Synthesis and Properties of Novel Tetraalkyl Cationic Lipids

Jérôme Gaucheron,*,† Tabitha Wong,† Kim F. Wong,† Norbert Maurer,† and Pieter R. Cullis†,‡

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3 and Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8. Received December 20, 2001

The synthesis, physical properties, and transfection potencies of two representives of a new class of divalent, tetraalkyl cationic lipids is described. These cationic lipids are dimers of *N*,*N*-Dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) joined by a hydrocarbon tether three or six carbons in length (TODMAC3 and TODMAC6, respectively). It is shown that TODMAC6 can display improved transfection properties in comparison to DODAC when formulated into plasmid DNA−cationic lipid complexes. These improved transfection potencies are observed at cationic lipid to DNA charge ratios of two or higher. It is also shown that TODMAC6 exhibits equivalent or improved ability (as compared to DODAC) to induce nonbilayer structure in mixtures with anionic lipid. This is consistent with the hypothesis that the ability of cationic lipids to induce nonbilayer structures when mixed with anionic lipids is correlated to their transfection potency. Complexes containing TODMAC3 on the other hand exhibit lower transfection potencies than achieved with DODAC, behavior that is consistent with steric effects limiting the formation of ion pairs with anionic lipids. It is concluded that TODMAC6 exhibits potential as a transfection agent for in vitro and in vivo use and that the design of cationic lipids according to their ability to induce nonbilayer structure provides a useful guide for synthesis of new cationic lipids.

INTRODUCTION

Plasmid DNA-cationic lipid complexes are the leading nonviral gene therapy vectors used in the clinical setting (*1−4*). However the levels of gene expression that can be achieved using these complexes are substantially lower than can be achieved using viral vectors. Considerable effort has therefore been devoted to synthesizing new cationic lipids that have improved transfection properties over those currently available (*5−10*). These efforts are largely phenomenological due to the lack of understanding of the mechanism whereby cationic lipids facilitate the intracellular delivery of macromolecules such as plasmid DNA. In this regard recent work from this laboratory suggests that the ability of cationic lipids to facilitate intracellular delivery is related to their ability to induce the nonbilayer hexagonal H_{II} phase in combination with anionic lipids (*11*). In particular this proposal suggests that the most potent cationic lipids are those that are the most effective inducers of H_{II} phase organization.

The ability of lipids to adopt the H_{II} phase has been related to their dynamic "molecular shape" properties. Within this framework lipids with a small cross-sectional area in the headgroup region and a larger acyl chain cross-sectional area exhibit a "cone" shape compatible with H_{II} phase organization (*12*). In this regard a direct method to increase the cone-shaped character of a given lipid is to increase the number of alkyl chains associated with the headgroup. In this work we describe the synthesis and transfection properties of a new class of cationic lipids that contain four alkyl chains. It is shown that these cationic lipids can exhibit improved transfection properties compared to dialkyl cationic lipids.

Earlier studies have shown *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) to be quite an effective DNA transfection agent (*13*, *14*). We thus chose a synthetic route to generate a molecule with two quaternary ammonium groups and four oleyl chains, essentially a DODAC dimer. The procedure involved alkylating dimethylamines with oleyl bromide. The polymorphic phases of these new cationic lipids were monitored by ^{31}P NMR of these lipid mixtures containing 1,2-dipalmitoyl-sn-glycero-3-[phospho-L-serine] (DPPS). The in vitro transfection potency was measured and compared with that of DODAC.

EXPERIMENTAL PROCEDURES

Materials and Reagents. All organic solvents and reagents including the Dragendorff reagent and ninhydrin reagent were obtained from Sigma-Aldrich Canada (Oakville, ON). DOPE and DPPS were purchased from Avanti Polar Lipids (Alabaster, AL). The cationic lipid DODAC was a gift from Dr. Steven Ansell of Inex Pharmaceuticals (Vancouver, BC). The plasmid pCMV-luc (5650 bp, CMV promoter, coding for luciferase) was a gift from Inex Pharmaceuticals (Vancouver, BC).

