260 nm (4.63 mg·mL^{-1}). The integrity and purity of the pDNA was verified by agarose gel electrophoresis and a 260 nm/280 nm absorption ratio of 1.8. B16F0 cells were cultured to confluence using DMEM supplemented with 10% fetal bovine serum, 50 units·mL^{-1} penicillin, 50 μg·mL^{-1} streptomycin in a 5% CO$_2$ at 37°C. Approximately 50,000 cells in 0.5 mL growth media were added to each well of 48 well plates. After 12 to 14 hours later, the serum media were removed and the cells were transfected with 250 μL lipoplexes per well. The amount of plasmid DNA was kept constant at 1 μg per well, while the amount of cationic lipid was increased appropriately to achieve cationic lipid to DNA charge ratios of 1 : 1, 2 : 1 and 4 : 1. The cells were incubated for 4 hours, after which time the lipoplexes were removed and the serum free media were replaced with complete growth media. After an additional incubation of 44 h, the cells were washed with cold phosphate-buffered saline (PBS) pH 7.4 twice and lysed with 150 μL lysis buffer (0.1 M Tris pH 7.2, 0.1% w/v Triton X) per well. Beta-galactosidase activity was quantified by an ONPG assay. The cytotoxicity of cationic lipids was evaluated by an MTT assay as described elsewhere [14].

2.3. EtBr Displacement Studies

A Cary Eclipse spectrofluorometer was used to assess the level of displacement of ethidium bromide (EtBr) from the pDNA (pUC19). Briefly, 0.8 μg of EtBr and 22.5 μg of pDNA were diluted to 3 mL in either Tris buffer pH 7.2 or in serum free media (SFM). Aliquots of 11.4 μl of cationic lipid (1.2 mM) were added to the solution until there was either a complete quenching of the EtBr or no apparent change in the fluorescence intensity. All samples were scanned at an excitation wavelength of 515 nm and the emission collected at the wavelength between 550 - 605 nm (excitation and emission slit widths were set at 5 nm). All experiments were conducted at 22°C. The EtBr emission intensity f, was plotted against the lipid to DNA charge ratio R, and the results were fitted either linearly or parabolically within a 95% confidence level. The interaction of MTC was shown to be a special case of cooperative interaction and was simulated by **Eq.1**:

$$f = f_{min} + \frac{f_{max} - f_{min}}{1 + \left(\dfrac{R}{R_{1/2}}\right)^n} \tag{1}$$

where f_{min} and f_{max} are the lowest and highest normalized fluorescence intensities, respectively, R, is the +/– charge ratio, $R_{1/2}$ is the charge ratio at which 50 % of plasmid DNA is neutralized or compacted by cationic lipid and the exponent n denotes the steepness of the curve. Data were fitted by the method of non-linear least-squares using the Microsoft Excel Solver function, through minimization of the square residuals with 3 adjustable parameters (f_{min}, f_{max} and $R_{1/2}$) at 5% tolerance.

2.4. Gel Retardation Assay

0.8% Agarose solution was prepared in 40 mM TAE (Tris base, glacial acetic acid and EDTA) pH 7.4, followed by the addition of 10 μL EtBr (10 mg mL^{-1}). The lipoplexes were prepared by keeping the pDNA amount constant at 0.2 μg while the amount of cationic lipid (0.6 mM) was varied to produce lipid to pDNA charge ratios of 0.5, 1, 2, 4, 6 and 8. Naked pDNA (0.2 μg) was used as a control in the first and last lanes. To each sample 1 μL of a 6X gel loading buffer (0.25% w/v bromophenol blue, 0.25% w/v Xylene cyanol FF, 30% v/v glycerol in water) was added and the volume was brought to 11 μL with 40 mM TAE buffer pH 7.4. The samples were allowed to sit for 10 - 15 minutes before they were loaded onto the wells of the agarose gel. Afterwards, an appropriate amount of running buffer (TAE) was used to submerge the gel and the gel was subjected to an electrophoretic field of 90 V for 35 min. The results of the gel electrophoresis were assessed using a Bio-Rad Mini Transilluminator.

