Synthesis and in Vitro Evaluation of Glutamide-Containing Cationic Lipids for Gene Delivery

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A novel series of cationic amphiphiles based on dialkyl glutamides with cationic pyridinium head group were synthesized as potential gene delivery agents. Four cationic lipids with glutamide as linker and varying chain lengths were tested for their transfection efficiency in three cell lines. The DNA−lipid complexes were characterized for their ability to bind to DNA, protection from nuclease digestion, size, ζ-potential, and toxicity. All four lipids demonstrated efficient transfection in MCF-7, COS, and HeLa cells, and the reporter gene expression was much higher with DOPE as the helper lipid in the formulation when compared to cholesterol. Among these 14-carbon lipids, lipid **2** has shown the highest transfection efficiency, complete protection of DNA from nuclease digestion, and low toxicity. Interestingly, lipid **2** has also shown remarkable enhancement in transfection in the presence of serum.

INTRODUCTION

Carriers that can deliver nucleic acids into cells have immense potential to investigate the properties of gene sequences and in gene therapy (*1*−*4*), and broadly fall into viral and nonviral classes. Among the nonviral carriers, the successful strategies are mainly based on cationic lipids, cationic peptides, or polymers and cationic dendrimers (*5*). Cationic lipids have several advantages including simplicity of method, clinical grade manufacturing, lack of ability to elicit immune response, and capacity to carry large fragments of DNA (*6*, *7*). Several clinical trials employing cationic lipids are in progress (*8*).

Cationic lipids are amphiphilic in nature and carry a positive charge at physiological pH (*9*, *10*). The cationic nature of the lipids allows them to bind to the nucleic acids and also to the negatively charged cell membrane. Besides being cationic, the long hydrophobic moiety of these lipids allows self-assembly into vesicles to entrap the nucleic acids, thus protecting DNA from nucleases (*9*). The synthesis, chemistry, and biology of the cationic lipids have been extensively reviewed (*10*−*15*). Essentially, a cationic lipid has a cationic head group that is connected to a hydrophobic moiety by a linker. Glycerol, with ether or ester bonds with fatty acids, is the most popular linker, though other groups such as phosphonates and aromatic groups have also been used (*16*). We report here the synthesis and transfection efficiencies of cationic lipids based on glutamic acid as the linker. These glutamide-derived dialkyl lipids form self-assembled lamellar and non-lamellar structures based on intermolecular hydrogen bonding among amide moieties (*17*−*20*). Four cationic pyridinium amphiphiles with different alkyl chain lengths (C12 to C18) with glutamide as a linker were synthesized. The physical properties of these lipids have been characterized and were tested for their DNA binding and transfection ability in the presence of serum on three cell lines.

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EXPERIMENTAL PROCEDURES

Plasmid/Chemicals. pCMV.SPORT-β-gal plasmid, Lipofectamine, and Lipofectin were purchased from Invitrogen. DOTAP, cholesterol, and DOPE were purchased from Avanti Polar Lipids, Inc. Dulbecco's Modified Eagle's Medium (DMEM) and cholesterol was obtained from Sigma Chemicals, U.S.A. All other reagents used were of high-quality grade.

Preparation of Liposomes. The methods employed here have been described earlier (*21*, *22*). In the preparation of lipid−DNA complex (Lipoplex) cationic lipids were either mixed with DOPE or cholesterol as the helper lipid. Liposomes were prepared at 1:1 (mol/mol) ratio of cationic lipid and helper lipid, by drying the chloroform stock solutions in 1.5 mL glass tubes under nitrogen gas. Tubes were dried under vacuum to remove traces of chloroform for about 2 h. Sterile MilliQ was added to dried lipid film and hydrated overnight by incubating the tubes at 55 °C in water bath. The sample was vortexed for about 3 min and sonicated using a microprobe until the solution became clear.

Cell Culture. MCF-7, COS-1, and HeLa cells were maintained in DMEM containing 10% fetal bovine serum and 500 μg/mL penicillin, 600 μg/mL streptomycin, and 1 mg/mL kanamycin.