Analytical Techniques. The cationic lipids were purified by column chromatography using silica gel 60 (Merck, 70−230 mesh) and dichloromethane (CH_2Cl_2)/methanol (MeOH) mixtures. Unless stated otherwise, the ratios describing the composition of solvent mixtures represent relative volume fractions. The reaction progress was followed by thin-layer chromatography (TLC) on silica plates F_{254} (Merck). The following developing systems were used: UV light, H_2SO_4/EtOH (1/1 v/v), Dragendorff reagent (Sigma), ninhydrin reagent (Sigma). ^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained employing a Bruker MSL 200 spectrometer operating at 200.13,

* To whom correspondence should be addressed. Phone: (604) 822−2649. Fax: (604) 822−4843. E-mail: jerome_gaucheron@yahoo.fr.

† University of British Columbia.

‡ Inex Pharmaceuticals Corporation.

10.1021/bc0101259 CCC: $22.00 © 2002 American Chemical Society
Published on Web 04/05/2002

50.3, and 81.02 MHz, respectively. Deuterated chloroform ($CDCl_3$) was used as the solvent in the NMR experiments. Chemical shifts were measured relative to $CHCl_3$ (δ 7.24 ppm) for ^1H, relative to $CDCl_3$ (δ 77.16 ppm) for ^{13}C. The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are expressed in ppm and listed as follows: shift in ppm (multiplicity, coupling, integration and assignment). Elemental analyses were carried out by Microanalytical Service (Department of Chemistry, University of British Columbia).

Synthesis of *N,N,N,N*-Tetraoelyl-*N,N*-dimethyl-1,3-propanediammonium Chloride (TODMAC3). A solution of *N,N*-dimethyl-1,3-propanediamine (0.253 g, 2.5 mmol) and oleyl bromide (5 g, 15.1 mmol) in toluene (20 mL) and a solution of tetrabutylammonium hydrogensulfate (0.4 g, 1.2 mmol) in 1 M sodium hydroxide (20 mL) were stirred under reflux. After 4 days, 2.5 g oleyl bromide (7.5 mmol) was added and the reaction continued for another 5 days. The water phase was extracted two times with toluene. The combined organic phases were evaporated. The residue was dissolved in CH_2Cl_2 and washed six times with 3% hydrochloric acid and three times with saturated aqueous NaCl. The organic phase was evaporated and the remaining material purified by silica gel chromatography (CH_2Cl_2/MeOH from 10/0 to 9/1). **TODMAC3** was obtained as a pale yellow oil at a yield of 1.15 g (0.98 mmol, 39%). The product was washed six times with 3% hydrochloric acid and three times with saturated aqueous NaCl again.

TLC (CH_2Cl_2/MeOH 9/1, H_2SO_4/EtOH): $R_f = 0.43$. ^1H NMR ($CDCl_3$): δ 0.87 (t, $^3J = 6.6$ Hz, 12H, CH_3); 1.08−1.48 (broad s, 88H, $CH_3(CH_2)_6CH_2CH{=}CHCH_2(CH_2)_5$); 1.48−1.77 (m, 10H, CH_2CH_2N); 1.80−2.12 (m, 16H, $=CHCH_2$); 3.22−3.80 (m, 18H, CH_3, CH_2N); 5.20−5.45 (m, 8H, $=CH$). ^{13}C NMR ($CDCl_3$): δ 14.1 (CH_3CH_2); 22.7 (CH_3CH_2); 27.2 ($=CHCH_2$); 22.5, 26.4, 29.1, 29.2, 29.3, 29.5, 29.7 ($CH_3(CH_2)_2(CH_2)_4CH_2CH{=}CHCH_2(CH_2)_6$, NCH_2-CH_2CH_2N); 31.9 ($CH_3CH_2CH_2$); 49.0 (NCH_3); 61.5 (NCH_2); 129.6, 130.1 ($=CH$). Anal. ($C_{77}H_{152}N_2Cl_2$) H, N, C: calcd, 78.58; found, 79.50.

Synthesis of *N,N,N,N*-Tetraoelyl-*N,N*-dimethyl-1,6-hexanediammonium chloride (TODMAC6). TODMAC6 was synthesized using the same procedure as described above for TODMAC3 by substituting *N,N*-dimethyl-1,6-hexanediamine for *N,N*-dimethyl-1,3-propanediamine.

