On the disulfide-linker strategy for designing efficacious cationic transfection lipids: an unexpected transfection profile

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Abstract Herein, employing a previously reported disulfide-linker strategy, we have designed and synthesized a novel cationic lipid 2 with a disulfide-linker and its non-disulfide control analog lipid 1. The relative efficacies of lipids 1 and 2 in transfecting CHO, COS-1 and MCF-7 cells were measured using both reporter gene and whole cell histochemical staining assays. In stark contrast to the expectation based on the disulfide-linker strategy, the control non-disulfide cationic lipid 1 showed phenomenally superior in vitro transfection efficacies to its essentially transfection incompetent disulfide counterpart lipid 2. Results in DNase I protection experiments and the electrophoretic gel patterns in the presence of glutathione, taken together, are consistent with the notion that the success of the disulfide-linker strategy may depend more critically on the DNase I sensitivity of the lipoplexes than on the efficient DNA release induced by intracellular glutathione pool.

Keywords: Disulfide-linker; Intracellular reduction; DNA transfection; Lipoplexes; Lipofection; Transgene expression

1. Introduction

Developing clinically successful gene therapy approach for treating myriads of inherited diseases critically depends upon the biosafety and gene transfer efficacies of the vectors used for delivering the therapeutic genes into the body cells [1–3]. The contemporary transfection vectors are broadly classified into two major categories: viral and non-viral. Recombinant retroviral vectors are remarkably efficient in transfecting body cells [4,5]. However, retroviral vectors are potentially capable of: generating replication-competent virus through recombination events with the host genome; inducing inflammatory and adverse immunogenic responses; producing insertional mutagenesis through random integration into the host genome, etc. [6–9]. Additional major disadvantages associated with the use of viral vectors include their incapability of transfecting non-dividing cells, limited insert-size and difficulty of large-scale production [4–9]. Conversely, cationic lipids, because of their least immunogenic nature, robust manufacture, ability to deliver large pieces of DNA, and ease of handling and preparation techniques, are finding increasing uses as the alternative non-viral vectors of choice in gene therapy [10–17].

Currently believed key mechanistic steps in cationic lipid mediated transfection pathways include formation of nano-size lipoplexes (liposome:DNA complexes), endocytotic cellular uptake of lipoplexes, escape of DNA from endosomes into the cell cytoplasm, trafficking of the endosomally released DNA into the cell nucleus and finally transgene expression [18–20]. Such complex transfection pathway makes rational design of efficient cationic transfection lipids an arduous task. A number of recent structure-activity investigations [21–24], including our own [25–30], have thrown significant new insights into the various architectural elements of cationic lipids necessary for overcoming the above mentioned cellular barriers involved in lipofection process.

Inefficient release of DNA from lipoplexes into the cell cytoplasm is believed to be one of the major impeding factors behind the generally poor transfection efficacies of cationic lipids. Towards this end, Tang and Hughes pioneered the use of the disulfide bond as the linker functionality of cationic transfection lipids [31,32]. The rationale behind this elegant approach was to ensure collapsing of the lipid:DNA complex inside the cell cytoplasm after reduction of the disulfide-linker by the intracellular glutathione pool. Exploitation of such intracellular disulfide reduction strategy has also been demonstrated recently in the area of antisense peptide nucleic acids (PNAs) delivery [33]. Inspired by this disulfide-linker strategy, we designed and synthesized a novel cationic disulfide lipid **2** towards further enhancing the in vitro gene transfer property of its novel non-disulfide counterpart lipid **1**, an efficient cationic transfection lipid recently designed in our laboratory. In the present investigation, we report on the strikingly unexpected relative efficacies of lipids **1** and **2** in transfecting CHO, COS-1 and MCF-7 cells, measured using both reporter gene and whole cell histochemical staining assays. Surprisingly, the control non-disulfide cationic lipid **1** showed phenomenally superior efficacies to its disulfide-linker counterpart lipid **2** in transfecting all the three cells. As delineated below, results in DNase I protection experiments and the electrophoretic gel patterns in the presence of glutathione, taken together, are consistent with the notion that the success of the

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; FBS, fetal bovine serum; DCC, dicyclohexyl carbodiimide; NHS, *N*-hydroxy succinimide; DMF, dimethyl formamide; DCM, dichloromethane; HCl, hydrochloric acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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disulfide-linker strategy may depend more critically on the DNase I sensitivity of the lipoplexes than on the efficient DNA release induced by the intracellular glutathione pool.

2. Materials and methods

2.1. Materials

Dicyclohexyl carbodiimide (DCC), *N*-hydroxy succinimide (NHS) and 1,2-ethylenediamine were procured from Merck, India. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60–120 mesh). Cell culture media, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), β-galactosidase enzyme and cholesterol were purchased from Sigma, St. Louis, USA. NP-40, antibiotics and agarose were purchased from Hi-media, India. LipofectAmine was purchased from Invitrogen life technologies, USA. Unless otherwise stated all the other reagents purchased from local commercial suppliers were of analytical grades and were used without further purification. ^{1}H NMR spectra were recorded on a Varian FT 200 MHz, AV 300 MHz or Varian Unity 400 MHz. The FABMS analyses were performed on a Micromass AUTOSPEC-M mass spectrometer (Manchester, UK) with OPUS V3, 1X data system. Data were acquired by liquid secondary ion mass spectrometry (LSIMS) using *meta*-nitrobenzyl alcohol as the matrix.

