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Evaluation of the influence of ionization states and spacers in the thermotropic phase behaviour of amino acid-based cationic lipids and the transfection efficiency of their assemblies

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ABSTRACT

The influence of both the ionization states and the hydrocarbon chain spacer of a series of amino acid-based cationic lipids was evaluated in terms of gene delivery efficiency and cytotoxicity to the COS-7 cell line and compared with that of Lipofectamine™ 2000. We synthesized a series of amino acid-based cationic lipids with different ionization states (i.e., $-NH_2$, $-NH_3^+Cl^-$ or $-NH_3^+TFA^-$) in the lysine head group and different hydrocarbon chain spacers (i.e., 0, 3, 5 or 7 carbon atoms) between the hydrophilic head group and hydrophobic moieties. In the 3-carbon series, the cationic assemblies formed a micellar structure in the presence of $-NH_3^+Cl^-$ and a vesicular structure both in the presence of $-NH_2$ and $-NH_3^+TFA^-$. Differential scanning calorimetry (DSC) data revealed a significantly lower (8.1 °C) gel-to-liquid crystalline phase transition temperature for cationic assemblies bearing $-NH_3^+TFA^-$ when compared to their $-NH_2$ counterparts. Furthermore, the zeta potential of cationic assemblies having $-NH_3^+TFA^-$ in the hydrophilic head group was maximum followed by $-NH_3^+Cl^-$ and $-NH_2$ irrespective of their hydrocarbon chain spacer length. The gene delivery efficiency in relation to the ionization states of the hydrophilic head group was as follows: $-NH_3^+TFA^- > -NH_3^+Cl^- > -NH_2$.

1. Introduction

Delivery of foreign DNA to living cells is an invaluable technique for treating various complicated genetic disorders and has become an important aspect of nano-biomedical engineering research. However, the strategies available for introducing foreign DNA into living cells are limited because of their low transfection efficiencies and associated high level of cytotoxicity. The nonviral gene delivery vectors have many advantages over their viral counterparts, including the lack of specific immune response, relatively low cytotoxicity, ability to deliver large pieces of DNA, ease of handling and preparation techniques, and well defined physical and chemical compositions (Li and Huang, 2000, 2006; Ma et al., 2007; Mintzer and Simanek, 2009; Wiethoff et al., 2004). The principal disadvantages of nonviral gene delivery vectors are their relatively low transfection efficiency and short duration of gene expression (Li and Huang, 2000; Miller, 1998). To date, a range of nonviral gene delivery vectors such as liposomes (Chien et al., 2005; Felgner et al., 1987, 1994), micelles (Itaka et al., 2003; Vigneron et al., 1996), dendrimers (Haensler and Szoka, 1993; Kukowska-Latallo et al., 1996) and cationic polymers (Boussif et al., 1995; Wagner et al., 1990) have been investigated as potential carriers for plasmid DNA.

A detailed study on the structure-activity relationship of cationic lipids has been performed by many research groups (Karmali et al., 2004, 2006; Koynova and Tenchov, 2009). Important cationic lipids such as *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) (Felgner et al., 1987), didecyldimethylammonium bromide (DDAB) (You et al., 1999), 1,2-dioleoyl-3-dimethylamonium propane (DODAP) (Jeffs et al., 2005), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulphate (DOTAP) (Leventis and Silvius, 1990), 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-L-propanaminium trifluoroacetate (DOSPA) or di-octadecyl-amido-glycyl-spermine (DOGS) (Behr et al., 1989) are

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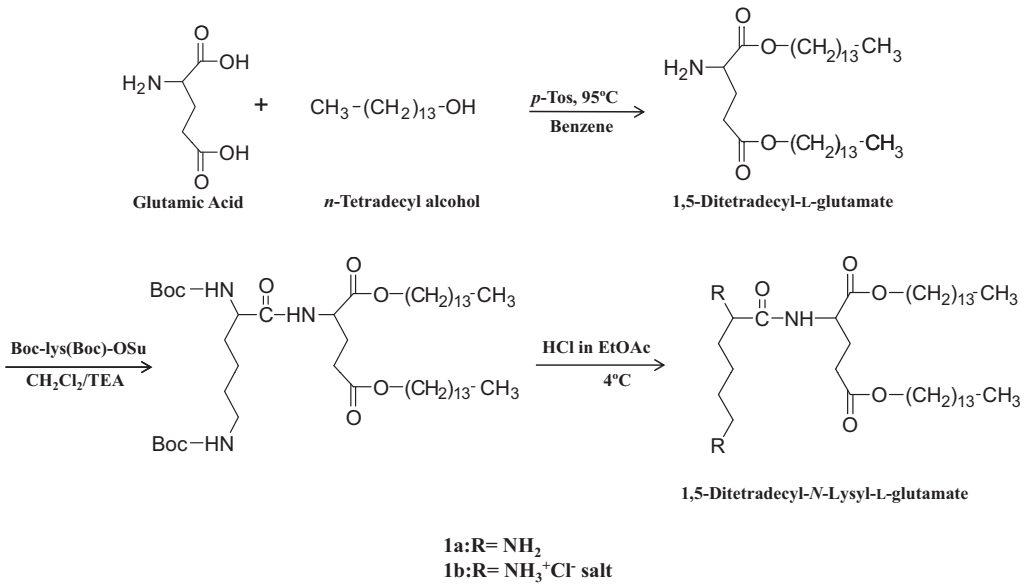


Scheme 1. Syntheses of the amino acid-based cationic lipids with different ionization states.