Cell Transfections. For quantitating the transient transfections in cell lines, the β-gal activity was measured 24 h after the transfections (*23*). Lipoplexes with different lipid/DNA charge ratios were prepared by mixing different amounts of liposomes with plasmid DNA (0.3 μg per well) in DMEM and incubated at room temperature for about 15 min. Complexes were incubated with cells for about 3 h, after which lipoplex-containing medium was replaced with 10% serum containing DMEM medium. After 24 h, the medium was removed, and cells were washed with PBS and lysed with 50 μL lysis buffer (250 mM Tris-HCl pH 8.0 containing 0.5% NP40) for 10 min at room temperature. Cell lysate was used for protein estimation

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by Lowry's method and 50 μL was used for the β-galactosidase activity. Absorbance at 410 nm was read in a SPECTRA Max ELISA plate reader. β-Galactosidase activity was calculated from a standard graph constructed from commercial β-galactosidase enzyme. β-Galactosidase activity was normalized against milligrams of cell protein. For transient transfections done in the presence of varying serum concentrations, CLDC was prepared in the absence of serum, and serum was added to the complex at the required final concentration, after which they were added to the cells. Data is represented as β-gal activity per well ($\sim$20 000 cells).

Binding of Lipid to DNA and DNaseI Protection Assay. pCMVβ-gal was complexed with cationic lipids at charge ratios varying from 0.3:1 to 3:1 (lipid/DNA) in order to measure the DNA binding ability and, second, to evaluate the susceptibility of the DNA in these complexes to DNaseI digestion. In a typical binding assay, 0.6 nmol of DNA was complexed with lipids in MQ water to obtain the above charge ratios and incubated for 25 min at room temperature (RT) prior to electrophoresis on an agarose gel. For the DNaseI protection assay, the complexes were prepared in 0.5× PBS (pH 7.5). Subsequently, the complexes were treated with 0.2 units of DNaseI and incubated at RT for 20 min, followed by heat inactivation at 60 °C for 7 min. The reactions were then halted with EGTA added to a final concentration of 30 mM. Lipids from the lipoplex were extracted with a phenol−chloroform mixture. Equal volumes of the aqueous phase was treated with DNA loading dye and electrophoresed on a 0.8% agarose gel in TAE buffer at a constant voltage (80 V). The gel was stained with ethidium bromide postelectrophoresis and the bands visualized on a transilluminator.

Cytotoxicity Assay. MCF-7 cells were grown in 96-well plates at about 70% confluency (*24*). After incubating the lipoplexes with the cells for 3 h, the lipoplexes were removed from the well, washed once with PBS, and replaced with 100 μL of DMEM containing 10% serum and antibiotics per well. To this, 100 μL of MTT (5 mg/mL in PBS) solution was added to each well, and the cells were incubated in the incubator for additional 2 h. At the end of 2 h, the medium was removed, and 100 μL of DMSO/methanol (1:1 vol/vol) was added per well. The plate was gently rocked and kept at room temperature for 5 min and then read at 540 nm in the SPECTRA Max ELISA plate reader. Data were represented as percentage cell viability.

Lipoplex Size. The sizes of lipoplexes were measured by photon correlation spectroscopy on a Zeta sizer 3000HSA (Malvern, U.K.) in DMEM and HEPES buffer, pH 7.4, using a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated by using the 200/5 nm polystyrene polymer (Duke Scientific Corporation, Palo Alto, CA).

Synthesis of Lipids. Structures and the synthesis scheme of glutamide-derived lipids having cationic pyridinium head groups with different alkyl chain lengths are shown in Figure 1. The synthesis processes of lipid **1** are given below.

N′,N″-Didodecyl-N-benzyloxycarbonylglutamide (2C12-Gln-(Z)). *N*-Benzyloxycarbonylglutamic acid (Glu(Z)) (7.0 g, 25 mmol), *n*-dodecylamine (12 mL, 53 mmol), and triethylamine (8.9 g, 63 mmol) were dissolved in THF (400 mL) (*23*). The solution was cooled to 0 °C, and diethylphosphorocyanidate (DEPC) (10 mL, 64 mmol) was added to the solution and stirred for 1 h at this temperature. After stirring for 1 day at room temperature, the solution was concentrated in vacuo, and the residue was dissolved in 350 mL of chloroform. The solution was washed with 10% $NaHCO_3$, 0.1 mol/L HCl, and water. The solution was dried over Na_2SO_4, concentrated, and finally recrystallized from ethanol, which gave a white solid powder: yield 10.7 g (68%, 17 mmol); mp 141−143 °C; FT-IR (KBr)/3287, 2917, 2850, 1686, 1637, and 1539 cm^{-1}; ^1H NMR