TLC (CH_2Cl_2/MeOH 9/1, H_2SO_4/EtOH): $Rf = 0.61$. ^1H NMR ($CDCl_3$): δ 0.85 (t, $^3J = 6.6$ Hz 12H, CH_3); 1.00−1.46 (broad s, 92H, $CH_3(CH_2)_6CH_2CH{=}CHCH_2(CH_2)_5$, $N(CH_2)_2(CH_2)_2(CH_2)_2N$); 1.46−1.74 (m, 10H, CH_2CH_2N); 1.80−2.12 (m, 16H, $=CHCH_2$); 3.14−3.75 (m, 18H, CH_3, CH_2N); 5.20−5.45 (m, 8H, $=CH$). ^{13}C NMR ($CDCl_3$): δ 14.1 (CH_3CH_2); 22.7 (CH_3CH_2); 27.2 ($=CHCH_2$); 21.0, 22.5, 23.9, 26.4, 29.4, 29.6, 29.8 ($CH_3(CH_2)_2(CH_2)_4CH_2$-$CH{=}CHCH_2(CH_2)_6$, $NCH_2(CH_2)_4CH_2N$); 31.9 (CH_3CH_2-CH_2); 49.0 (NCH_3); 61.2, 62.7 (NCH_2); 129.7, 130.2 ($=CH$). Anal. ($C_{80}H_{158}N_2Cl_2$)·H_2O C, H, N.

^{31}P Nuclear Magnetic Resonance Spectroscopy. Lipid mixtures containing 40−50 mg DPPS and appropriate molar ratios of cationic lipids (DODAC, TODMAC3, TODMAC6) were prepared by drying from chloroform solution, placed in 10 mm NMR tubes, and hydrated with 1.5 mL of distilled water. Free induction decays (FIDs) corresponding to 1000 scans were obtained by using a 3.0 μs 60° pulse with a 1 s interpulse delay and a spectral width of 25000 Hz. A gated two-level proton decoupling was used to ensure sufficient decoupling with minimum sample heating. An exponential multiplication corresponding to 50 Hz of line broadening was applied to the FIDs prior to Fourier transformation. The sample temperature (±1 °C) was regulated using a Bruker B−VT1000 variable temperature unit. Chemical shifts were referenced to 85% phosphoric acid as an external standard.

Preparation of Large Unilamellar Vesicles (LUV). Mixtures of cationic lipids and DOPE in chloroform were dried under a stream of nitrogen gas, and the residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with distilled water. LUVs were obtained by extruding the lipid dispersions 10 times through two stacked 100-nm pore size polycarbonate filters (Costar Nuclepore polycarbonate membrane, Northern Lipids Inc. extruder) (*15*). Phospholipid concentrations were determined by the inorganic phosphorus assays according to Fiske and Subbarow (*16*).

Preparation of Lipoplexes. Lipoplexes were prepared in distilled water at pDNA concentrations ranging from 12.5 to 25 μg pDNA/ml at positive-to-negative charge ratios between 0.5 and 5. Plasmid DNA was added under vortexing to cationic LUVs (cationic lipid/DOPE 1:1). In the absence of the helper lipid DOPE, lipoplexes were prepared by addition of the cationic lipids from ethanol solutions to an aqueous solution containing the pDNA. The amount of ethanol in these formulations was between 0.4 and 2.3%. The lipoplexes were incubated at room temperature for 30 min, diluted to a final concentration of 2.5 μg pDNA/mL with serum-free media (Dulbecco's Modified Eagle's Medium, DMEM), and subsequently applied to BHK cells.