usually used to prepare cationic liposomes. The cationic liposomes offer positively charged interfaces for effective complexation with DNA to form lipoplexes *via* electrostatic interaction and protect DNA from digestion by intracellular DNases (Bhattacharya and Bajaj, 2009). The commercially available transfection reagent Lipofectamine™ 2000 is a proprietary formulation of cationic lipids and a polymer. Cationic lipids comprise a cationic head group and a hydrophobic moiety such as hydrocarbon chains (Behr et al., 1989; Sen and Chaudhuri, 2005) or a cholesterol derivative (Bajaj et al., 2007b; Choi et al., 2001). Numerous reports have confirmed that the gene transfer efficiency of cationic liposomes is influenced by their molecular architecture, including the length of the hydrophobic alkyl chain (Felgner et al., 1994), nature of the head group (Heyes et al., 2002; Majeti et al., 2004; Obata et al., 2008) as well as the characteristics of the linker and spacer functionalities used to covalently tether the polar head group and nonpolar tail (Ghosh et al., 2002; Obata et al., 2009; Rajesh et al., 2007). Recently, it has been reported that the tocopherol based cationic lipid with three hydroxyl groups in its head group region has better transfection efficiency than the commercial formulation (i.e., Lipofect) (Kedika and Patri, 2011). Bhattacharya et al. reported the influence of spacers on the transfection efficiency of gemini cholesterol-based cationic amphiphiles and their serum compatibility (Bajaj et al., 2007a, 2008). We have also previously reported the influence of different spacers such as hydrophobic and hydrophilic parts between the cationic head group and hydrophobic moieties in cationic liposomes and confirmed that a suitable hydrocarbon chain spacer enhances the transfection efficiency of cationic assemblies by many fold (Obata et al., 2009). Specifically, the length of the spacer was shown to influence (i) the hydration level and organization of the corresponding liposomes prepared from cationic gemini amphiphiles, (ii) difference in lipid hydration level and (iii) DNA conformation upon formation of the complex (Luciani et al., 2007). It was also reported that the counterions influence cationic lipid-mediated (i.e., quaternary trimethylammonium cytofectin (DOTAP)) transfection of plasmid DNA (Aberle et al., 1996). However, until now, a detailed study has not been performed with regard to the morphology, size, zeta potential and phase transition temperature of these cationic assemblies.

Our data demonstrate that the ionization states of the cationic head group play a significant role in determining the properties of amino acid-based cationic assemblies such as size, zeta potential, overall morphology and phase transition temperature. In particular, the phase transition temperature of cationic assemblies determines their membrane fusion potential. This is because the higher the phase transition temperature the higher the membrane rigidity, which in turn lowers the membrane fusion potential and, consequently, the transfection efficiency. In this study, we designed and synthesized a series of amino acid-based cationic lipids with different ionization states (i.e., $-NH_2$, $-NH_3^+Cl^-$ or $-NH_3^+TFA^-$) in the hydrophilic head group moiety and different hydrocarbon chain spacers (i.e., 0, 3, 5 or 7 carbon atoms) between the hydrophilic head group and the hydrophobic moiety to investigate the influence of ionization states of the cationic head group and hydrocarbon chain spacer on the transfection efficiency. Cationic assemblies prepared from the cationic lipids were characterized with regard to the size, morphology, zeta potential and phase transition temperature. The transfection efficiencies of these cationic assemblies were also evaluated using the COS-7 cell line both in the absence and presence of serum (10% FBS) and compared with that of Lipofectamine™ 2000.

2. Materials and methods

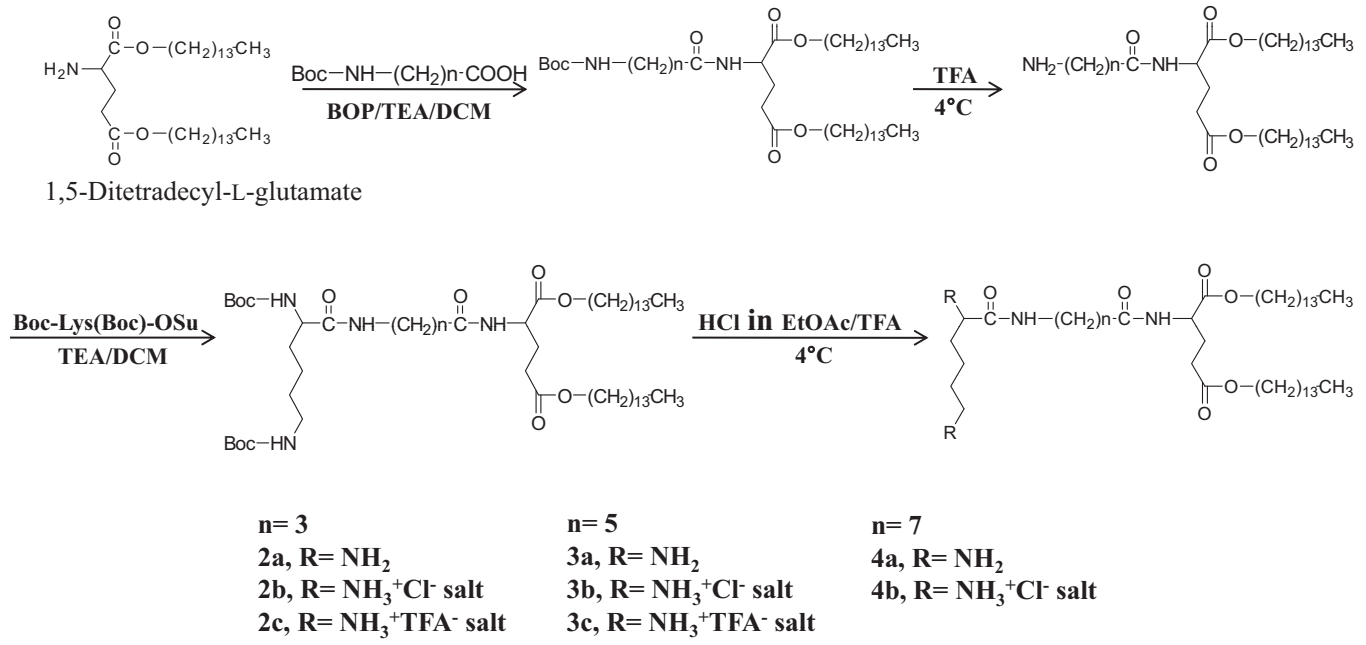
2.1. Materials

The following reagents were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan): 4-aminobutyric acid and 6-amino hexanoic acid. L-Glutamic acid, *p*-toluene sulfonic acid mono hydrate (*p*-Tos), benzotriazol-L-yloxytris (dimethylamino) phosphonium hexafluorophosphate (BOP) and tetradecyl alcohol were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). *N*,*N*′-Di-Boc-L-lysine hydroxysuccinimide ester (Boc-Lys(Boc)-OSu) and di-tert-butyl dicarbonate were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). 8-Aminocaprylic acid was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Syntheses

2.2.1. Syntheses of cationic lipids

We synthesized all the cationic lipids according to the protocol of Obata et al. (2008, 2009) with some modifications in the deprotection step as shown in Schemes 1 and 2 (detailed synthetic procedures are given in Supplementary information). Deprotection



Scheme 2. Syntheses of the amino acid-based cationic lipids with different ionization states and different hydrocarbon chain spacers.

with HCl (4 M) in ethyl acetate followed by neutralization with saturated sodium carbonate solution gave **1a**, **2a**, **3a** and **4a**. Deprotection with HCl (4 M) in ethyl acetate followed by removal of excess HCl under reduced pressure gave **1b**, **2b**, **3b** and **4b**. Deprotection with TFA at 4 °C overnight followed by removal of excess TFA gave **2c** and **3c**.