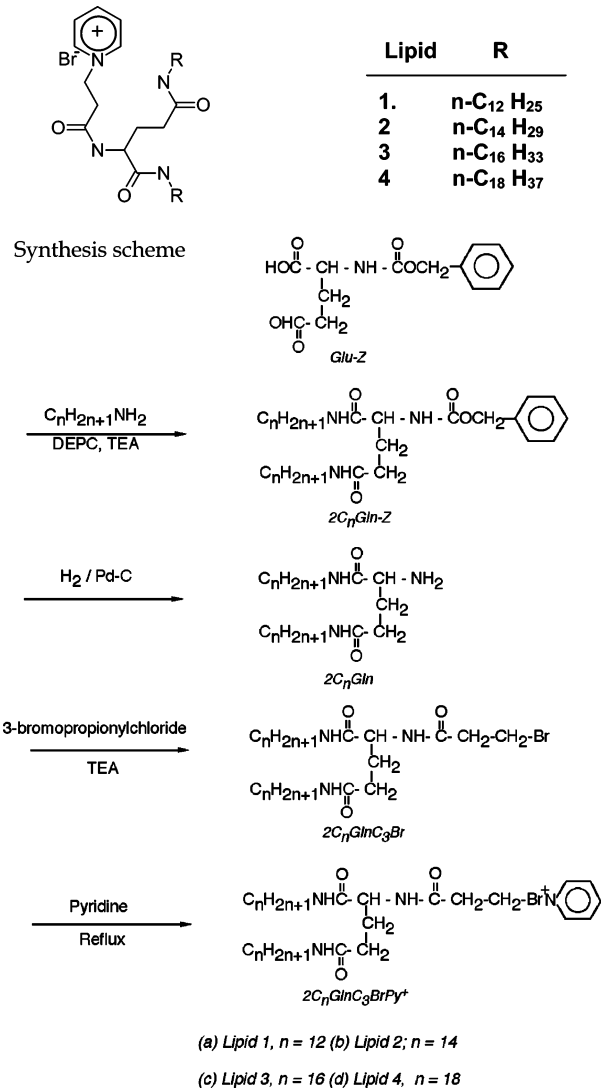

Figure 1. Structures and the synthesis scheme of the glutamide lipids used in this study.

($CDCl_3$) δ 0.862−0.896 (t, 6H, CH_3), 1.257 (m, 36H, CH_3-(CH_2)$_9$), 1.486 (m, 4H, CH_2CH$_2$NHC(=O)), 1.980 (m, ^{1}H, C*HCH_2), 2.091 (m, 1H, C*HCH_2), 2.297 (m, 2H, C*HCH$_2$-CH_2C(=O)), 3.221 (m, 4H, CH_2NHC(=O)), 4.144 (m, 1H, C*H), 5.098 (s, 2H, CH_2Ph), 7.328 (s, 5H, C$_6H_5$). Anal. Found: H, 10.9; C, 71.8; N, 6.75; Calcd. for $C_{37}H_{65}N_3O_4$: H, 10.9; C, 72.1; N, 6.82.

N′,N″-Didodecylglutamide (2C$_{12}$-Gln). 2C$_{12}$-Gln(Z) (10.0 g, 16 mmol) was dissolved in 400 mL ethanol with heating, and Pd carbon (1 g) was added to the solution. H_2 gas was bubbled slowly into the solution for 10 h at 60 °C. Pd carbon was removed by filtration. The solution was concentrated, recrystallized from methanol, and dried in vacuo to give a white solid powder: yield 6.76 (88%, 14 mmol); mp 117.0−118.5 °C; FT-IR (KBr)/3318, 2934, 2874, 1647, and 1553 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, CH_3), 1.26 (m, 36H, CH_3(CH_2)$_9$), 1.50 (m, 4H, CH_2CH$_2$NHC(=O)), 1.95 (m, 2H, C*HCH_2CH$_2$C-(=O)), 2.31 (m, 2H, C*HCH$_2$CH_2C(=O)), 3.23 (m, 4H, CH_2-NHC(=O)), 3.41 (m, 1H, C*H). Anal. Found: H, 12.2; C, 72.1; N, 8.74. Calcd. for $C_{29}H_{59}N_3O_2$: H, 12.3; C, 72.3; N, 8.72.

N′,N″-Didodecyl-N-bromopropionylglutamide (2C$_{12}$-GlnC$_3$Br). 2C$_{12}$-Gln (1.45 g, 3.0 mmol) was dissolved in THF (100 mL); 3-bromopropionylchloride (0.77 g 4.5 mmol) and triethylamine (0.91 g, 9.0 mmol) were also added into the solution. The solution was cooled to 0 °C and stirred for 1 h at this temperature. After being stirred for 1 day at room temperature,