2.5. Langmuir Monolayer Studies

Cationic lipid monolayers were studied at the air/water

interface using the Langmuir film balance technique as described elsewhere [16]. Compression isotherms of the cationic lipids were obtained using a Langmuir film balance (model KSV 1000, KSV instruments LTD, Finland) equipped with a Wilhelmy plate for measuring the surface pressure. The Teflon microtrough (24,225.0 mm^2) is equipped with two barriers that allow for symmetric compression. Each run was followed by a thorough wash of both trough and barriers with ethanol and a final rinse with distilled water. The Wilhelmy plate was heated over a blue flame for about 30 seconds before each run. In a typical experiment, 15 μL (30.0 nmol) of cationic lipid (2 mM dissolved in chloroform) was spread onto the subphase (40 mM Tris buffer pH 7.2) maintained at 22˚C, with a Hamilton® micro syringe and allowed to sit for 15 min before the start of each run. The barriers were compressed at a constant speed of 10 mm·min^{-1}. All experiments were conducted at a temperature of 22˚C, while each run was repeated at least three times for reproducibility. Independent and dependent variables collected by the instrument were imported into an Excel spreadsheet and the compressibility modulus, K, was calculated from the first derivative of the monolayer surface pressure-trough Area isotherms using **Eq.2**:

$$K = -A\left(\frac{\partial \Pi}{\partial A}\right)_T \tag{2}$$

Equilibrium parameters at the onset of monolayer collapse were determined at the peak point of the K-Π isotherm.

2.6. pK_a Studies

Buffer solutions (2 mL) made from 40 mM Tris and 40 mM MES having a pH from 1.2 to 13.2 were transferred into plastic cuvettes together with 0.1 mL of lipid dispersions (0.6 mM for cationic lipid (CL) alone and 2 mM for CL/DOPC/Chol 0.2/0.9/0.9 molar ratio) and 6 μL of TNS (0.1 mM) for CL alone and 20 μL TNS (0.1 mM) for CL/DOPC/Chol 0.2/0.9/0.9 mol/mol/mol). The lipid to probe molar ratio was kept constant at 100 : 1. The static fluorescence intensity of these samples was registered at an excitation wavelength of 321 nm (slit width 5 nm) and an emission wavelength of 445 nm (slit width 5 nm) on a Cary Eclipse fluorescence spectrophotometer at 25˚C. The emission intensity of TNS was plotted against the pH and the results were fitted using a modified version of the Henderson-Hasselbalch (Equation 3), using the

$$f = f_{min} + \frac{f_{max} - f_{min}}{1 + 10^{c \cdot (pH - pKa)}} \tag{3}$$

Microsoft Excel Solver through minimization of the sum of the squared residuals with four adjustable parameters at a 5% tolerance level [17]. The parameter, f is the

calculated TNS fluorescence, f_{max} and f_{min} are the maximum and minimum fluorescence values of TNS and c is a constant that adjusts the slope of the curve.

2.7. Particle Size and Electrophoretic Mobility Studies

A Malvern Zetasizer Nano Series instrument was used to determine the size and zeta potential of the pure cholesterol derivatives and corresponding lipoplexes at +/– charge ratios of 1 : 1, 2 : 1 and 4 : 1. Experiments were performed in both filtered Tris buffer (using 0.2 μm membrane filter) and SFM at 22˚C. Lipoplexes were prepared by adding pDNA into 200 μL of 0.6 mM lipid dispersion to produce the desired lipid to pDNA charge ratios and then diluted to 1.5 mL with Tris buffer (40 mM, pH 7.2) or SFM [17].

3. RESULTS AND DISCUSSION

3.1. *In Vitro* Transfection and Cytotoxicity Studies

Regardless of charge ratio, the MAC mediated highest transfection activity while the quaternary ammonium salt, MTC, exhibited the lowest potency among its peers (**Figure 1(a)**). The invariable β-gal expression levels by MAC could be due to decreased cell viability with increasing charge ratio. Impressively, unlike DC-Chol which is transfection incompetent in the absence of DOPE, the MDC and EDC that bear methyl and ethyl substitution at the carbamoyl nitrogen, respectively, mediated significant transfection activity proportional to charge ratio. More specifically, at +/– charge ratio of 1, the activity mediated by the cationic lipids decreased with polar head methyl substitution, while at +/– charge ratio of 2 and 4 the transfection activity mediated by MMC, MDC and EDC was essentially indistinguishable. MDC exhibited the lowest toxicity maintaining greater than 70% cell viability, whereas MMC was quite cytotoxic at all charge ratios (**Figure 1(b)**).