2.3. Preparation of the cationic assemblies

The lyophilized lipid powder (10 mg) was hydrated in 1 mL HEPES buffer (20 mM, pH 7.4) for 12 h and extruded through a LIPLEX EXTRUDER (Northern Lipids Inc.; Vancouver, Canada) with membrane filters (Whatman polycarbonate membrane filters with pore sizes of 0.2 and 0.1 μm) at 60 °C. The cationic assemblies were prepared from all the synthetic lipids. The lipid concentration of the cationic assemblies was determined by HPLC analysis (Meyer et al., 2000).

2.4. Microscopic observation of the cationic assemblies

The morphology of the cationic assemblies was analysed by transmission electron microscopy (TEM). The cationic assembling dispersion (5 μL) was mixed with 1% (5 μL) phosphotungstic acid (pH 7.4) in a microcentrifuge tube and incubated for 3 min. Then a drop of the sample dispersion was placed on a 150 mesh copper grid and excess dispersion was carefully removed with filter paper and examined under transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan). Electron micrographs recorded on imaging plates were scanned and digitized by an FDL 5000 imaging system (Fujifilm, Tokyo, Japan).

2.5. Preparation of lipoplexes

The cationic assemblies (0.6–6 μg lipid) were mixed with 0.2 μg pGL4.75 [hRluc/CMV] plasmid vector (4281 bp; Promega, Madison, WI) encoding Renilla luciferase. The mixed solutions were gently agitated and incubated at room temperature for 20 min. The solutions were then diluted with an appropriate volume of Dulbecco's modified Eagle's medium (DMEM) with or without fetal bovine serum (10% FBS) to analyse the gene transfer efficiency. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used as the gold standard for gene transfer studies according to the manufacturer's guidelines.

2.6. Measurement of size distribution and zeta potential of the cationic assemblies and the lipoplexes

A dispersion of the cationic assemblies (5 μL) containing 10 mg/mL lipid or a dispersion of lipoplexes (0.6–6 μg lipid and 0.2 μg pDNA) were diluted with 20 mM HEPES buffer (1 mL, pH 7.4). The mean particle diameter was then measured with a dynamic light scattering spectrophotometer (N4 PLUS Submicron Particle Size Analyzer, Beckman-Coulter Inc.; Fullerton, FL). The data represents an average diameter and standard deviation (SD) from measurements made in triplicate.

The zeta potential of cationic assemblies and lipoplexes were measured with a Zetasizer (Zetasizer 4; Malvern, UK). The liposome (0.1 mg/mL lipid) and the lipoplex (0.6–6 μg lipid and 0.2 μg pDNA) dispersions in 20 mM HEPES buffer (pH 7.4) were loaded in a capillary cell mounted on the apparatus and measured five times at 37 °C. The data represents an average zeta potential and standard deviation (SD) from measurements made in triplicate.

2.7. Calorimetric analysis of the cationic assemblies

The gel-to-liquid crystalline phase transition temperature (T_c) of the cationic assemblies was investigated by differential scanning calorimetry (DSC). The dispersion of cationic assemblies (20 μL, 10 mg/mL) was added to a silver pan and sealed. A reference pan was mounted with 20 μL of 20 mM HEPES buffer (pH 7.4). Both the pans are then placed in a DSC cell compartment. The measurement was started from 0 °C and the temperature was raised at a rate of 2 °C/min up to 80 °C. The phase transition temperature was estimated from the DSC curve.

2.8. Gel retardation assay of the lipoplexes

The lipoplexes prepared from different ratios of lipid (0.6–6 μg) to plasmid DNA (0.2 μg) were loaded onto a 1% seaplaque GTG agarose gel (Takara Bio. Inc., Otsu, Japan) containing ethidium bromide (EtBr). The gel was run at 90 V for 30 min and visualized on a UV transilluminator.

2.9. Transfection of cells

COS-7 cells (transformed African green monkey kidney fibroblast cells) were utilized for evaluating the gene expression efficiencies of various cationic assemblies. The cells were seeded into a 96 well plate at a density of 1×10^4 cells/well and cultured with DMEM containing 10% FBS in an atmosphere of 5% CO_2 at 37 °C overnight. The medium in each well of the cell culture dish was exchanged with lipoplexes (100 μL) containing varying amounts of cationic assemblies (0.6–6 μg) and 0.2 μg of plasmid DNA both in the absence and presence of serum (i.e., 10% FBS). After incubation under the same conditions for 24 h, the cells were washed twice with ice-cold PBS solution and lysed using lysis buffer provided with a luciferase assay kit (Promega, Madison, WI). The luciferase activity of 10 μL aliquot of the cell lysate was measured using a Microplate Luminometer (Promega, Madison, WI). The protein concentration of the cell lysate was determined by a standard protein assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA) protocol. The luciferase activity in each sample was normalized to the relative light unit (RLU) per microgram of protein. The data represent the average gene expression efficiency and a standard deviation (SD) value from independent measurements performed in triplicate. Furthermore, the gene expression efficiency of the lipoplexes was compared with that of Lipofectamine™ 2000 (Invitrogen).

2.10. Toxicity of the cationic assemblies

The cytotoxicity of the cationic assemblies with different ionization states and hydrocarbon chain spacers was investigated in COS-7 cells. The cells were seeded into a 96 well plate at a density of 1×10^4 cells/well and cultured overnight in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 at 37 °C. The cell culture medium was exchanged with complete DMEM (100 μL) containing varying concentrations of liposomes. After incubation for 24 h, the old medium was exchanged with fresh DMEM (110 μL) containing tetrazolium salt (WST-1) and further incubated for 30 min. Finally, the absorbance of formazan produced by succinate tetrazolium-reductase in living cells was measured at 440 nm using a microplate reader (Perkin Elmer Japan Co. Ltd., Tokyo, Japan).