Table 1. Elemental Analysis and Average Mass of Different Glutamide-Derived Cationic Lipids

	elemental analysis		average mass (MALDI-TOF-MS)	
	calculated	found	calculated	found
		Lipid **1**		
C%	63.8	63.5		
N%	8.0	7.95	615.9	616.9
H%	9.7	9.75		
		Lipid **2**		
C%	65.4	64.0		
N%	7.4	7.3	672.0	673.4
H%	10.0	9.8		
		Lipid **3**		
C%	66.8	65.9		
N%	6.9	6.8	728.2	730.2
H%	10.3	10.5		
		Lipid **4**		
C%	68.1	67.0		
N%	6.4	6.3	784.3	785.5
H%	10.6	10.7		

the solution was concentrated, and the residue was dissolved in chloroform. The solution was washed with 10% $NaHCO_3$, 0.1 mol/L HCl, and water. The solution was dried over Na_2SO_4, concentrated, and finally recrystallized from ethyl acetate, which gave a white solid powder: yield 0.93 g (49%, 1.51×10^{-3} mol); mp 164−166 °C; FT-IR (KBr)/3316, 2933, 2875, 1648, and 1555 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.73 (t, 6H, CH_3), 1.23 (m, 36H, $CH_3(CH_2)_9$), 1.33 (m, 2H, C*HC$H_2$$CH_2$C(=O)), 1.43 (m, 4H, C$H_2$$CH_2$NH), 2.43 (m, 2H, C*$HCH_2CH_2$C(=O)), 2.83 (t, 2H, C(=O)C$H_2$$CH_2Br^-$), 2.93 (m, 4H, CH_2CH_2NH), 3.28 (t, 2H, C(=O)$CH_2CH_2Br^-$), 4.73 (m, 1H, C*H). Anal. Found: H, 10.3; C, 63.1; N, 6.94. Calcd. for $C_{32}H_{62}N_3O_3Br$: H, 10.13; C, 62.32; N, 6.81.

*N′,N″-Didodecyl(pyridinium-N-propionyl)glutamide Bromide ($2C_{12}$-$GlnC_3Py^+Br^-$), Lipid **1**.* $2C_{12}$-$GlnC_3Br$ (0.50 g, 0.81 mmol) was dissolved in 50 mL of pyridine and stirred at reflux temperature for 2 days. After cooling to room temperature, a white crystal appeared, and unreacted pyridine was removed by filtration. The white crystal was dissolved in methanol and filtered. The white crystals in this step were dried in vacuo: yield 0.31 g (54%, 0.44 mmol); mp 180−182 °C; FT-IR (KBr)/3295, 2928, 2865, 1650, and 1541 cm^{-1}; ^1H NMR (CD_3OD) δ 0.87−0.91 (t, 6H, CH_2), 1.28 (m, 36H, $(CH_2)_3(CH_2)_9$), 1.47 (m, 4H, C$H_2$$CH_2$C(=O)), 1.80−1.85 (m, 2H, C*HC$H_2$$CH_2$C(=O)), 1.94−1.99 (t, 2H, C*HCH_2CH_2C(=O)), 2.16−2.20 (t, 2H, C(=O)C$H_2$$CH_2Py^+$), 3.02−3.04 (t, 2H, C(=O)CH_2CH_2Py^+), 3.13−3.15 (m, 4H, CH_2CH_2C(=O)), 4.19−4.22 (m, 1H, C*H), 8.07−8.10 (m, 2H, pyridine ring), 8.56−8.60 (t, 1H, pyridine ring), 8.98−9.00 (d, 2H, pyridine ring). Anal. Found: H, 9.85; C, 62.8; N, 7.75. Calcd. for $C_{37}H_{67}N_4O_3Br$: H, 9.70; C, 63.86; N, 8.05. The others (lipids **2**, **3**, and **4**) were also synthesized, and the structures of the compounds in each step were characterized using procedures employed in the synthesis of lipid **1**. The results of elemental analysis, melting points, and MALDI-TOF-MS data for all four lipids are provided in Table 1.

RESULTS AND DISCUSSION

DNA Binding of the Lipids 1−4. To quantify the binding strength of the four lipids to DNA, the lipid-bound DNA was separated from the free or unbound DNA by agarose gel electrophoresis. Figure 2 shows that, in phosphate-buffered saline (PBS), complete binding of DNA was observed with all four lipids when the helper lipid was DOPE. With cholesterol as the helper lipid, only lipids **1** and **4** has shown complete binding, while the binding of lipids **2** and **3** to DNA was comparatively lower. Also, the presence of lipid at charge ratio of 2:1 was sufficient to achieve complete binding. However, when the complexes were prepared in water, all the lipids bind DNA strongly, indicating an ionic basis of DNA interaction with the cationic lipids (data not reported).