3.2. pK_a Studies

The pK_a of cationic lipids is conventionally determined in mixed lipid vesicles using the membrane potential indicator TNS [17-21]. In this investigation, an attempt was made to determine the apparent pK_a of cationic lipids from pure assemblies in the absence of any co-lipids. As shown in **Table 1**, the pK_a of the primary amine derivative MAC is slightly lower than that of the secondary amine derivative MMC. Further methyl substitution on the amine polar head drastically decreases the pK_a by one unit (MDC), while the apparent pK_a of the ethyl carbamoyl tertiary amine derivative EDC, is even lower reaching
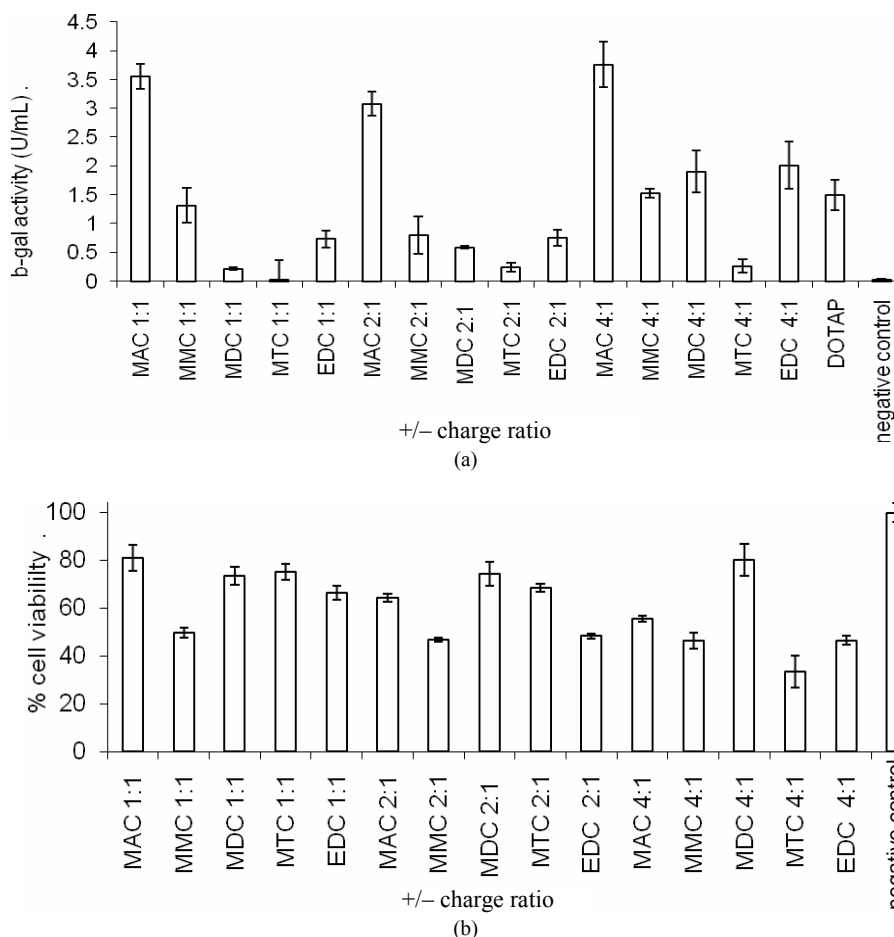
Figure 1. (a) Transfection activities and (b) Cytotoxicity studies of MAC, MMC, MDC, MTC and EDC at different +/− charge ratios in mouse melanoma cells (B16F0). Results are the average of three different experiments. In the control experiment, plasmid DNA was added to the cells without cationic lipids.

Table 1. Apparent pKa of cationic lipids determined at 25°C in pure cationic assemblies and in the presence of DOPC and cholesterol (CH) at a molar ratio of 2 : 9 : 9.