3. Results

3.1. Syntheses of the amino acid-based cationic lipids

We synthesized a series of amino acid-based cationic lipids with different ionization states (–NH_2, –$NH_3^+Cl^-$, or –$NH_3^+TFA^-$) and a hydrocarbon chain spacer containing 0, 3, 5 or 7 carbon atoms. All the synthetic cationic lipids comprised 1,5-ditetradecyl-L-glutamate as the hydrophobic moiety. To introduce the hydrocarbon chain spacer, aminoalkanoic acid derivatives with different carbon numbers were conjugated with 1,5-ditetradecyl-L-glutamate *via* an amide linkage as shown in Scheme 2. Then a protected lysine moiety was introduced as a cationic head group. We obtained **1a**, **2a**, **3a** and **4a** lipids by deprotection with HCl (4 M) in EtOAc followed by neutralization and lipids **1b**, **2b**, **3b** and **4b** by deprotection with HCl (4 M) in EtOAc followed by removal of excess HCl under reduced pressure. Cationic lipids **2c** and **3c** were prepared by deprotection with TFA followed by removal of excess TFA under reduced pressure. All the synthetic compounds, including intermediates as shown in Schemes 1 and 2, were characterized by ^{1}H NMR, ^{13}C NMR, FT-IR (**2a**–**2c** as representative), X-ray photoelectric spectroscopy (**2a**–**2c** as representative), and ESI-MS (all the data are provided in supplementary information).

Table 1
Size, zeta potential and phase transition temperature of the cationic assemblies. Values shown are the averages obtained from 3 (size), 5 (zeta potential) and 3 (DSC) measurements.

Liposome	Size (nm)	Zeta potential (mV)	T_c (°C)
1a	191 ± 50	ND	ND
1b	76 ± 36	+20 ± l	41.0
2a	88 ± 37	+33 ± 1	32.6
2b	20 ± 9	+41 ± 6	–
2c	91 ± 41	+60 ± 6	24.5
3a	104 ± 30	+29 ± 1	36.1
3b	70 ± 27	+41 ± 3	32.3
3c	104 ± 22	+44 ± 4	27.0
4a	ND	ND	37.4
4b	ND	ND	–

3.2. Cationic assembling structures

The cationic assemblies from all synthetic amino acid-based cationic lipids were prepared by an extrusion method. We found that the size, morphology and zeta potential of the cationic assemblies were influenced by the difference in the ionization states and the hydrocarbon chain spacer length (Table 1). The size of assemblies prepared from **2b** was only 20 nm and formed a micellar structure, which was supported by the TEM images (Fig. 1b). We speculate that the smaller size is due to the higher hydrophilicity when compared to **2a** and **2c**. The bigger the hydrophilic moiety, the larger the volume of the hydrophilic part that readily forms a micellar structure. However, the highest zeta potential (+60 mV) for **2c** is due to the elevated protonation ratio and the relatively large size of the TFA counterion when compared to its HCl counterpart. The cationic assemblies prepared from **2c** and **3b**-**3c** form highly stable vesicular structures (Fig. 1c–e). However, the cationic assemblies prepared from **1a** and **1b** have a tendency to aggregate (Fig. 1a) due to the lack of a proper balance between the hydrophilic and hydrophobic moieties. The cationic assemblies prepared from **4a** and **4b** form either tube-like or rod-like structures (Fig. 1f–g) rather than vesicular structures.

3.3. Physicochemical characterization of lipoplexes

The size and zeta potential of the lipoplexes composed of **2b**–**2c** and **3b**–**3c** were determined (Table 2a–d). The size of the lipoplexes composed of **2c** and pDNA increased with the increasing ratios of lipid-to-pDNA from 3/1 to 5/1. However, any further increase in the lipid-to-pDNA ratio resulted in a decrease in the size of the lipoplexes. The 91 nm unilamellar vesicles of **2c** formed 281 nm lipoplexes at a lipid-to-pDNA ratio of 10/1. The zeta potential of the lipoplexes composed of **2c** increased sharply as the lipid-to-pDNA ratio increased from 3/1 to 5/1. However, a further increase in the lipid-to-pDNA ratio from 5/1 to 30/1 resulted in only a gradual rise of zeta potential up to +53 mV. A similar trend involving a sudden increase in size and zeta potential was also observed for lipoplexes composed of **3c** at a lipid-to-pDNA ratio of 5/1. Further study is required to characterize the lipoplexes in the presence of serum.