2.2. Syntheses

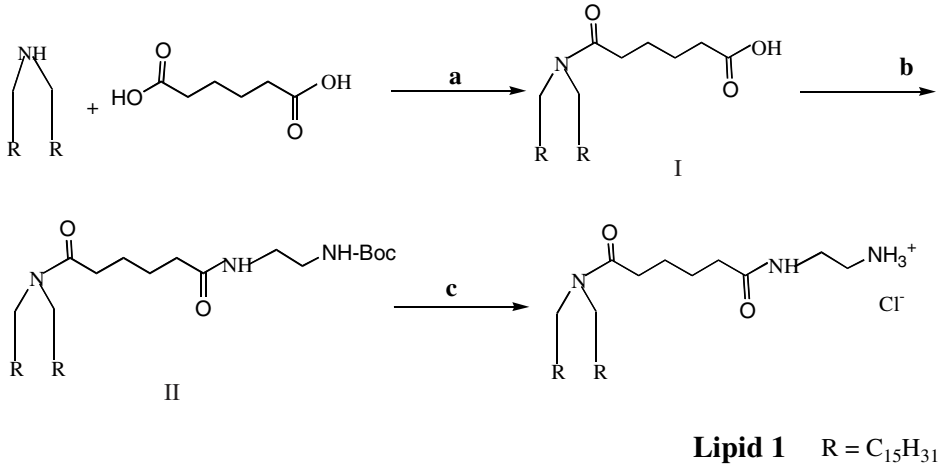
The synthetic procedures for preparing lipids **1** and **2** are depicted schematically in Fig. 1. Detailed experimental procedures are delineated below.

Synthesis of lipid **1**

*6-(Di-n-hexadecylamino)-6-oxohexanoic acid (***I***, Fig. 1):* Di-*n*-hexadecyl amine (2 g; 4.3 mmol) in dichloromethane (DCM; 25 ml) was added to dimethyl formamide (DMF; 5 ml) solution containing adipic acid (0.69 g; 4.73 mmol), NHS (0.55 g; 4.73 mmol) and the mixture was cooled to 0 °C. DCC (0.98 g; 4.73 mmol, dissolved in 20 ml DCM) was added dropwise to the cold solution. The reaction mixture was stirred overnight at room temperature. The solvent mixture was removed under vacuum and the residue was finally taken in DCM (50 ml). The solution was washed with water (3 × 50 ml), dried over anhydrous sodium sulfate and filtered. DCM was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue, using 60–120 mesh silica size and 1–2% methanol in chloroform (v/v) as the eluent, afforded the pure title intermediate 1.3 g (52%, $R_f = 0.4$ in 5% methanolic chloroform, v/v).

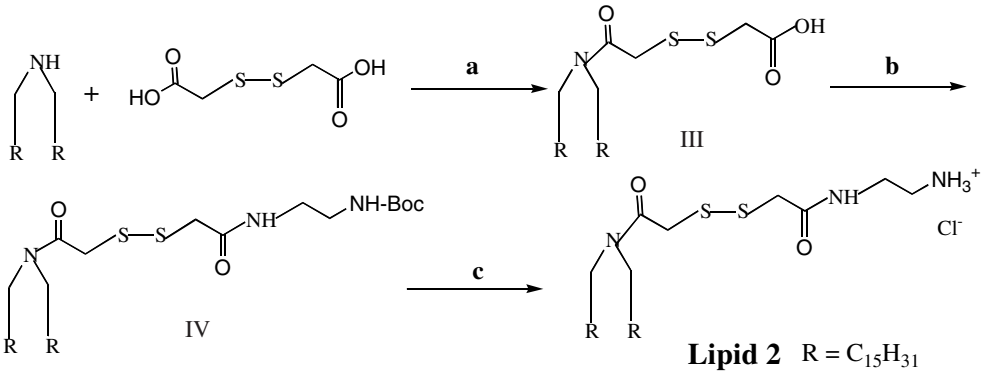
^{1}H NMR (200 MHz, CDCl$_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$–]; 1.1–1.3 [m, 52H, –$(CH_2)_{13}$–]; 1.4–1.6 [m, 4H, –NCO–CH_2–$(CH_2)_2$–CH_2–COOH]; 1.6–1.7 [m, 4H, –CH_2–CH_2–NCO–]; 2.2–2.4 [m, 4H, –NCO–CH_2–$(CH_2)_2$–CH_2–COOH]; 3.1–3.3 [m, 4H, –CH_2–CH_2–NCO–].