Composition	pK_a	Composition	pK_a
MAC	7.19 ± 0.06	MAC/DOPC/CH	8.83 ± 0.055
MMC	7.24 ± 0.06	MMC/DOPC/CH	8.82 ± 0.16
MDC	6.17 ± 0.09	MDC/DOPC/CH	7.54 ± 0.07
EDC	6.04 ± 0.03	EDC/DOPC/CH	7.37 ± 0.14
DC	6.76 ± 0.075	-	-

the value of 6.04. This trend, MMC > MAC > MDC > EDC can be explained by the lipid hydration that precedes the inductive effect [22,23]. Generally, the amine basicity is increased with methyl substitution due to the electron donation from the methyl groups. However, in aqueous solution, the increased electron density and basicity of the methyl substituted nitrogen is compromised by the low solvation energy of the increasingly hydrophobic substituted nitrogen. Interestingly, the pK_a of the DC is much higher than that of MDC (6.76 versus 6.17) but significantly lower than the structural isomer MMC.

Thus, moving the methyl group from the amine head to the carbamoyl group increases the pK_a by 0.5 units and converts an ineffectual lipid into a transfection potent one.

As with the pure assemblies, mixed assemblies containing primary and secondary amine derivatives were more basic by 1.3 and 1.5 pH units than those containing the tertiary amine derivatives MDC and EDC, respectively (**Figure 2** and **Table 1**). Furthermore, due to the increased molecular separation, the pK_a of all cationic lipids in mixed vesicles composed of 90% DOPC and
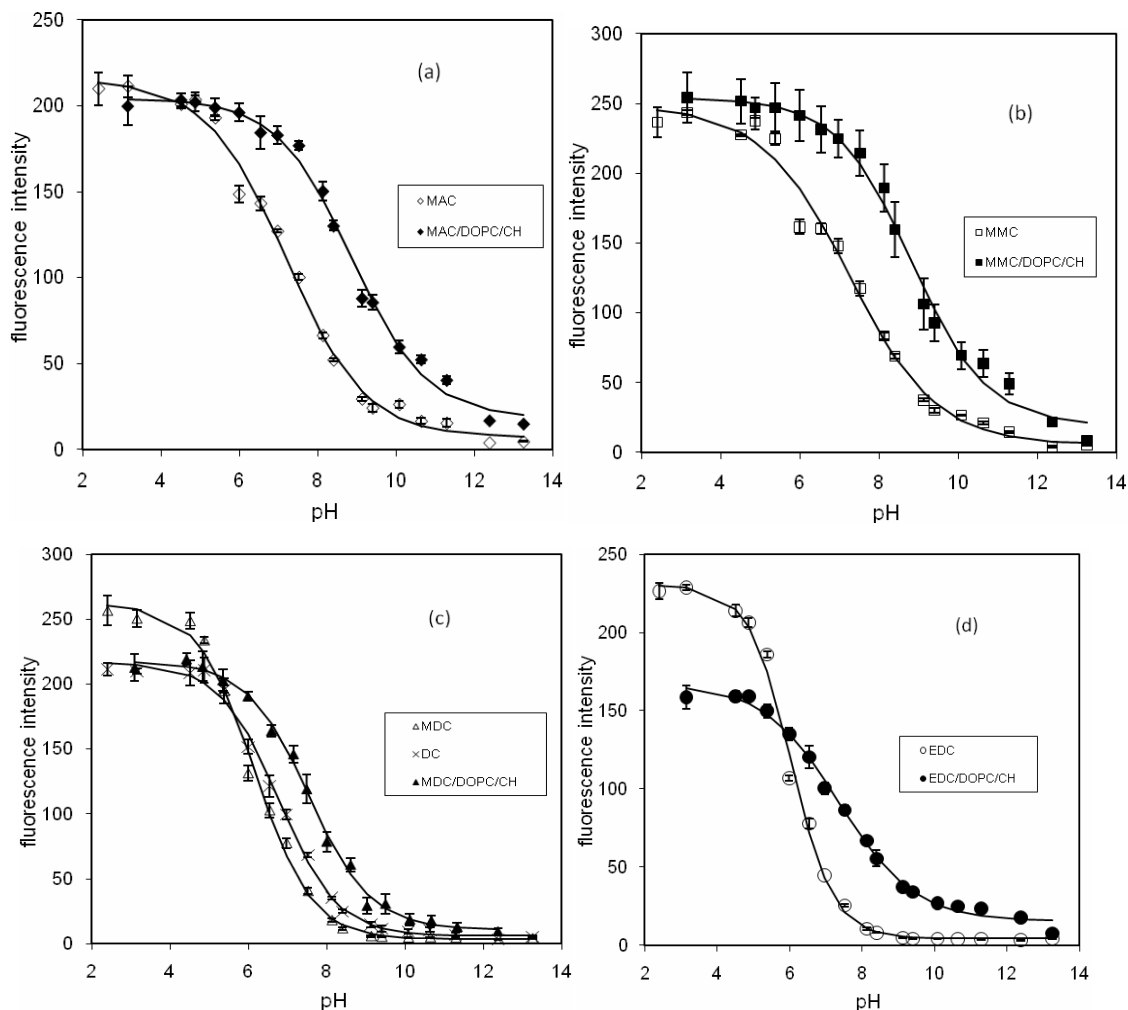
Figure 2. Fluorescence intensity of TNS as a function of bulk pH in pure and mixed cationic assemblies at 25˚C. Results are the average of three independent experiments with the error bars representing standard uncertainties.