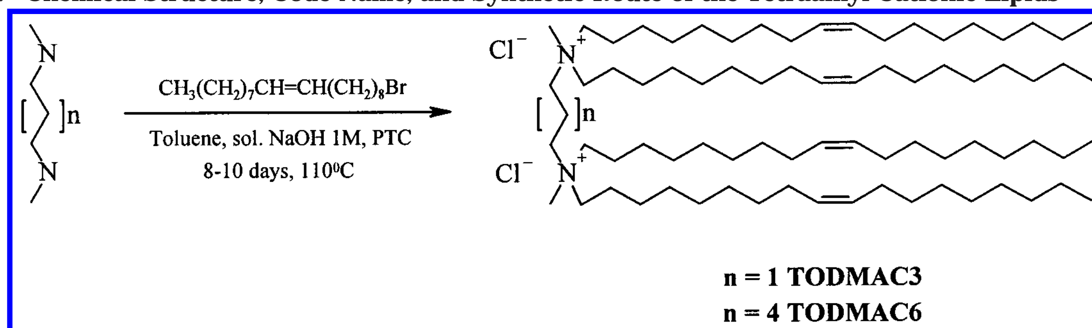
In Vitro Transfection of BHK Cells. BHK cells, grown in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, were seeded in 96-well plates at a density of 1×10^4 cells per well 24 h before transfection. The next day media was removed and replaced with 100 μL of lipoplexes dispersed in serum-free DMEM (0.25 μg pDNA/100 μL). After 4 h, lipoplexes were replaced with serum-containing DMEM and cells further incubated for 20 h. After that the culture medium was aspirated, the cells washed twice with 100 μL of PBS and then lysed through addition of 50 μL of lysis buffer (0.1% Triton X-100 in PBS). Luciferase activity was measured with a Dynex luminometer (Dynex model ML3000) using the Luciferase Assay Kit from Promega. The total protein concentration per well was determined using the BCA assay from Pierce. Means and standard deviations were calculated from three independent experiments.

Statistical Analyses. Statistical analyses were performed using a Student t test. The difference between two means was considered as significant when the probability p is 0.05.

RESULTS

Rationale for and Synthesis of the Cationic Lipids TODMAC3 and TODMAC6. As indicated in the Introduction, cationic lipids with a small cross-sectional area in the headgroup region would be expected to give rise to improved transfection properties compared to lipids with larger headgroup areas, assuming the cross-sectional area in the alkyl chain region remains constant (*11*). One method to vary the headgroup area is to create a dimer of cationic lipids joined together in the headgroup region by a tether of variable length. We chose to work with DODAC, a cationic lipid with excellent transfection properties, and construct dimers joined by hydrocarbon

Scheme 1. Chemical Structure, Code Name, and Synthetic Route of the Tetraalkyl Cationic Lipids



tethers three and six carbons long (TODMAC3 and TODMAC6, respectively).

These cationic lipids were synthesized by nucleophilic substitution of secondary amines using oleyl bromide under phase-transfer catalytic condition as outlined in Scheme 1. The reaction was performed in toluene and 1 M sodium hydroxide in the presence of a phase-transfer catalyst for 10 days at 100 °C. The strongly basic amines required high alkaline reaction conditions so that they remained deprotonated to react with oleyl bromide. The yields were 39 and 85% for TODMAC3 and TODMAC6, respectively. The lower yield for TODMAC3 is probably due to steric hindrance as a result of the close proximity of the two positive charges. The diquaternary ammonium chlorides were characterized by ^{1}H and ^{13}C NMR as well as by elemental analysis. The NMR resonances are consistent with the diquaternary structure of the cationic lipids (see above Experimental Procedures).

Polymorphic Phase Properties of Mixtures of TODMAC3 and TODMAC6 with DPPS. The ability of cationic lipids to induce the hexagonal H_{II} phase in combination with anionic lipids correlates well with their transfection properties in plasmid DNA–cationic lipid complexes. The relative ability of cationic lipids to induce the H_{II} phase can be ascertained by measuring the bilayer-to-nonbilayer transition temperature (T_C) of the cationic lipid in equimolar mixtures (with respect to charge) with an anionic phospholipid such as DPPS. The bilayer-to-nonbilayer transition can be conveniently monitored employing ^{31}P NMR techniques (*12*). Briefly, phospholipids that are in a bilayer organization exhibit a broad ($\geq$40 ppm) aymmetric ^{31}P NMR line shape with a low field shoulder and a high field peak whereas phospholipids in the hexagonal H_{II} phase exhibit a line shape with reversed asymmetry that is a factor of 2 narrower. Phospholipids in nonbilayer cubic phases that often occur in conjunction with H_{II} phase formation exhibit narrow "isotropic" ^{31}P NMR spectra. It should be noted that small (diameter $\leq$ 200 nm) phopholipid vesicles also exhibit isotropic spectra; however, in the results presented here the presence of such spectra will be attributed to nonbilayer structures as the temperature-dependent phase behavior was reversible.