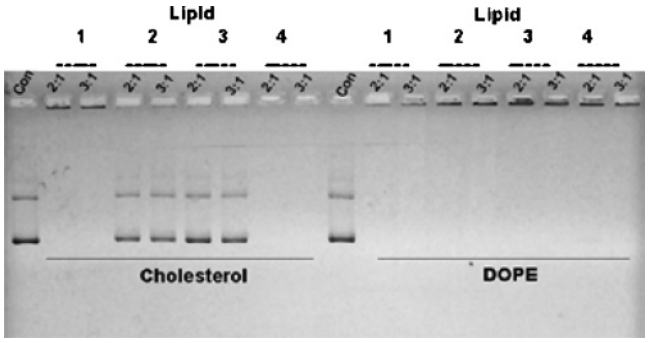

Figure 2. Binding of DNA with lipids **1**−**4**/0.8% agarose gel electrophoresis of plasmid pCMVβ-gal complexed with lipids **1**−**4** at two charge ratios, i.e., 2:1 and 3:1. The helper lipid is either cholesterol or DOPE. Free DNA was loaded in the first well and the tenth well.

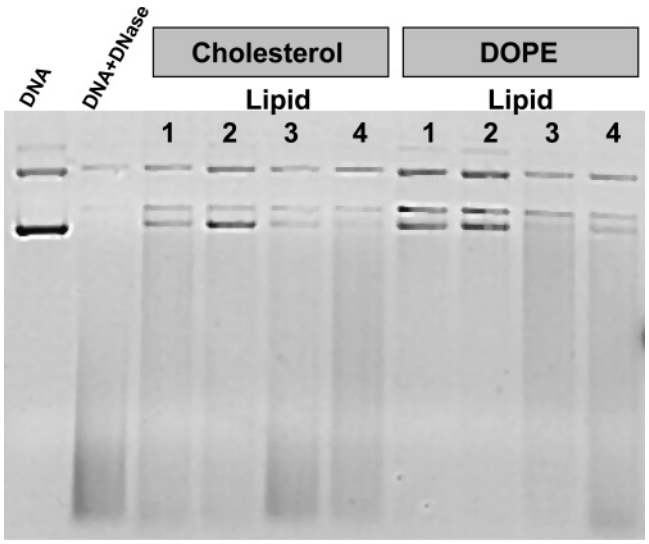

Figure 3. Sensitivity of DNA in lipid−DNA complexes to DNaseI/0.8% agarose gel electrophoresis of lipid−DNA complexes treated with DNaseI. Lane 1: Control plasmid pCMVβ-gal. Lane 2: Control DNA + DNaseI. Lanes 3−6: Lipids **1**−**4** with cholesterol as the co-lipid at 3:1 lipid/DNA. Lanes 7−10: Lipids **1**−**4** with DOPE as the co-lipid at 3:1 lipid/DNA.

In addition to the DNA binding assay, the susceptibility of bound DNA to DNaseI was performed to assess the protective ability of the lipid. Presence of DOPE as helper lipid offers better protection of DNA from DNase I digestion compared to cholesterol (Figure 3). Although all the eight formulations showed protection to DNA from DNaseI treatment to some extent, complete protection was seen only with lipid **2** with either of the helper lipids. Lipid **1** also showed compete protection with DOPE alone but not with cholesterol as the helper lipid.

Size of the Lipoplexes Prepared with Lipids 1−4 and Plasmid DNA. Transfection efficiency of lipoplexes was known to depend strongly on the size and the surface charge of the lipoplex (*25*, *26*). We measured these parameters on lipoplexes with DOPE as the helper lipid in DMEM, since this medium is relevant to the transfection protocol. The data presented in Table 2 demonstrate that the size of the complex increases with the increase in charge ratio with all four lipids. Chain length of the lipid does not influence the size of the complex with varying charge ratios, though longer chain-length lipids have resulted in vesicles of larger size. Surface charge as measured by ζ-potential has negative values across all the lipids and at all