*Tert-butyl-(2-[6-(di-n-hexadecylamino)-6-oxohexanoyl]aminoethyl)-carbamate (***II***, Fig. 1):* A mixture of **I** (1.1 g; 1.86 mmol), *N-tert*-butoxycarbonyl-1,2-ethanediamine (0.30 g; 1.86 mmol), and NHS (0.21 g; 1.86 mmol) was taken in 20 ml dry DCM and was cooled to



Lipid 1 R = $C_{15}H_{31}$

Reaction Conditions: (**a**) DCC, NHS, dry DMF, dry DCM, 16h; (**b**) N-Boc-1,2- ethanediamine, DCC, NHS, dry DCM, 16h; (**c**) 2M HCl in Dioxane, 2h



Lipid 2 R = $C_{15}H_{31}$

Reaction Conditions: (a) DCC, NHS, dry DMF, dry DCM, 16h; (b) N-Boc-1,2- ethanediamine, DCC, NHS, dry DCM, 16h; (c) 2M HCl in Dioxane, 2h

Fig. 1. Synthesis of lipids **1** and **2**.

0 °C. DCC (0.38 g; 1.86 mmol) dissolved in 20 ml dry DCM was added dropwise to the cold solution. The reaction mixture was stirred overnight at room temperature. The reaction mixture was taken in DCM (50 ml), washed with water (3 × 50 ml), dried over anhydrous sodium sulfate and filtered. DCM was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue, using 60–120 mesh silica size and 0.5–1% methanol in chloroform (v/v) as the eluent, afforded the pure title intermediate (1.0 g, 73.5%, $R_f = 0.6$ in 5% methanolic chloroform, v/v).

^1H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$–]; 1.2–1.3 [m, 52H, –$(CH_2)_{13}$–]; 1.4–1.5 [m, 13H, –O–CO–C(CH_3)$_3$, –NCO–CH_2–$(CH_2)_2$–CH_2–CO–NH]; 1.6–1.7 [m, 4H, –CH_2–CH_2–NCO–]; 2.2–2.4 [m, 4H, –NCO–CH_2–$(CH_2)_2$–CH_2–CO-NH]; 3.1–3.4 [bm, 8H, –CH_2–CH_2–NCO–, –NH–CH_2–CH_2–NH–CO–]; 5.5 [bm, 1H, *NH*Boc]; 7.1 [bm, 1H, –CO–N*H*–].

2-{[6-(Di-n-hexadecylamino)-6-oxohexanoyl]amino} ethanaminium (lipid **1***, Fig. 1):* Intermediate **II** (0.9 g, 0.35 mmol) was dissolved in 1 ml of 2 N hydrochloric acid (HCl) in dioxane and the solution was kept under stirring at room temperature overnight. The solvent was removed with nitrogen flush and the residue was kept under vacuum for 1 h. Silica gel column chromatography, using 60–120 mesh silica size and 8–10% methanol in chloroform (v/v) as the eluent, afforded pure title lipid **1** (0.72 g, 86%, $R_f = 0.3$ in 15% methanolic chloroform, v/v).

^1H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$–]; 1.2–1.3 [m, 52H, –$(CH_2)_{13}$–]; 1.5–1.8 [dm, 8H, –NCO–CH_2–$(CH_2)_2$–CH_2–CO–NH, –CH_2–CH_2–NCO-0]; 2.5 [m, 4H, –NCO–CH_2–$(CH_2)_2$–CH_2–CO–NH]; 3.2–3.4 [m, 6H, –CH_2–CH_2–NCO–, –CONH–CH_2–CH_2–NH_3^+]; 3.6–3.7 [bm, 2H, –CONH–CH_2–CH_2–NH_3^+]; 8.3 [bm, 1H, –CO–N*H*–].

FABMS: m/z: 637 (100%).

Synthesis of lipid **2**

*[2-(Di-n-hexadecylamino)-2-oxoethyl]di-sulfanylacetic acid (***III***, Fig. 1):* Di-*n*-hexadecyl amine (2.0 g; 4.3 mmol) in DCM (25 ml) was added to a solution of dithioglycolic acid (0.78 g; 4.73 mmol) and NHS (0.54 g; 4.73 mmol) in DMF (5 ml) and the mixture was cooled to 0 °C. DCC (0.89 g; 4.73 mmol) in DCM (20 ml) was added dropwise to the cold solution. The reaction mixture was stirred overnight at room temperature. The solvent mixture was removed under vacuum and the residue was finally taken in DCM (50 ml). The solution was washed with water (3 × 50 ml), dried over anhydrous sodium sulfate and filtered. DCM was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue, using 60–120 mesh silica size and 1–2% methanol in chloroform (v/v) as the eluent, afforded 1.45 g (53.6%) of the pure intermediate **III** ($R_f = 0.4$ in 5% methanolic chloroform, v/v).

^1H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$–]; 1.2–1.4 [m, 52H, –$(CH_2)_{13}$–]; 1.5–1.7 [bm, 4H, –CH_2–CH_2–NCO–]; 3.2–3.4 [m, 4H, –CH_2–CH_2–NCO–]; 3.6 [s, 2H, –CH_2–SS]; 3.8 [s, 2H, SS–CH_2–].