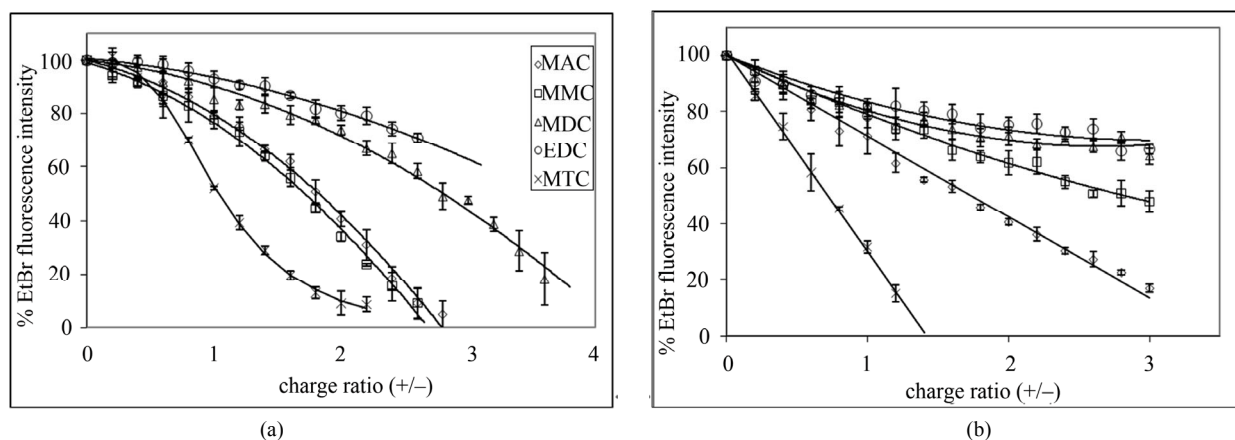**Figure 3.** EtBr displacement assay at 22˚C (a) in 40 mM Tris buffer pH 7.2; (b) in serum free media (SFM) of physiological ionic strength. Symbols are the average of 3 different experiments while solid lines represent data fitted by linear equations and parabolas (top panel). The interaction of MTC with plasmid DNA in 40 mM Tris pH 7.2 (top panel) was simulated by **Eq.2**, as described in Materials and Methods.
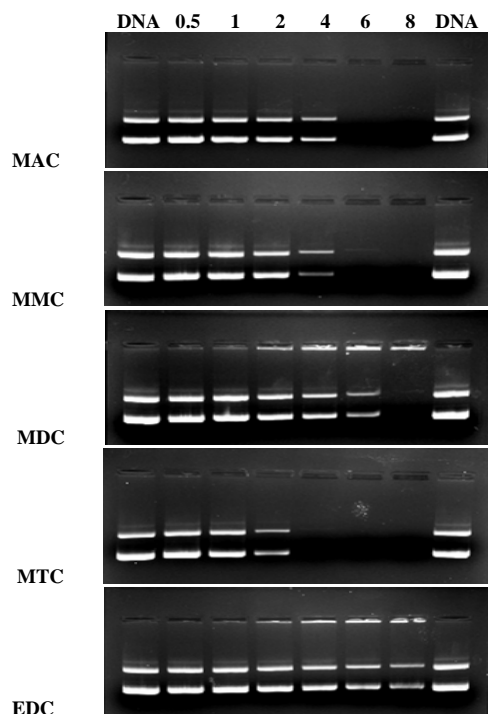
Figure 4. Representative agarose gel retardation studies in 40 mM Tris buffer pH 7.2 of MAC, MMC, MDC, MTC and EDC at different +/– charge ratios (no lipid, 0.5, 1, 2, 4, 6, 8, no lipid).