In the first series of experiments the temperature-dependent polymorphism of TODMAC6/DPPS mixtures was compared to the behavior of DODAC/DPPS mixtures. As shown in Figure 1, at 25 °C both mixures exhibited ^{31}P NMR line shapes indicative of bilayer structure. However at 30 °C the mixture containing TODMAC6 has largely adopted the H_{II} phase or a structure giving rise to a narrow isotropic ^{31}P NMR peak, whereas the dispersion containing DODAC remains in a bilayer organization at 35 °C, adopting the structure giving rise to the "isotropic" ^{31}P NMR peak at 30 °C. On the basis of this behavior it would be expected that TODMAC6 should exhibit transfection properties that are comparable to or slightly better than DODAC when used in plasmid DNA–cationic lipid complexes.

The ^{31}P NMR behavior of dispersions of TODMAC3/DPPS (0.5:1; mol:mol) is illustrated in Figure 2A. At temperatures as low as 15 °C an H_{II} component is clearly visible; however, the large bulk of the phospholipid remains in the bilayer organization and the proportion in the H_{II} phase does not increase as the temperature is raised. This behavior is consistent with a limited solubility of the TODMAC3 in the DPPS component in the lipid mixture. This may be due to steric constraints associated with the need to form ion pairs between the cationic lipid and two DPPS molecules in order to form the H_{II} phase. For example, if only one DPPS molecule was able to form an ion pair with each TODMAC3, 50% of the DPPS would be unpaired and thus remain in the bilayer organization. If this is the case it would be expected that TODMAC3/DPPS systems containing higher proportions of TODMAC3 should exhibit increased preference for nonbilayer structure. The phase properties of a mixture containing equimolar proportions of TODMAC3 and DPPS were therefore investigated and, as shown in Figure 2B, this system exhibited an increased preference for H_{II} phase structure. On the basis of this behavior TODMAC3 would not be expected to be as effective a transfection agent as DODAC on a charge for charge basis.

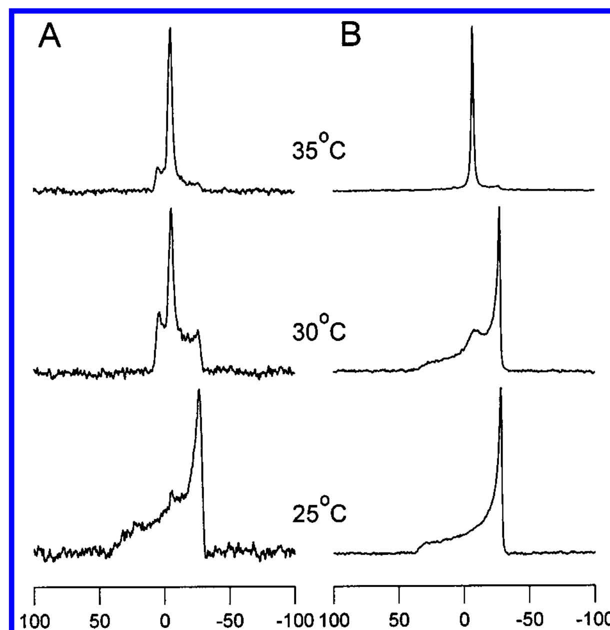


Figure 1. ^{31}P NMR spectrum obtained from an aqueous dispersion of TODMAC6/DPPS (0.5 molar ratio) (A) and of DODAC/DPPS (1 molar ratio) (B). The temperatures are indicated. Sample parameters and NMR parameters are described in Experimental Procedures.