*Tert-butyl-[2-{[(2-N,N-di-n-hexadecylamino-2-oxoethyl)disulfanyl]-acetyl} aminoethyl]carbamate (***IV***, Fig. 1):* A mixture of **III** (1.45 g; 2.3 mmol), *N-tert*-butoxycarbonyl-1,2-ethanediamine (0.41 g; 2.53 mmol), and NHS (0.27 g; 2.53 mmol) was taken in 20 ml dry dichloromethane and was cooled to 0 °C. DCC (0.48 g; 2.53 mmol) in DCM (20 ml) was added dropwise to the cold solution. The reaction mixture was stirred overnight at room temperature. The reaction mixture was taken in dichloromethane (50 ml), washed with water (3 × 50 ml), dried over anhydrous sodium sulphate and filtered. Dichloromethane was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue, using 60–120 mesh silica size and 1–2% methanol in chloroform (v/v) as the eluent, afforded 0.91 g (51.1%) of the pure intermediate **IV** ($R_f = 0.4$ in 5% methanolic chloroform, v/v).

^1H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$–]; 1.2–1.3 [m, 52H, –$(CH_2)_{13}$–]; 1.4 [s, 9H, –O–CO–C(CH_3)$_3$]; 1.5–1.7 [bm, 4H, –CH_2–CH_2–NCO–]; 3.21–3.37 [bm, 6H, –CH_2–CH_2–NCO–, –NH–CH_2–CH_2–NH–]; 3.4–3.5 [m, 4H, –CH_2–SS–, –NH–CH_2–CH_2–NH–]; 3.55 [s, 2H, –SS–CH_2–]; 5.5 [bm, 1H, N*H*Boc]; 8.1 [bm, 1H, –CO–N*H*–].

2-[({[2-(Dihexadecylamino)-2-oxoethyl]disulfanyl}acetyl)amino]-ethanaminium (lipid **2***, Fig. 1):* Compound **IV** (0.9 g, 1.2 mmol) was dissolved in 1 ml of 2 N HCl in dioxan and the solution was kept under stirring at room temperature overnight. The solvent was removed with nitrogen flush and the residue was kept under vacuum for 1 h. Silica gel column chromatography using 60–120 mesh silica size and 8–10% methanol in chloroform (v/v) as the eluent, afforded 0.76 g (93%) of pure lipid **2** ($R_f = 0.3$ in 15% methanolic chloroform, v/v).

^1H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3–$(CH_2)_{13}$-]; 1.2–1.3 [m, 52H, –$(CH_2)_{13}$–]; 1.5–1.7 [bm, 4H, –CH_2–CH_2–NCO–]; 3.17–3.40 [bm, 6H, –CH_2–CH_2–NCO–, –CONH–CH_2–CH_2–NH_3^+]; 3.47–3.80 [m, 6H, –CH_2–SS–CH_2–, –CONH–CH_2–CH_2–NH_3^+]; 8.1 [bm, 1H, –CO–N*H*–].

FABMS: m/z: 673 (70%).

2.3. Cells and cell culture

CHO (Chinese hamster ovary), COS-1 (SV 40 transformed African green monkey kidney cells) and MCF-7 (Human breast adenocarcinoma) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin and 20 μg/ml kanamycin in a humidified atmosphere containing 5% CO_2.

2.4. Preparation of plasmid DNA

pCMV-SPORT-β-gal plasmid was a generous gift from Dr. Nalam Madhusudhana Rao (Centre for Cellular and Molecular Biology, Hyderabad, India). Plasmid was amplified in DH5α strain of *Escherichia coli*, isolated by alkaline lysis procedure and finally purified by PEG-8000 precipitation as described previously [34]. The purity of plasmid was checked by A_{260}/A_{280} ratio (around 1.85) and 1% agarose gel electrophoresis.

2.5. Preparation of liposomes

Liposomes were prepared by the ethanol injection method. Briefly, 75 μl of a 5.4 mM ethanolic solution of cationic lipids **1**, **2** and Cholesterol (at 2:1 mole ratio) was rapidly injected into 1 ml of HEPES buffer (pH 7.4) under vortexing to give a final cationic lipid concentration of 0.4 mM. The liposomes were kept for 15 min at room temperature before transfection.

2.6. Transfection procedure

Cells were seeded at a density of 20 000 cells/well (for CHO and MCF-7) or 15 000 cells/well (for COS-1) in a 96-well plate usually 18–24 h before transfection. Plasmid DNA (0.30 μg diluted to 50 μl with plain DMEM) was complexed with varying amount of cationic liposomes (diluted to 50 μl with plain DMEM) for 15–30 min. The mole ratios (lipid:DNA) were varied from 0.5:1 to 4:1. Cells were washed twice with PBS buffer (pH 7.4, 100 μl each) and the lipid:DNA complex was added to the cells. After incubating for 3 h in a humidified atmosphere containing 5% CO_2 at 37 °C, 100 μl of DMEM containing 20% FBS was added to the cells. The medium was changed to complete medium containing 10% FBS after 24 h and the reporter gene activity was assayed 48 h after transfection. Cells were washed once with PBS buffer, pH 7.40 (100 μl), and lysed with 50 μl of lysis buffer (0.25 M Tris–HCl, pH 8.0, and 0.5% NP-40). The β-galactosidase activity per well was estimated by adding 50 μl of 2× substrate solution (1.33 mg/ml of ONPG, 0.2 M sodium phosphate, pH 7.30, and 2 mM magnesium chloride) to the cell lysate in a 96-well plate. Absorption of the product *ortho*-nitrophenol at 405 nm was converted to absolute β-galactosidase units by using a calibration curve constructed with pure (commercial) β-galactosidase enzyme. The transfection values reported are the average values from two replicate experiments performed in the same plate on the same day. Each transfection experiment was performed three times on three different days. The day to day variation in transfection efficiency was mostly within 2–3-fold and was dependent on the cell density and condition of the cells.