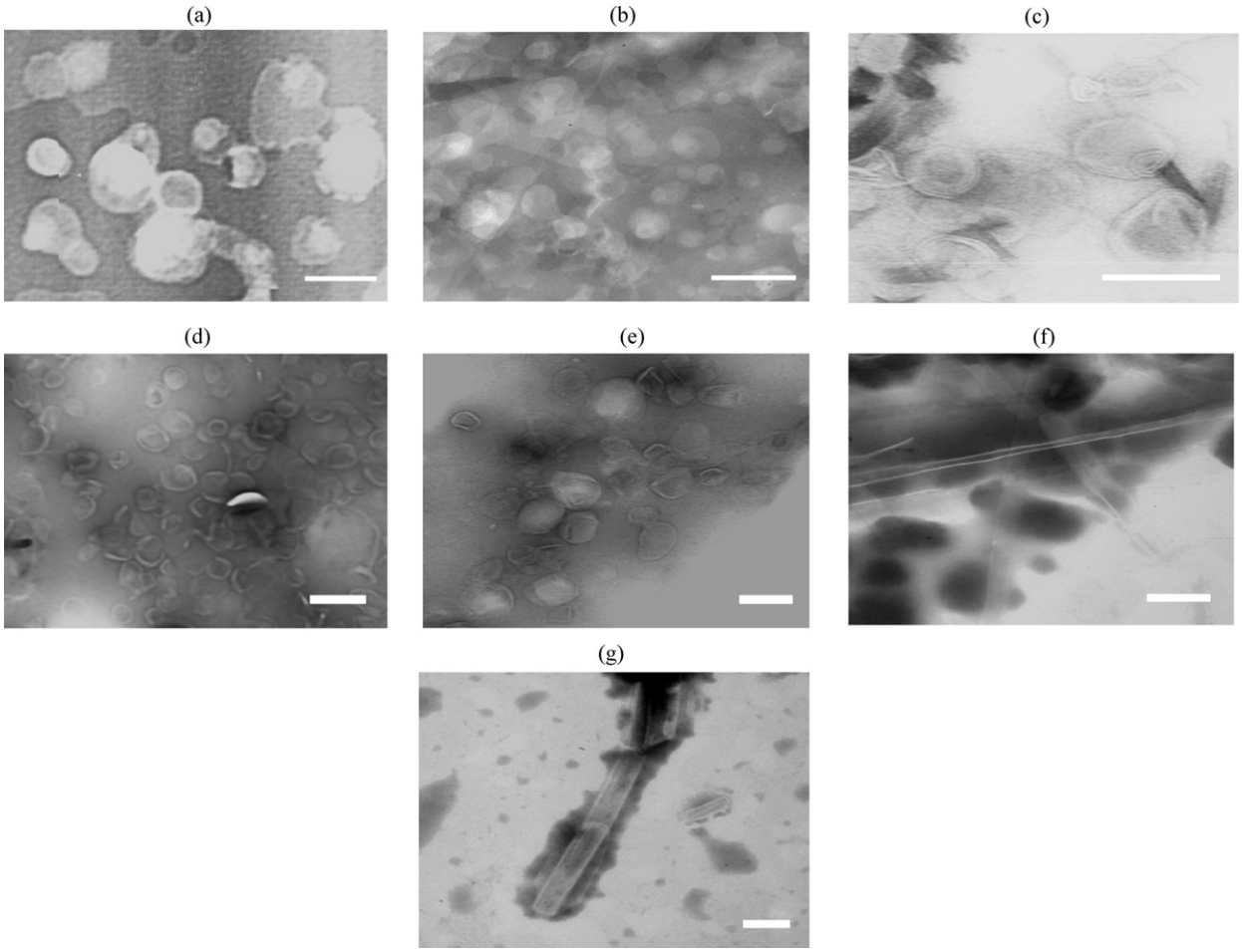


Fig. 1. Transmission electron microscopic images of the cationic assemblies constructed with (a) **1b**, (b) **2b**, (c) **2c**, (d) **3b**, (e) **3c**, (f) **4a** and (g) **4b**. The cationic assemblies were stained with 1% phosphotungstic acid. The scale bar indicates 100 nm.

3.4. Thermotropic phase behaviour of the cationic assemblies

The DSC measurement showed that the phase transition temperature of **2a** and **2c** were 32.6 and 24.5 °C, respectively (Table 1). However, there was no phase transition temperature detected for **2b**, which supports its micellar structure determined from DLS and TEM. The phase transition temperatures of **3a**, **3b** and **3c** were 36.1, 32.3 and 27 °C, respectively. Our results showed that the gel-to-liquid crystalline phase transition temperature (T_c) of the cationic assemblies was influenced by the ionization states of the hydrophilic moiety of the cationic lipids. Cationic assemblies with HCl-salt and TFA-salt showed phase transition temperatures lower than their neutral (i.e., NH_2) counterpart within the same hydrocarbon chain spacer series. The difference in phase transition temperature between **2a** and **2c** was 8.1 °C, which explains the superior transfection efficiency of **2c**. However, no significant difference was observed in the phase transition temperature of cationic assemblies through changing the hydrocarbon chain spacer length. The detailed thermal transition parameters (i.e., enthalpy and entropy) of the cationic assemblies (**2a**–**3c** as representative) have been determined (data are provided in supplementary information (Table S1)).

3.5. Lipid/DNA binding interactions

The representative electrostatic binding interaction between cationic assemblies (**2b**–**2c** and **3b**–**3c**) and pDNA at varying lipid-to-pDNA charge ratios was measured using conventional gel retardation assay (Fig. 2a–d). All the representative cationic assemblies were capable of completely inhibiting the electrophoretic mobility of pDNA from lipoplexes at a lipid-to-pDNA charge ratio of 10/1. At a lipid-to-pDNA charge ratio of 5/1, all the cationic assemblies exhibited moderately strong pDNA binding properties. However, at a lipid-to-pDNA charge ratio of 3/1, all the cationic assemblies showed poor pDNA binding affinity.

3.6. Transfection efficiency of the cationic assemblies

The reporter gene, *luc*, expression assay was used for evaluating the *in vitro* gene delivery efficiencies of **1a**–**1b**, **2a**–**2c**, **3a**–**3c** and **4a**–**4b** cationic assemblies in the COS-7 cell line (Fig. 3a–d) using pGL4.75[hRluc/CMV] plasmid vector encoding the enzyme luciferase. Lipoplexes composed of each cationic assembly and pDNA with ratios of 3/1 to 30/1 were transfected to the COS-7 cell line. The concentration of pDNA was kept constant at 2 μg/mL. In the 3-carbon series, **2c** showed the highest transfection efficiency followed by **2b** and **2a**. All three synthetic lipids showed relatively higher transfection efficiency than Lipofectamine™ 2000. The highest gene expression efficiency was observed at a **2c**-to-pDNA ratio of 10/1, which was 4-fold greater than that of Lipofectamine™ 2000 in the absence of 10% FBS. The transfection efficiency of Lipofectamine™ 2000 reduced 4-fold in the presence of 10% FBS. Therefore, **2c** showed 9-fold higher transfection efficiency when compared to Lipofectamine™ 2000 in the presence of 10% FBS.

In the C5 series, **3c** showed a maximum transfection efficiency followed by **3b** and **3a**. All the cationic assemblies (**3a**–**3c**) showed