Table 2. Monolayer properties determined at the onset of monolayer collapse with 40 mM Tris buffer pH 7.2 as the subphase at 22°C. Standard uncertainties were calculated from three to five independent experiments. The % ionization of cationic lipids in the monolayers were calculated from the measured pKa values included in **Table 1**.

Cationic lipid	MmA ($\mathring{A}^2$)	Π (mN m^{-1})	K (mN m^{-1})	% ionization
MAC	36.6 ± 0.3	42.5 ± 2.4	212 ± 21	49.3 ± 3.4
MMC	37.6 ± 1	41.9 ± 0.4	233 ± 48	52.4 ± 3.4
MDC	35.7 ± 1.1	42.6 ± 1.7	151 ± 12	8.60 ± 2
EDC	33.7 ± 1	37.3 ± 2.1	126 ± 10	6.50 ± 0.4
MTC	44.6 ± 0.4	32.0 ± 0.9	123 ± 11	100

Table 3. (+/– charge ratio at which 50% of plasmid DNA is compacted in 40 mM Tris buffer pH 7.2 and in Serum Free Media (SFM) at 22°C.

Cationic lipid	40 mM Tris buffer	SFM
MAC	1.8	1.7
MMC	1.7	2.7
MDC	2.8	-
EDC	3.5	-
MTC	1.1	0.7

cholesterol increased by 1.3 to 1.6 units. However, what is really interesting is the fact that 90% ionization takes place within ± 2 pH units from the pK_a value and not 1 unit as predicted by the Henderson-Hasselbalch equation. This expansion of the buffering region of cationic cholesterol assemblies was not observed with double-chained monovalent cationic lipids (results not shown) and it definitely extends the range of pH at which cationic lipid-DNA interaction occurs.

Interestingly, the transfection efficiency decreased with the increase in the methylation on the terminal nitrogen while it surprisingly increased with the increase in alkyl chain of the carbamoyl nitrogen. This trend reveals that higher ionization of a cationic lipid is not always correlated with a higher transfection activity.

3.3. Monolayer studies

As shown in **Table 2**, the trend in the mean molecular areas (MmA) of cationic lipids at the onset of collapse pressure is MTC > MMC > MAC > MDC > EDC. Looking at the MDC and EDC derivatives, the mean surface area decreased from 35.7 Å^2 molecule^{-1} for MDC to 33.7 Å^2 molecule^{-1} for EDC. Tighter packing of EDC as compared to MDC molecules is due to the increased van der Waals forces and the lower pK_a, both a consequence of the increased hydrophobicity of the molecule which is also reflected in lower monolayer collapse pressure and

magnitude of the compressibility modulus, K.

The trend in the MmA of cationic lipids as a function of amino group methyl substitution (MTC > MMC > MAC > MDC) verifies that cationic lipid molecular dimensions are directly proportional to the pK_a and degree of ionization in Tris buffer pH 7.2. Thus, although MDC is a bulkier tertiary amine, the increased ionization of the primary amine MAC at pH 7.2 (49% versus 9%; **Table 2**), encourages greater hydration and repulsion among the aligned molecules in the interface, thus effectively increasing its molecular dimensions as determined just before the monolayer collapse.