2.7. X-gal staining

Cells expressing β-galactosidase were histochemically stained with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously [35]. Briefly, forty eight hours after transfection with lipoplexes in 96-well plates, the cells were washed two times (2× 100 μl) with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) and fixed with 0.5% glutaraldehyde in PBS (225 μl). After 15 min incubation at room temperature, the cells were washed again with PBS three times (3× 250 μl) and were stained subsequently with 1.0 mg/ml X-gal in PBS containing 5.0 mM $K_3[Fe(CN)_6]$, and 5.0 mM $K_4[Fe(CN)_6]$

and 1 mM $MgSO_4$ for 2–4 h at 37 °C. Blue colored cells were identified by light microscopy (Leica, Germany). A minimum of 100 cells were counted to determine the percentage of cells expressing β-galactosidase.

2.8. DNA binding assay

The DNA binding ability of the cationic lipids **1** and **2** (Fig. 5A) was assessed by their gel retardation assay on a 1% agarose gel (pre-stained with ethidium bromide) across the varying lipid:DNA charge ratios of 0.5:1 to 4:1. pCMV-β-gal (1 μg) was complexed with the varying amount of cationic lipids in a total volume of 50 μl in HEPES buffer (pH 7.40) and incubated at room temperature for 30 min. 10 μl of 6× loading buffer (0.25% Bromophenol blue in 40%, w/v, sucrose in H_2O) was added to it and 20 μl from the resulting 60 μl solution was loaded on each well. The samples were electrophoresed at 90 V for 2 h and the DNA bands were visualized in a gel documentation unit.

2.9. DNase I sensitivity assay

Briefly, in a typical assay pCMV-β-gal (1 μg) was complexed with the varying amount of cationic lipids **1** and **2** (using indicated lipid:DNA charge ratios in Fig. 5C) in a total volume of 40 μl in HEPES buffer, pH 7.40, and incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with 10 μl DNase I (at a final concentration of 1 μg/ml) in the presence of 20 mM $MgCl_2$ and incubated for 20 min at 37 °C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM) and incubated at 60 °C for 10 min in a water bath. The aqueous layer was washed with 50 μl of phenol:chloroform:isoamylalcohol (25:24:1 mixture, v/v) and centrifuged at $10\,000 \times g$ for 5 min. The aqueous supernatants were separated, loaded (20 μl) on a 1% agarose gel (pre-stained with ethidium bromide) and electrophoresed at 90 V for 2 h.

2.10. Monitoring glutathione-induced DNA release from DNA–liposome complexes

One microgram of plasmid DNA was dissolved in 10 μl of 10 mM HEPES buffer (pH 7.4). Cationic liposomes with Lipids **1** or **2** were added to the pDNA solution to obtain final lipid:DNA (+/−) charge ratio of 4:1 (Fig. 5B). After the complexes were incubated for 30 min at room temperature, 10 μl of 50 mM of glutathione in 10 mM HBS (pH 7.3) was added to the mixture to reach a final 10 mM concentration of glutathione. The mixtures were incubated at 37 °C for 20 h. Released DNA was visualized by 1% agarose gel electrophoresis.

2.11. Cell viability assay

Cytotoxicities of the cationic lipids were assessed using MTT reduction assay as described earlier [36]. The cytotoxicity assay was performed in 96-well plates by maintaining the same ratio of cell to the amount of cationic lipid as in transfection experiments. Briefly, 3 h after the addition of lipoplexes, MTT (5 mg/ml PBS) was added to the cells and incubated for 3–4 h at 37 °C in a CO_2 incubator. Results are expressed as percent viability = $[A_{550}(\text{treated cells}) - \text{background}]/[A_{550}(\text{untreated cells}) - \text{background}] \times 100$.

2.12. Liposome and lipoplex size measurements

The nano-sizes of the liposomes and lipoplexes in HEPES buffer, pH 7.4, were measured by dynamic laser light scattering technique (Zetasizer 3000HAS, Malvern Instruments, UK). The system was calibrated by using the 199 ± 6 nm Nanosphere™ Size Standard (Duke Scientific Corp., Palo Alto, CA, USA) and DTS 0050 standard from Malvern.

3. Results and discussion

Lipid **1** (Fig. 1) was designed and synthesized in our on-going structure–activity program in the area of liposomal gene delivery [25–30] as a non-glycerol based mono-cationic lipid where the positively charged terminal amine group was separated from the hydrophobic aliphatic tails by a reasonably long spacer arm. Fig. 2A–C summarizes the efficacies of lipids **1** and **2** (used as cationic liposomes prepared in combination with cholesterol at a mole ratio of 2:1) in transfecting CHO, COS-1 and MCF-7 cells across the increasing lipid:DNA mole ratios