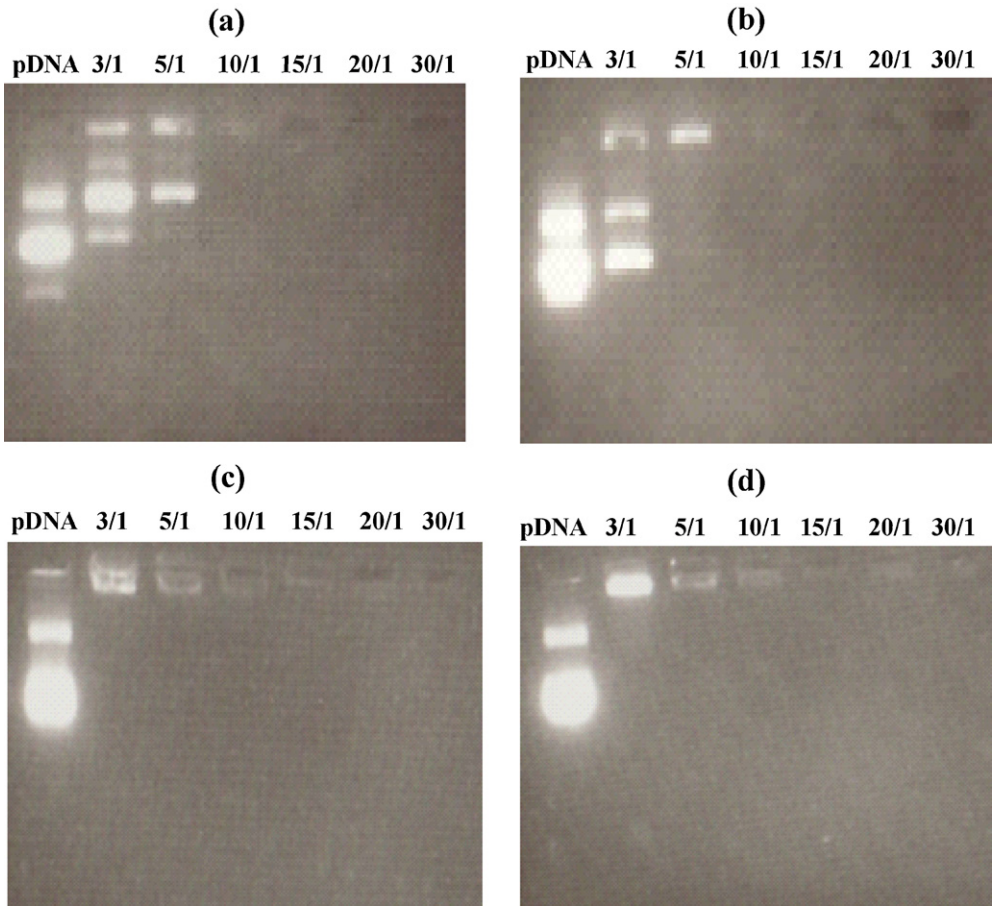


Fig. 2. Gel retardation assay of the lipoplexes constructed with the cationic assemblies and pDNA with various lipid-to-pDNA ratios (w/w). (a) **2b**, (b) **2c**, (c) **3b** and (d) **3c**.

higher transfection efficiency than Lipofectamine™ 2000 both in the absence and presence of 10% FBS at a lipid-to pDNA ratio of 10/1. The gene expression efficiency of **3c** was 2-fold higher in the absence of serum and 3-fold higher in the presence of serum (10% FBS) when compared to that of Lipofectamine™ 2000. There was no transfection efficiency observed for **1a**–**1b**, which are highly unstable and tend to aggregate into bigger particles. Likewise, **4a** and **4b** did not show any transfection efficiency due to their respective tube-like and rod-like structures.

3.7. Cytotoxicity

The cytotoxicity of cationic assemblies (**2b**–**2c** and **3b**–**3c**) was investigated up to 1500 μg/mL of the respective synthetic lipids (Fig. 4a and b). The results were expressed as a percentage of cell viability with respect to a control corresponding to untreated cells. All the cationic assemblies showed significantly lower cytotoxicity in the presence of 10% FBS when compared to Lipofectamine™ 2000. For example, more than half of the cells seeded into a culture dish survived even in the presence of 1 mg/mL of synthetic lipids.

4. Discussion

4.1. Influence of ionization states on the morphology of the cationic assemblies

Numerous studies have described the structure-activity relationship of amino acid-based cationic lipids. Specifically, several reports have investigated the influence of different spacers between the hydrophilic and hydrophobic moieties and different counterions in the polar head group in terms of gene delivery efficiency (Aberle et al., 1996; Bajaj et al., 2007a, 2008; Luciani et al., 2007; Obata et al., 2009). In the current study, we demonstrate that, in addition to the hydrocarbon chain spacer length, the nature of ionization states of the cationic head group plays a significant role in modulating gene delivery efficiencies of cationic lipids. Furthermore, the ionization states of the cationic head group influence morphology, zeta potential and phase transition temperature of the cationic assemblies.

We have synthesized a series of cationic lipids with different ionization states in the cationic head group (i.e., lysine) and different hydrocarbon chain spacer length (0, 3, 5 and 7 carbon atoms) whilst keeping the length of the hydrophobic moiety fixed at 14 carbons. The ionization states of the lysine hydrophilic moiety as the $-NH_2$, $-NH_3^+Cl^-$ and $-NH_3^+TFA^-$ form were controlled using different deprotection procedures. Cationic assemblies prepared from **2b** (size: 20 nm) formed a micellar structure (Fig. 1b) because of the presence of $-NH_3^+Cl^-$ in the hydrophilic head group despite having the same hydrophobic moiety and hydrocarbon chain spacer length as **2c** (size: 91 nm) that formed a multilamellar vesicular structure (Fig. 1**c**). Moreover, **4a** and **4b** formed either tube-like or rod-like structures (Fig. 1f–g) due to the presence of a larger hydrocarbon chain spacer (i.e., 7 carbon atoms) that resulted in the imbalance between the hydrophilic and hydrophobic moieties. The cationic assemblies prepared from **1a** and **1b** have a tendency to aggregate due to the lack of a proper balance between the hydrophilic and hydrophobic moieties. Herein, we confirmed that the synthetic amino acid-based cationic lipids with different ionization states and different hydrocarbon chain spacers formed various assembling structures like micellar, vesicular, and rod-like shapes. Indeed,