3.4. EtBr Displacement and Exclusion Studies

To better understand the transfection activity profile of the cationic lipids, their ability to bind and compact pDNA was assessed using the EtBr displacement assay. The cationic lipids displaced EtBr from pDNA in 40 mM Tris pH 7.2 in the following order: MTC > MMC > MAC > MDC > EDC (**Figure 3(a)**). Thus, the more basic the lipid and the more expanded the molecular dimensions, the more efficient is its interaction with plasmid DNA. This trend was also observed with a gel retardation assay, although none of the lipids was able to completely retard the pDNA at the exact ratio as determined in the EtBr displacement assay (**Figure 4**). In the high ionic strength serum free media (SFM), the interaction cooperativity observed in 40 mM Tris was lost and the order of interaction was changed to MTC > MAC >

MMC. MDC and EDC failed to significantly condense the plasmid DNA (**Figure 3(b)**). Most importantly, the charge ratio at which 50% of EtBr is displaced from pDNA decreased from 1.1 in 40 mM Tris to 0.7 for MTC, remained the same around 1.7 to 1.8 for the MAC and increased from 1.7 to 2.7 for MMC (**Table 3**). Evidently, the increased ionic strength of the media did not adversely affect the electrostatic interaction of the more hydrophilic quaternary ammonium salt MTC and primary amine derivative MAC, but it seriously reduced the reactivity of the more lipophilic secondary and tertiary amine derivatives MMC and MDC and EDC, respectively, due to reduced hydration.

3.5. Particle Size and Electrophoretic Mobility Studies

Cationic lipid dispersions in the absence of pDNA were in general below 200 nm in 40 mM Tris buffer pH 7.2. The smallest particle diameter was exhibited by the quaternary ammonium derivative MTC presumably due to increased ionization and polar head expansion. Owing to reduced hydration, the particle size of cationic lipid dispersions and corresponding zeta potential increased and decreased, respectively, in the high ionic strength SFM (**Table 4**). The lipoplexes exhibited a very different, but predictable, zeta potential and particle size distribution (**Table 5**). Lipoplex particles were slightly bigger than the size of cationic lipid dispersions in the absence of pDNA in 40 mM Tris and SFM, until the point of charge neutrality where a big jump in the lipoplex size was observed. Specifically, as previously determined by the EtBr displacement assay, complete charge neutralization occurs at +/– charge ratio of 2 and 4 for MTC and MAC and MMC, respectively. A drastic increase in lipo-

Table 4. Hydrodynamic characterization of lipid dispersions (in the absence of DNA) carried out in 40 mM Tris buffer pH 7.4 and serum free media (SFM) at 22˚C. Standard uncertainties were calculated from the average values measured from three separate experiments.

Composition	Hydrodynamic Diameter (nm)		PDI		Zeta Potential (mV)	
	40 mM Tris	SFM	40 mM Tris	SFM	40 Mm Tris	SFM
MAC	160 ± 6	258 ± 78	0.26 ± 0.02	0.15 ± 0.01	+53 ± 3	+19.5 ± 1
MMC	113 ± 7	160 ± 9	0.24 ± 0.01	0.26 ± 0.02	+55 ± 1	+24 ± 2
MDC	169 ± 14	183 ± 11	0.39 ± 0.07	0.36 ± 0.04	+45 ± 1.5	+21 ± 3
EDC	203 ± 26	302 ± 83	0.45 ± 0.03	0.40 ± 0.01	+45 ± 2.6	+22 ± 2
MTC	77 ± 5	98 ± 3	0.26 ± 0.04	0.24 ± 0.02	+31 ± 7	+23 ± 1

Table 5. Particle size and zeta potential of lipoplexes at various charge ratios in Tris 40 mM buffer pH 7.2 and in Serum Free Medium (SFM) at 22˚C. Standard uncertainties were calculated from the average values measured from three independent experiments.