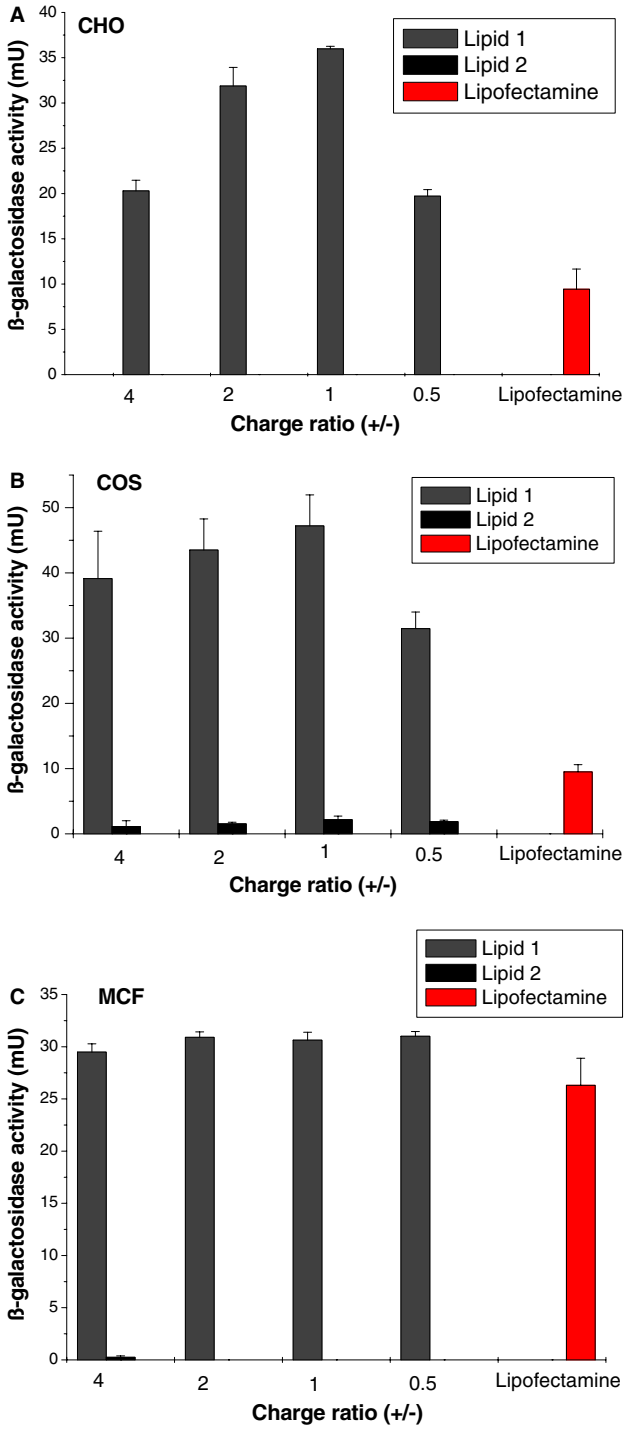


Fig. 2. Transfection efficiencies of cationic lipids **1** and **2** and lipofectamine in CHO (A), COS-1 (B) and MCF-7 (C) cells. The β-galactosidase activities in each well were converted to an absolute β-galactosidase milliunits using standard curve obtained with pure (commercial) β-galactosidase. All the lipids were tested on the same day and the data shown are the average values of three replicate experiments performed on the same day ($n = 3$). Each transfection experiment was performed three times on three different days.

0.5:1.0–4.0:1.0 (using pCMV-SPORT-β-gal plasmid as the reporter gene). After observing the remarkably high efficacies of lipid **1** in transfecting CHO, COS-1 and MCF-7 cells (Fig. 2A–C), the elegant disulfide-linker strategy pioneered by Tang and

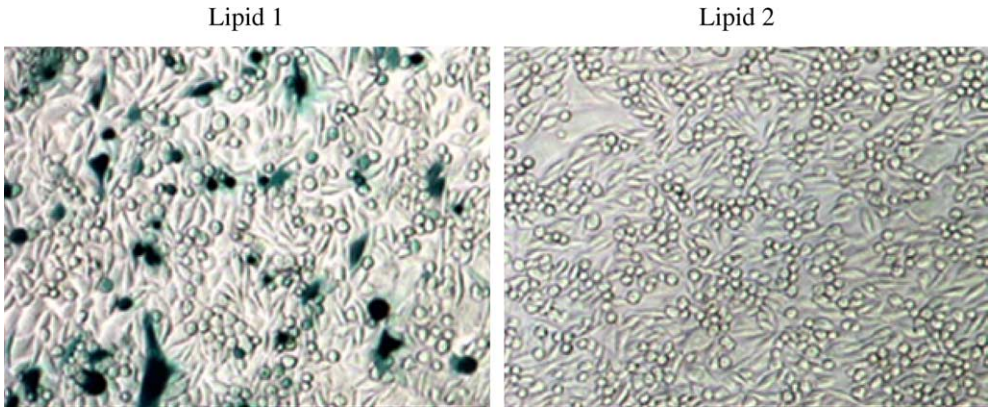


Fig. 3. Histochemical X-gal staining of transfected CHO cells with lipids **1** and **2** at lipid:DNA mole ratio of 1:1. Cells expressing β-galactosidase were stained with X-gal as described in the text.