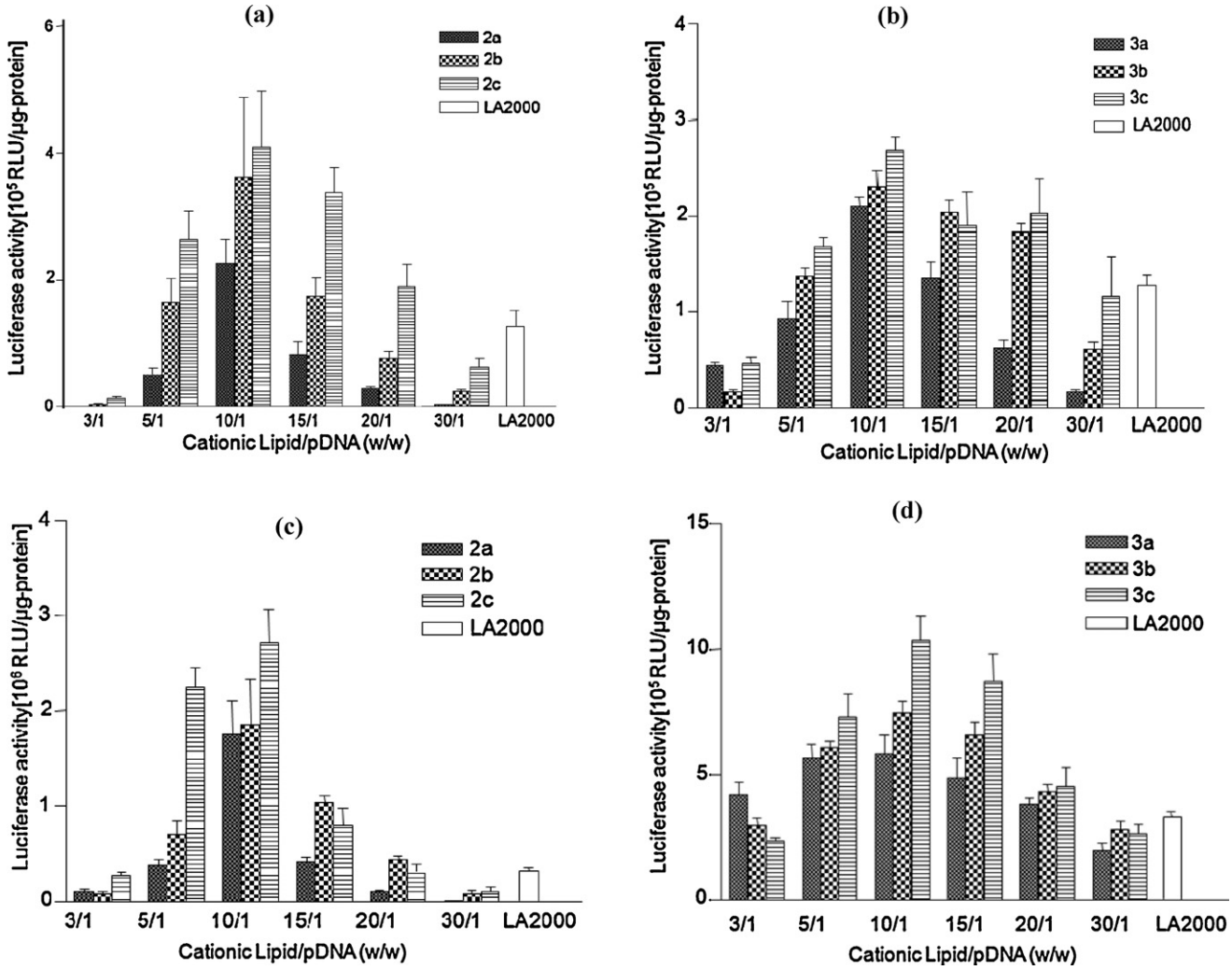


Fig. 3. Transfection efficiencies of the amino acid-based cationic assemblies **2a**–**2c** and **3a**–**3c** were compared with that of Lipofectamine™ 2000 in COS-7 cell line in the absence of serum (a) and (b) and in the presence of 10% FBS (c) and (d). Units of luciferase activity were plotted against the various lipid-to-pDNA ratios (w/w). The data presented are the average values of three independent experiments performed on three different days ($n = 5$).

previous reports suggest that assemblies formed from amino acid-based lipids can adopt various different structures such as vesicles, tubes, fibers or ribbons as a result of slight structural variations (Obata et al., 2008; Shimizu, 2003; Yamada et al., 1998). Moreover, the multilamellar structure of **2c** was confirmed by small angle X-ray scattering (SAXS) analysis (Supplementary information (Fig. S23 and Table S2)). Interestingly, without performing any chemical modification to the lipid scaffold, the change of ionization states can control the morphology of the cationic assembling structure.

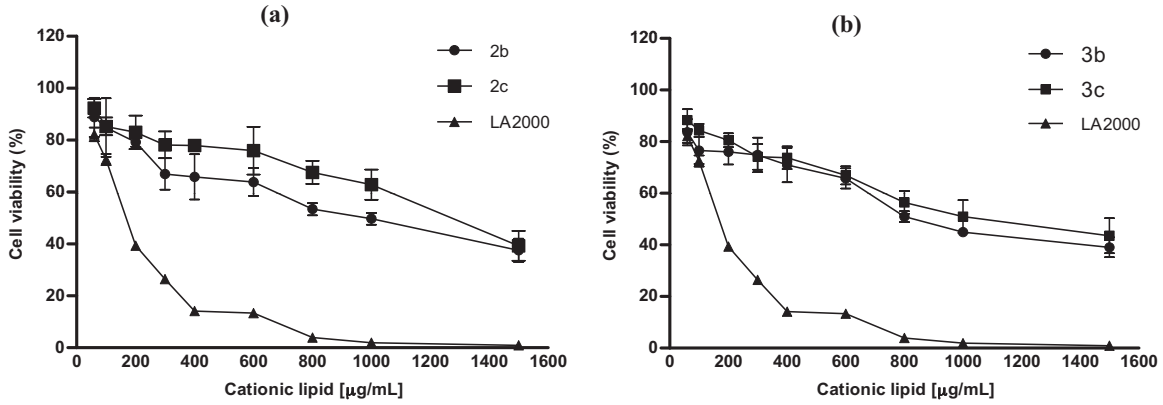


Fig. 4. Cytotoxicity of the amino acid-based cationic assemblies with different ionization states and hydrocarbon chain spacer was compared with that of Lipofectamine™ 2000. (a) **2b** and **2c**; (b) **3b** and **3c**. The cationic assemblies were added to COS-7 cells in the presence of 10% FBS and incubated for 24 h. Cytotoxicity was estimated through WST-1 assay. The data presented are the average values of three independent experiments performed on three different days ($n = 5$).

Table 2
Size distribution and zeta potential of the lipoplexes constructed with (a) **2b**, (b) **2c**, (c) **3b** and (d) **3c**. Values shown are the averages obtained from 3 (size) and 5 (zeta potential) measurements.