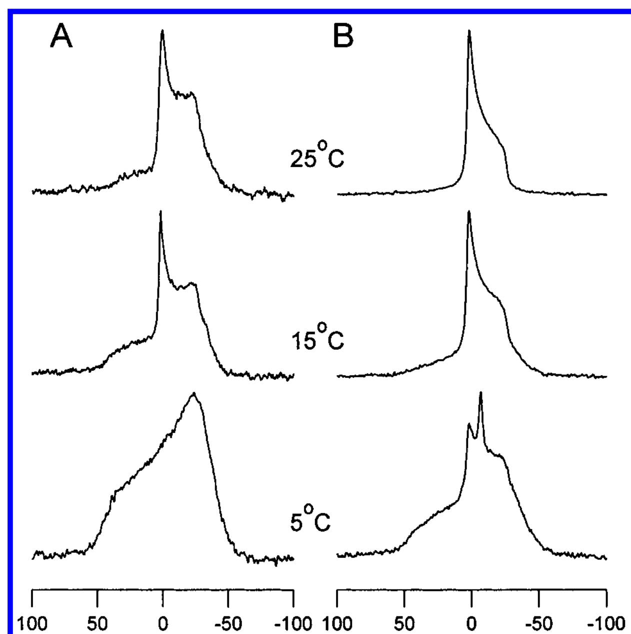


Figure 2. ^{31}P NMR spectrum obtained from an aqueous dispersion of TODMAC3/DPPS (0.5 molar ratio) (A) and of TODMAC3/DPPS (1 molar ratio) (B). The temperatures are indicated. Sample parameters and NMR parameters are described in Experimental Procedures.

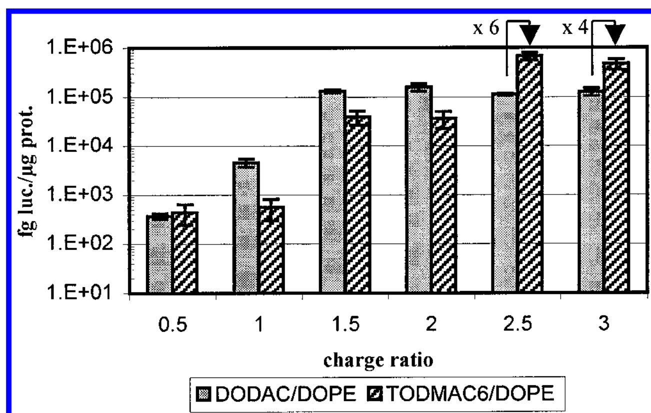


Figure 3. Transfection efficiency in BHK cells of the TODMAC6/DOPE lipoplexes, as compared with, DODAC/DOPE lipoplexes. The data presented correspond to a concentration of 0.25 μg DNA/well. The given means ± SEM were calculated from three independent experiments.

In Vitro Transfection Properties of Plasmid DNA–Cationic Lipid Complexes Containing TODMAC3 and TODMAC6. Plasmid DNA cationic lipid complexes are usually formed by mixing plasmid DNA with preformed vesicles containing the cationic lipid mixed with equimolar amounts of the "helper" lipid dioleoylphosphatidylethanolamine (DOPE) (*17, 18*). However, it was found that vesicles could not be prepared consisting of TODMAC3/DOPE mixtures, as lipid films prepared of this lipid mixture were extremely difficult to hydrate and formed clumps that could not be extruded. Vesicles of TODMAC6/DOPE could be readily prepared, and the transfection properties of complexes formed employing TODMAC6 and DODAC and containing the L018 plasmid coding for the luciferase gene were tested in vitro using baby hamster kidney (BHK) cells. Figure 3 illustrates the transfection properties of complexes prepared at cationic lipid-to-DNA charge ratios (positive to negative) ranging from 0.5 to 5. It may be observed that the transfection potency of complexes containing TODMAC6 are comparable to or slightly lower than complexes

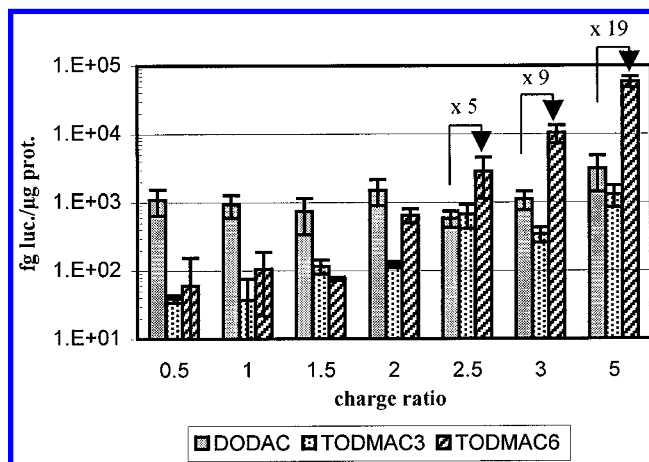


Figure 4. Transfection efficiency in BHK cells of the TODMAC6 and TODMAC3 lipoplexes, as compared with, DODAC lipoplexes. The data presented correspond to a concentration of 0.25 μg DNA/well. The given means ± SEM were calculated from three independent experiments.