Hughes [31,32] appeared as the first choice to us for further enhancing the gene transfer properties of the mono-cationic lipid **1**. With this view in mind, we designed and synthesized lipid **2** as the disulfide-linker analog of lipid **1**. Based on the rationale of the disulfide-linker strategy, our expectation was that the intracellular glutathione pool would reduce the disulfide bond of lipid **2** inside the cytoplasm thereby inducing its improved transfection efficacies via significant cytoplasmic release of plasmid DNA. In stark contrast to such expectations, lipid **2** was found to be essentially incompetent compared to lipid **1** in transfecting COS-1, CHO and MCF-7 cells (Fig. 2A–C). The whole cell histochemical X-gal staining of representative CHO cells (Fig. 3) further confirmed the strikingly unexpected relative transfection profiles of lipids **1** and **2** observed in the reporter gene assay (Fig. 2A–C).

Towards gaining insights into whether the dramatically unexpected in vitro DNA transfection profiles of lipids **1** and **2** were due to their varying inherent toxicity profiles, MTT-based cell viability assays were performed in representative CHO cells across the entire range of lipid:DNA mole ratios used in the actual transfection experiments. Per cent cell viabilities of both lipids **1** and **2** were found to be remarkably high upto lipid:DNA charge ratio 4:1 (>80% cell viability, Fig. 4). Thus, the phenomenally unexpected relative transfection efficacies of lipids **1** and **2** (Figs. 2 and 3) are unlikely to originate from varying cell cytotoxicities of the lipids. Next, with a view to understand whether or not the surprising relative transfection profiles could originate due to varying lipid:DNA binding interactions, we performed the conventional gel retardation assays by loading lipoplexes having lipid:DNA charge ratios across the range 4:1–0.5:1 on a 1% agarose gel. Intensities of free unassociated DNA bands for lipoplexes **1** were found to be significantly less than those for lipoplexes **2** at lipid:DNA charge ratios 4:1, 2:1 and 1:1 (Fig. 5A). Such electrophoretic gel pattern (Fig. 5A) supports the notion that relatively poor lipid:DNA binding interactions might play an important role in abolishing the in vitro gene transfer properties of lipid **2**.

Although lipid:DNA binding interactions in lipoplex **2** were, in general, found to be weaker than those for lipoplex **1**, such interactions were not too week for lipoplex **2** at higher lipid:DNA charge ratios of 4:1 and 2:1 (Fig. 5A). The gel patterns indicated that the lipid:DNA binding interactions were approximately similar in lipoplex **2** with 4:1 lipid:DNA charge ratio and lipoplex **1** with 1:1 lipid:DNA charge ratio (Fig. 5A).

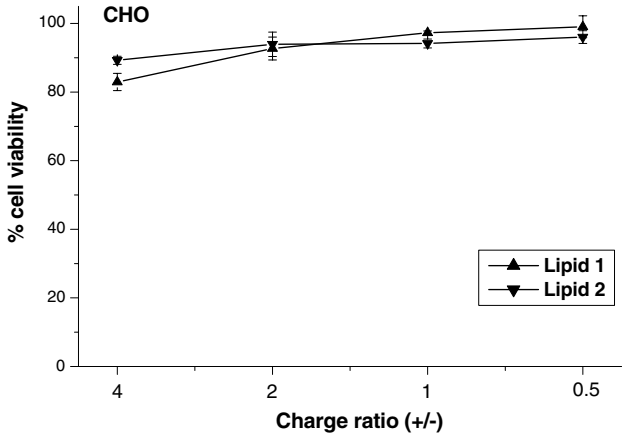


Fig. 4. Representative percent cell viabilities of cationic lipids **1** and **2** in CHO cells using MTT-based assay. The absorption obtained for reduced formazan formed in cells untreated with cationic lipids was taken to be 100. The toxicity assays were performed as described in the text. The data presented are average values of duplicate experiments ($n = 2$). Results were expressed as percent viability = $[A_{550}$(treated cells) – background$]/[A_{550}$(untreated cells) – background$] \times 100$.

In spite of such similar lipid:DNA interactions, what appeared very surprising to us is that lipid **1** was highly competent at 1:1 lipid:DNA charge ratio in transfecting all three cell lines (with efficacies better than or comparable to that of LipofectAmine, most widely used commercially available liposomal transfection kits) and yet lipid **2** was essentially transfection incompetent at 4:1 lipid:DNA charge ratio (Fig. 2). Since lipid **2** was found to be essentially incompetent in transfecting all three cells across the entire lipid:DNA charge ratios (Fig. 2), next we decided to check whether or not the disulfide-linker of lipoplex **2** is reducible by glutathione. Towards this end, we performed a representative gel retardation assay in the presence of 10 mM glutathione using lipoplexes **1** and **2** with lipid:DNA charge ratio of 4:1. Consistent with the rationale of the disulfide-linker strategy, significant DNA was released from lipoplex **2** when incubated for 20 h in the presence of 10 mM glutathione and the gel patterns for lipoplex **1** were found to be completely insensitive to glutathione (Fig. 5B). Thus, the possibility of inefficient cytoplasmic reduction by the intracellular glutathione pool is unlikely to play any key role behind the severely compromised transfection properties of lipid **2**.