Cationic lipid	+/– charge ratio	Hydrodynamic Diameter (nm)		Polydispersity index		Zeta Potential (mV)	
		40 mM Tris	SFM	40 mM Tris	SFM	40 mM Tris	SFM
MAC	1/1	204 ± 4	199 ± 7	0.26 ± 0.01	0.20 ± 0.01	−52 ± 2.3	−27 ± 3
MAC	2/1	328 ± 40	247 ± 14	0.30 ± 0.03	0.22 ± 0.01	−51 ± 1.5	−31 ± 3
MAC	4/1	1173 ± 714	731 ± 266	0.71 ± 0.4	0.34 ± 0.1	−6.35 ± 31.3	−33 ± 4
MMC	1/1	200 ± 59	250 ± 25	0.25 ± 0.04	0.26 ± 0.01	−50 ± 2.6	−29 ± 2
MMC	2/1	174 ± 32	273 ± 14	0.18 ± 0.07	0.25 ± 0.02	−49 ± 1.5	−31 ± 3
MMC	4/1	1137 ± 68	658 ± 166	0.53 ± 0.04	0.32 ± 0.16	+36 ± 4	−35 ± 1.3
MDC	1/1	229 ± 23	260 ± 25	0.28 ± 0.01	0.26 ± 0.02	−55 ± 2	−30 ± 2.3
MDC	2/1	236 ± 26	304 ± 31	0.31 ± 0.05	0.32 ± 0.08	−55 ± 2.6	−31 ± 2
MDC	4/1	411 ± 26	383 ± 35	0.27 ± 0.03	0.28 ± 0.07	−52 ± 0.4	−31 ± 2
EDC	1/1	454 ± 31	418 ± 112	0.19 ± 0.03	0.26 ± 0.09	−61 ± 2.6	−29 ± 2
EDC	2/1	323 ± 78	448 ± 149	0.26 ± 0.03	0.20 ± 0.05	−60 ± 2.6	−31 ± 0.2
EDC	4/1	421 ± 172	484 ± 116	0.20 ± 0.08	0.29 ± 0.01	−59 ± 2	−33 ± 2
MTC	1/1	270 ± 97	737 ± 437	0.37 ± 0.1	0.54 ± 0.16	−38 ± 0.6	−25 ± 2
MTC	2/1	1350 ± 1077	3782 ± 925	0.70 ± 0.2	0.62 ± 0.15	−30 ± 11	−18 ± 6.5
MTC	4/1	172 ± 13	475 ± 236	0.33 ± 0.05	0.41 ± 0.06	+39 ± 1	+26 ± 1

plex particle size was observed close to these charge ratios (**Table 5**). Charge neutralization in lipoplexes composed of these cationic lipids was verified in 40 mM Tris pH 7.2, where the zeta potential reverses sign and becomes positive or it is very close to zero as the charge ratio increases from 1 : 1 to 2 : 1 and 4 : 1.

In summary, it was found that increased hydration of cholesterol-based cationic lipids correlates with increased ionization, increased polar head group dimensions due to higher repulsive forces among the lipids and greater interaction with plasmid DNA, as shown by EtBr displacement and exclusion assays. Thus, hydration was found to be the primary factor controlling ionization of cationic lipids and their interaction with DNA. Factors affecting the hydration of the cationic lipids, such as increased ionic strength, were shown to adversely affect primarily the ionization and DNA interaction of the more hydrophobic tertiary (MDC, EDC) and secondary (MMC) amine derivatives. Furthermore, ionization and pK_a of the cholesterol-based cationic lipids increased tremendously by 1.3 to 1.5 pH units in the presence of other lipids due to increased spacing, improved hydration and reduced electronic overlap. No correlation was found between the pK_a of cationic assemblies and the transfection activity. Also, the similarity in lipoplex particle size and zeta potential precludes predicting a cationic lipid trend in mediating transfection activity. However, comparison of this work with our published report on secondary carbamate isomers AC, MC, DC and TC [14] substantiates the following: Firstly, methylating or ethylating the carbamoyl nitrogen of DC decreases the pK_a of cationic assemblies and their interaction with plasmid DNA, but it substantially increases their transfection activity in the absence of the helper lipid DOPE. Secondly, the transfection activity of the tertiary carbamate analog MMC is significantly lower than that of the secondary carbamate MC, presumably due to toxicity issue. Thirdly, the transfection activity of the primary amine derivatives, AC and MAC, was highest, while the quaternary ammonium derivatives, TC and MTC were essentially transfection incompetent despite their highly efficient interaction with plasmid DNA.

4. CONCLUSIONS

Our success of measuring the apparent pK_a of pure cationic assemblies and demonstrating that membrane ionization in pure cationic assemblies is much lower than that in mixed membranes was followed by the unfortunate realization that transfection activity is not correlated with the pK_a of basic cationic lipids. The appropriate test to build structure-activity relationships is still to be discovered, but as our studies indicated, the key lies in the three analogs MMA, DC and MDC which their physico-

chemical properties and their transfection activities differ drastically from each other. Rather a delicate balance between the hydrophilicity and hydrophobicity of these cationic lipids must be controlling their transfection potency.

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