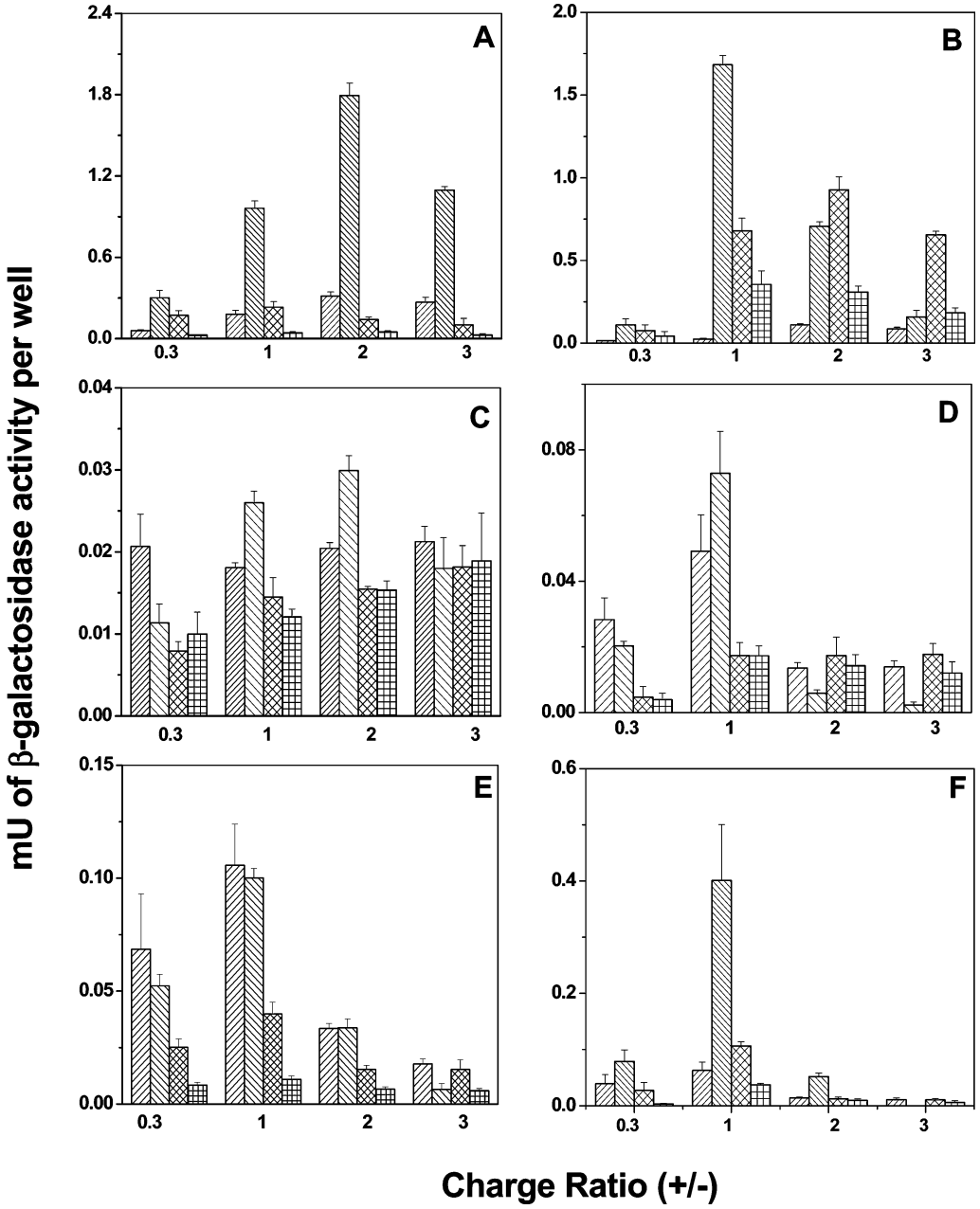

Figure 4. Transfection efficiencies of lipids **1**−**4** on MCF-7 (A, B), HeLa (C, D), and COS (E, F) cell lines. The four lipids were tested with either cholesterol (A, C, E) or DOPE (B, D, F) as the helper lipid. Bar fills are as follows: Lipid **1**, left slanting lines; lipid **2**, right slanting lines; lipid **3**, angled mesh; and lipid **4**, square mesh.

Table 2. Size of the Lipoplexes of Lipids 1−4:

charge ratio (±)	size, nm			
	0.3	1	2	3
lipid **1**	214 ± 4.7	242 ± 1.5	361 ± 14.7	956 ± 65.5
lipid **2**	267 ± 6.3	894 ± 592	367.7 ± 24	1036 ± 109
lipid **3**	441 ± 25	719 ± 52	1379 ± 41	2043 ± 91
lipid **4**	489 ± 13	561 ± 39	913 ± 60	1426 ± 113

charge ratios except in the case of **1** at 3:1 charge ratio (data not reported). Generally, lipoplexes achieve maximum size when the net charge is zero, and the size decreases when the particle is either net positive or net negative (*27*−*29*). The net charge with glutamide lipids even at 3:1 charge ratio was negative in DMEM, suggesting charge neutralization did not occur at these charge ratios. Though the cationic charge is far in excess compared to the charge on the nucleic acids, the negative ζ-potential indicates that components of the medium DMEM may bind to the complexes and render them anionic. However, if the potential measurements were made in water, the potential was found to be positive above a 1:1 charge ratio (data not reported).