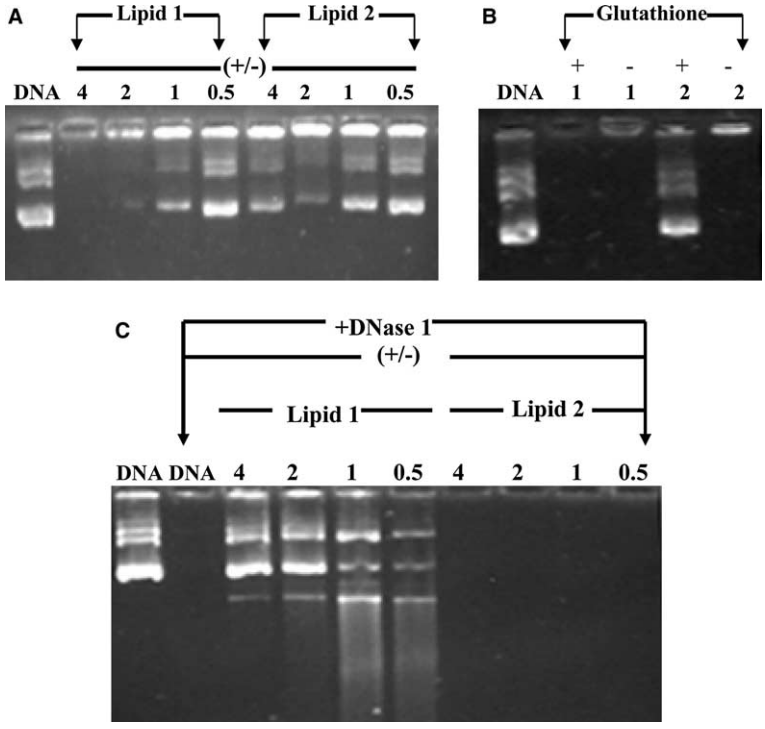


Fig. 5. Electrophoretic gel retardation and DNase I sensitivity assays. (A–C) depict gel patterns observed with pure lipoplexes, lipoplexes in the presence of 10 mM glutathione and lipoplexes treated with DNase I, respectively. The lipid:DNA charge ratios used in gel retardation assays with pure lipoplex (A) and in DNase I sensitivity assay (C) are indicated at the top of each lane. A lipid:DNA charge ratio of 4:1 was used in gel retardation assay in the presence of glutathione (B). The details of treatment are as described in the text.

Finally, with a view to getting some insights into the relative DNase I sensitivity profiles of lipoplexes **1** and **2**, DNase I protection experiments across the entire range of the lipid:DNA charge ratios were carried out by incubating the lipoplexes with DNase I. After the free DNA digestion by DNase I, the total DNA (both digested and inaccessible DNA) was separated from the lipid and DNase I (by extracting with organic solvent) and loaded onto a 1% agarose gel. Fig. 5C depicts the electrophoretic gel patterns observed for lipids **1** and **2** in DNase I sensitivity assays. Band intensities of any inaccessible, and therefore undigested, DNA associated with transfection incompetent lipoplexes prepared from lipid **2** were practically invisible compared to those associated with the most transfection efficient lipoplexes made from lipid **1** across the range of lipid:DNA charge ratios of 4:1 to 0:5:0.1 (Fig. 5C). Such gel patterns in DNase I sensitivity assays indicate that the plasmid DNA associated with lipid **2** is extremely susceptible to degradation by cellular DNase I than the DNA complexed to lipid **1**. Taken together, the findings in the DNase I protection experiments (Fig. 5C) and gel retardation assays (Figs. 5A and B) are consistent with the notion that transfection incompetency of lipid **2** is likely to originate, in part, from extreme DNase I sensitivities of the lipoplex **2**. However, the origin of such strikingly contrasting DNase I sensitivities for lipoplexes **1** and **2** remains elusive at this stage of investigation.

The nano-sizes of the lipoplexes prepared from lipids **1** and **2** across the varying lipid:DNA charge ratios (4:1–0.5:1) were measured using dynamic laser light scattering technique and the sizes of more than 90% of the lipoplex population were found to vary within 130–240 nm (data not shown). Based on such similar lipoplex size range and the same mono-cationic nature of both lipids **1** and **2**, the endocytotic cellular uptake efficiencies for the lipoplexes **1** and **2** are unlikely to be dramatically different. Interestingly, the cellular uptake of plasmid DNA complexed with a transfection efficient cholesterol-based disulfide-linker containing cationic lipid has been reported earlier to be even less than that of lipoplexes prepared from the corresponding non-disulfide transfection-inefficient counterpart [32]. Thus, correlating cellular uptake efficiency and transfection efficacies are not that straightforward. Flow cytometry experiments involving the use of fluorescently labeled plasmid DNA need to be carried out in the future to measure the relative cellular uptake efficiencies of lipoplexes **1** and **2**. However, given the extreme DNase I sensitivity of lipid **2**, flow cytometric technique may also fail to provide much useful insights towards this end. To conclude, results in our DNase I protection experiments and the electrophoretic gel patterns in the presence of glutathione indicate that the success of the disulfide-linker strategy may depend more critically on the DNase I sensitivity of the lipoplexes than on the efficient DNA release induced by intracellular glutathione pool.

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