containing DODAC at charge ratios or two or lower, but are greater at higher charge ratios.

To test the relative transfection potencies of DODAC, TODMAC6, and TODMAC3 a different procedure was used to associate the cationic lipid with the plasmid to form the complexes. In this method the cationic lipid dissolved in ethanol is introduced into an aqueous solution of the plasmid and DOPE is not present. The transfection properties of complexes formed in this manner are shown in Figure 4. It may be noted that at charge ratios of two and lower DODAC exhibits superior tranfection properties; however, at charge ratios above two TODMAC6 exhibits transfection properties that are superior to both DODAC and TODMAC3. Specifically, for charge ratios ≥2.5, TODMAC6 complexes gave rise to luciferase expression levels 5–20-fold higher than could be achieved with DODAC or TODMAC3.

DISCUSSION

This study describes the synthesis and transfection properties of a new class of tetraalkyl cationic lipids designed to increase the ability of cationic lipids to induce the hexagonal H_{II} phase. There are three points of interest, the first of which concerns the physical properties of these lipids in relation to their molecular shape characteristics. A second area concerns the correlation between the ability to induce H_{II} organization and the observed transfection properties. Finally, it is of interest to discuss the utility of the TODMAC3 and TODMAC6 cationic lipids and their derivatives as transfection agents. We discuss these points in turn.

The physical properties of TODMAC3 and TODMAC6 are quite different. TODMAC6 readily forms bilayer structures, both in the presence and absence of DOPE, on dispersion in an aqueous medium, whereas TODMAC3 does not. Further, when mixed with equimolar (with respect to charge) amounts of DPPS, TODMAC6 forms a homogeneous dispersion that collectively adopts nonbilayer structure in the region of 30 °C. TODMAC3 on the other hand is able to interact with only a limited subset of the DPPS but is able to induce the H_{II} phase for that subset at temperatures as low as 15 °C. At higher TODMAC3/DPPS ratios (2:1 on a charge basis), TODMAC3 is able to induce apparently complete H_{II} organization at temperatures of 25 °C or higher. These results are consistent with steric effects that limit the ability of

TODMAC3 to interact with more than one DPPS molecule due to the small size of the TODMAC3 headgroup.

The correlation between the ability of TODMAC6 and TODMAC3 to induce nonbilayer structure and their transfection properties is of obvious interest for the design of cationic lipids with optimized transfection capabilities. TODMAC6/DPPS and DODAC/DPPS liposomes exhibit bilayer−nonbilayer transitions that are close together, in the region of 30−35 °C. On this basis it would be expected that plasmid DNA−cationic lipid complexes containing TODMAC6 or DODAC should exhibit roughly equivalent transfection properties. For the complexes containing DOPE this is largely true, although somewhat higher transfection potency is observed for the complexes containing TODMAC6 at higher charge ratios. For the complexes prepared in the absence of DOPE the TODMAC6, complexes are not as effective transfection agents at charge ratios less than two but can be substantially more potent at charge ratios above two. In the case of TODMAC3 the tranfection potency of the complexes (prepared in the absence of DOPE) only become comparable to complexes containing DODAC at charge ratios above two. This could be interpreted as reflecting a need for increased amounts of TODMAC3 in order to bind to endogenous anionic lipid due to steric effects inhibiting these interactions. Overall these results suggest that while the ability to induce nonbilayer structure correlates roughly with transfection potency, other factors can also influence transfection potencies substantially. These other effects include the method of constructing the complexes, steric barriers inhibiting the formation of neutral ion pairs between cationic and anionic lipids, the ability of the cationic lipid to dissociate from the plasmid DNA following uptake into the cell, and the lipid composition of the endosomal membrane.