Transfection Studies with Lipids 1−4. We have tested the four dialkyl pyridinium glutamides for their transfection efficiencies with two helper lipids in three different cell lines, viz., MCF7, COS, and HeLa. Since non-cationic lipid additives in the formulations were known to enhance the transfection efficiency, we have used either DOPE or cholesterol as the helper lipid to investigate if the transfection efficiency is helper-lipid-dependent (*30*). Since the transfection efficiency depends on the ratio of the positive "lipid" charges to negative phosphate charges in the DNA, we have also tested the efficiency at various charge ratios (*23*). The reporter gene expression as measured by monitoring *β*-galactosidase activity is shown in Figure 4A−F to compare transfection efficiency among the three cell lines. MCF-7 cells showed maximum transfection compared to COS and HeLa cells. Between the two helper lipids, DOPE enhanced

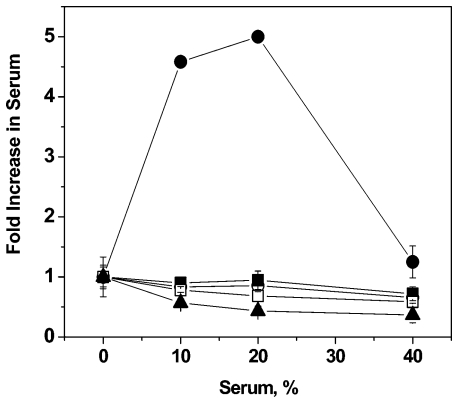

Figure 5. Transfection efficiency of lipid **2** in MCF-7 cells in the presence of serum. Transfection efficiency (fold increase) of lipid **2** + DOPE (●), with varying concentrations of serum compared with Lipofectamine (1 mg/mL) used at 1 μL (■), 2 μL (□), 4 μL (▲), and 8 μL (○) with 0.9 μg of DNA.

transfection in COS and HeLa cell lines compared to cholesterol; however, such differences were not apparent in the MCF-7 cell line where both the helper lipids elicited similar transfection efficiencies. Though the optimum charge ratio was dependent on the lipids and also on cell lines, a charge ratio of approximately 1:1 or 2:1 was found to be optimal. Very high (3:1) or low (0.3:1) charge ratios were not effective in transfection (*31*). Lipid **2** showed maximum transfection in all three cell lines at a 1:1 charge ratio with DOPE as the helper lipid. The observed maximum transfection with a 14-carbon lipid is in agreement with the reported literature that a 12- to 16-carbon length hydrophobic domain is optimally suited for transfection (*25*, *32*). Lack of transfection with shorter-chain lipids was suspected to be due to poor vesicle-forming abilities and toxicity to the cells. Long-chain lipids would probably result in stable and rigid vesicles unsuitable for transfection. Some lipids were reported to be efficient when the chain length was 18-carbon (*16*).

Transfection Efficiency in the Presence of Serum. Serum stability of the lipoplexes is very important for their in vivo efficacies (*33*, *34*). We have tested the transfection efficiency of lipids, initially in the presence of 10% serum, and found that only lipid **2** shows significant transfection efficiencies in the presence of serum (Figure 5). The other lipids showed decreased transfection efficiency in the presence of serum (data not shown). Serum contains nucleases, which would degrade the plasmid DNA, and also contains several proteins, including albumin, which would reduce the cell association properties of the lipoplex (*35*). We further tested lipid **2** with both the helper lipids in MCF-7 cells, in the presence of 0−40% serum along with a commercial transfecting agent Lipofectamine (Figure 5). We observed nearly a 5-fold increase in the transfection efficiency with lipoplexes with lipid **2** and DOPE in the presence of 20% serum (Figure 5). Unlike the small decrease in transfection efficiency observed with Lipofectamine, the transfection efficiency of lipid **2** has in fact enhanced by 5-fold in the presence of serum. Such increases in transfection efficiency by cationic lipids in the presence of serum have not been reported to our knowledge. This suggests that lipid **2** demonstrates a very interesting stability to serum and also a serum-mediated enhancement of transfection efficiency.

Toxicity of the Formulations on MCF-7. Since toxicity of the formulations to the cells is critical for transfection, we have tested the viability of MCF-7 cells in the presence of formulations prepared with the four lipids along with two helper lipids. Lipoplexes with cholesterol were found to be very toxic to the cell by decreasing the cell viability by more than 50% even at

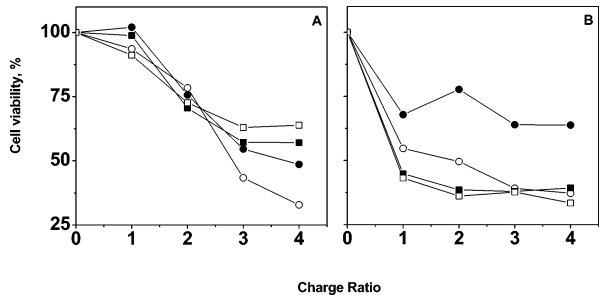

Figure 6. Cytotoxicity of lipids **1**−**4** on MCF-7 cells. Toxicity, measured as percent viability, was evaluated with lipids **1** (●), **2** (○), **3** (■), and **4** (□) with DOPE (A) and cholesterol (B) as helper lipids. The absorption values obtained using reduced formazon with cells in the absence of lipids were taken to be 100.

a 1:1 charge ratio. In comparison, DOPE marginally affects the viability at a 1:1 charge ratio and by 20% at a 2:1 charge ratio (Figure 6).

Four dialkyl glutamides of varying chain lengths were synthesized and characterized. The data presented here demonstrate that these lipids efficiently transfect three cell lines tested in this study. These lipids have significant binding to DNA at charge ratios above 2:1 when the helper lipid in the formulation is DOPE. In line with this observation, the DNaseI protection was also higher in the presence of DOPE compared to cholesterol. Cholesterol apparently does not form stable lipoplexes strongly for occluding the DNA inside the lipoplex. Even at a 3:1 charge ratio, the lipoplexes have a net negative charge leading to a continuous increase in size. Among the four lipids, lipid **2** with DOPE as the helper lipid shows very promising transfection efficiencies on all three cell lines tested. The efficiency of the cationic lipids with chain length in the range 12−16 carbons in length is well-documented, and often 14-carbon-length lipids are most efficient among several cationic lipids with differing chemistries (*15*). The balance between the stability of the liposome in the medium and the readiness of the liposome to release the DNA is important for good transfection efficiency (*36*). Short-chain lipids may not result in stable liposomes, and long-chain lipids may form very stable particles for release of the DNA. Lipid **2** with DOPE as the helper lipid has good DNA binding ability, provides DNaseI protection, and is also less toxic. All these properties together elicited high transfection efficiencies. The converse is true with cholesterol in most of the cases. The size of the lipoplexes does not show any correlation with the transfection efficiency, indicating that factors in addition to size play a role in lipoplex and cell interactions. The role of biophysical properties of the lipid−DNA complex in its association and uptake by cells is still poorly understood (*37*, *38*). The remarkable enhancement of transfection of lipoplexes prepared with lipid **2** in the presence of serum suggests the potential of this lipid for in vivo transfections.

Tetraalkyldiglutamides with long spacers were synthesized by alkylating the free amino group into tertiary amine (*39*). These lipids with charge on the glutamate, called as lipopeptides, were shown to transfect cells with efficiencies lower than Lipofectin on 293 cells. Pyridinium head groups have been made popular with the publication of the Saint series of lipids, though the pyridinium was bonded to a linker at C3 and an R group was attached to the nitrogen (*40*−*42*). Cationic lipids with a heterocyclic trimethylpyridinium as the polar head linked to two alkyl chains via a propyl group have shown good transfection in both in vitro and in vivo experiments (*43*). Unlike the Saint lipids, in the lipids described here, the pyridine is N-linked to the linker, i.e., glutamic acid. Dialkyl glutamides were tested for their ability to form supramolecular structures due to the presence of a chiral center (*19*). The intermolecular hydrogen

bonding among the amide moieties of the lipids contributes to self-assembly and thus stabilizes the liposome (*17*, *44*). As a linker molecule, glutamic acid offers a synthetic flexibility to design various cationic lipids. To the best of our knowledge, this is the first report where dialkyl glutamides have been shown to transfect several cell lines efficiently.

CONCLUSIONS

Four cationic lipids based on glutamide as a linker were synthesized. Formulations with DOPE as the helper lipid show excellent transfection in three different cell lines. Although DNA binding and protection from nuclease, size, or surface potential measurements of lipoplexes of these lipids show variation, there is no apparent correlation with their transfection abilities. Very remarkably, one of the lipids, lipid **2,** demonstrates enhanced transfection in the presence of serum, suggesting its physiological relevance and suitability for in vivo applications.

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