The final area of discussion concerns the utility of TODMAC6 and TODMAC3 as transfection agents. It would appear that TODMAC3 has limited advantages over currently available cationic lipid such as DODAC. The utility of TODMAC3 is further compromised by the inability to incorporate DOPE into complexes containing TODMAC3 due to the inability to form vesicular structures. However, TODMAC6 appears to have interesting potential, particularly at charge ratios above two. Current work is focused on exploring this potential when incorporated into "stabilized plasmid-lipid particles" (SPLP) (*19−21*) for in vivo applications for treatment of systemic disease.

ACKNOWLEDGMENT

This research was supported by the Canadian Institutes of Health Research.

LITERATURE CITED

(1) Nishikawa, M., and Huang, L. (2001) Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum. Gene Ther. 20*, 861−70.

(2) Pedroso de Lima, M. C., Simoes, S., Pires, P., Faneca, H., and Duzgunes, N. (2001) Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Deliv. Rev. 47*, 277−94.

(3) Welsh, M. J., and Zabner, J. (1999) Cationic lipid mediated gene transfer of CFTR: safety of a single administration to the nasal epithelia. *Hum. Gene Ther. 10*, 1559−72.

(4) Maurer, N., Fenske, D. B., and Cullis, P. R. (2001) Developments in liposomal drug delivery systems. *Expert Opin. Biol. Ther. 1*, 1−25.

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

(6) Miller, A. D. (1998) Cationic Liposomes for gene therapy. *Angew. Chem., Int. Ed. 37*, 1768−1785.

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

(8) Wang, J., Guo, X., Xu, Y., Barron, L., and Szoka, F. C., Jr. (1998) Synthesis and characterization of long chain alkyl acyl carnitine esters. Potentially biodegradable cationic lipids for use in gene delivery. *J. Med. Chem. 41*, 2207−2215.

(9) Gao, H., and Hui, K. M. (2001) Synthesis of a novel series of cationic lipids that can act as efficient gene delivery vehicles through systematic heterocyclic substitution of cholesterol derivatives. *Gene Ther. 8*, 855−63.

(10) Hyvonen, Z., Plotniece, A., Reine, I., Chekavichus, B., Duburs, G., and Urtti, A. (2000) Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery. *Biochim. Biophys. Acta 1509*, 451−66.

(11) Hafez, I. M., Maurer, N., and Cullis, P. R. (2001) On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther. 8*, 1188−96.

(12) Cullis, P. R., and de Kruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta 559*, 399−420.

(13) Schoen, P., Chonn, A., Cullis, P. R., Wilschut, J., and Scherrer, P. (1999) Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. *Gene Ther. 6*, 823−32.

(14) Lam, A. M., and Cullis, P. R. (2000) Calcium enhances the transfection potency of plasmid DNA−cationic liposome complexes. *Biochim. Biophys. Acta 1463*, 279−90.

(15) Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta 858*, 161−8.

(16) Fiske, C. H., Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem. 66*, 375−379.

(17) Farhood, H., Serbina, N., and Huang, L. (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta 1235*, 289−295.

(18) Hui, S. W., Langner, M., Zhao, Y., Ross, P., Hurley, E., and Chan, K. (1996) The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys. J. 71*, 590−599.

(19) Tam, P., Monck, M., Lee, D., Ludkovski, O., Leng, E. C., Clow, K., Stark, H., Scherrer, P., Graham, R. W., and Cullis, P. R. (2000) Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Ther. 7*, 1867−74.

(20) Zhang, Y. P., Sekirov, L., Saravolac, E. G., Wheeler, J. J., Tardi, P., Clow, K., Leng, E., Sun, R., Cullis, P. R., and Scherrer, P. (1999) Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. *Gene Ther. 6*, 1438−47.

(21) Wheeler, J. J., Palmer, L., Ossanlou, M., MacLachlan, I., Graham, R. W., Zhang, Y. P., Hope, M. J., Scherrer, P., and Cullis, P. R. (1999) Stabilized plasmid-lipid particles: construction and characterization. *Gene Ther. 6*, 271−81.

BC0101259