Ratio (2b/pDNA)	Size (nm)	Zeta potential (mV, ζ)
(a)		
3/1	194 ± 39	−33 ± 2
5/1	3550 ± 255	2 ± 1
10/1	385 ± 90	32 ± 1
15/1	152 ± 24	39 ± 3
20/1	134 ± 26	42 ± 4
30/1	114 ± 19	43 ± 3
Ratio (2c/pDNA)	Size (nm)	Zeta potential (mV, ζ)
(b)		
3/1	219 ± 22	−25 ± 1
5/1	2600 ± 256	12 ± 1
10/1	281 ± 90	42 ± 2
15/1	245 ± 71	49 ± 2
20/1	188 ± 45	53 ± 3
30/1	162 ± 21	52 ± 2
Ratio (3b/pDNA)	Size (nm)	Zeta potential (mV, ζ)
(c)		
3/1	325 ± 71	−1 ± 2
5/1	4220 ± 298	23 ± 1
10/1	414 ± 138	36 ± 3
15/1	198 ± 56	39 ± 6
20/1	177 ± 42	42 ± 3
30/1	134 ± 29	41 ± 4
Ratio (3c/pDNA)	Size (nm)	Zeta potential (mV, ζ)
(d)		
3/1	176 ± 22	−25 ± 2
5/1	2570 ± 256	−1 ± 2
10/1	580 ± 90	26 ± 4
15/1	378 ± 71	42 ± 7
20/1	341 ± 45	44 ± 3
30/1	181 ± 21	43 ± 2

4.2. Influence of ionization states on the lipoplex properties and plasmid DNA binding affinity to the cationic assemblies

Gel retardation assay was performed to confirm the ability of the cationic assemblies (**2b**–**2c** and **3b**–**3c** as representative) to form lipoplexes with pDNA. When the lipid-to-pDNA ratio was more than 3/1, no free pDNA was observed for **2c**, **3b** and **3c** (Fig. 2b–d). By contrast, an additional amount of lipid was required to form the lipoplexes for **2b** (i.e., lipid-to-pDNA ratio >5/1) (Fig. 2a) due to its micellar structure as determined from TEM images, DLS and DSC data.

The particle size and zeta potential of lipoplexes have been investigated in detail because they have revealed to be strongly related with the lipoplex ability to influence gene delivery (Almofti et al., 2003; Cardoso et al., 2011; Rao and Gopal, 2006). The maximum transfection efficiency for **2a**–**2c** and **3a**–**3c** was obtained at a lipid-to-pDNA ratio of 10/1 (Fig. 3a–d). The size and zeta potential of lipoplexes exhibiting the highest gene expression efficiency at a lipid-to-pDNA ratio of 10/1 were 281 nm and +42 mV, respectively, for **2c**, and 580 nm and +26 mV, respectively, for **3c**. Importantly, higher zeta potentials were found for **2c**/pDNA complexes in comparison to those for **2b**/pDNA complexes across the entire lipid-to-pDNA ratios of 3/1 to 30/1 (Table 2a and b). This is presumably because of the less hydrated head group of **2c** liposomes, which reduces the shielding of the positive charges (Rajesh et al., 2007). All the lipoplexes formed at a lipid-to-pDNA ratio of 5/1 showed the largest hydrodynamic size due to the lowest zeta potential when compared to the other lipid-to-pDNA charge (+/−) ratios which resulted in the aggregation of lipoplex particles and, in turn, low cellular uptake. Moreover, the lipoplexes formed at the higher lipid-to-pDNA ratios (i.e., ≥10/1) showed lower transfection efficiency due to a higher N/P ratio. Previous studies have shown that the higher N/P ratio resulted in a lower transfection efficiency and higher cytotoxicity of the carriers (Zhao et al., 2009). On the contrary, our data demonstrate that a higher N/P ratio does not contribute to the higher cytotoxicity of the gene delivery vehicle. However, the maximum gene delivery efficiency was found at an optimum N/P ratio. Furthermore, the efficiency of lipoplexes to enter the cell by endocytic pathways (Khalil et al., 2006), namely clathrin- and caveolin-mediated endocytosis, which are involved in the formation of coated vesicles and flask-shaped invaginations, respectively, has been shown to largely depend on particle size (Hoekstra et al., 2007). The zeta potential of the lipoplexes is also reported to be a critical factor for lipoplex stability and interaction with the cell surface as well as preventing interaction with serum proteins and resistance to nuclease degradation *in vivo* (Rao and Gopal, 2006; Simoes et al., 2005). All the complexes prepared at a lipid-to-pDNA ratio of 3/1 showed a negative zeta potential, thereby explaining their low transfection efficiency. The highly transfection efficient complexes showed a positive zeta potential that facilitates interaction with the negatively charged cell surface.

4.3. Relationship between the lipid structure and phase transition temperature

The gene expression efficiency of a lipoplex is generally known to depend on the phase transition temperature (T_c) of the cationic assemblies (Radler et al., 1997; Zantl et al., 1999). T_c and membrane fluidity are inversely proportional (Obata et al., 2008). Thus, membrane rigidity increases with increasing phase transition temperature. The higher membrane fusion potential was confirmed with the cationic assemblies having the lower phase transition temperature (Rajesh et al., 2007). Lipid membranes with high fluidity tend to promote fusion with a biological membrane leading to elevated gene expression efficiency (Obata et al., 2008; Radler et al., 1997; Zantl et al., 1999). Our data demonstrated that the phase transition temperature of **2c** (24.5 °C) was lower than that of **2a** (32.6 °C). Therefore, we speculated that **2c** would have the highest membrane fusion potential leading to the highest cellular uptake of pDNA into the COS-7 cell line. We assume that the lower phase transition temperature of **2c** cationic assemblies originates from the reduced hydration of the head group. This arises from the presence of the $-NH_3^+TFA^-$ form as the ionization state when compared to $-NH_2$ form in **2a**. The $-NH_3^+TFA^-$ form moiety stabilizes the water structure and favours smaller lipid head group volumes. It was reported that the smaller lipid head group leads, in particular, to a preference for H_{II} phase relative to L_α and to a decrease of the L_α-to-H_{II} transition temperature (Koynova et al., 1989). However, the $-NH_3^+Cl^-$ form stimulates interfacial hydration resulting in the larger head group volume and *vice versa* (Koynova et al., 1989). The larger head group volume might also shield the significant coulomb repulsion among the positively charged head groups, thereby imparting increased rigidity to the liposomal membrane through more compact packing of the amphiphiles in the liposomal aggregates (Rajesh et al., 2007). Moreover, the phase transition temperature of **3b** and **3c** was 32.3 and 27.0 °C, respectively, and the transfection efficiency of **3c** was slightly higher than **3b** at a lipid/pDNA ratio of 10/1. Therefore, the C5 series also demonstrate the influence of ionization states on transfection efficiency.

Previous studies have shown that spacers significantly influence the phase transition temperature of cationic gemini amphiphiles (Bhattacharya and Bajaj, 2007; Bhattacharya and De, 1999). However, our data did not demonstrate any significant influence of spacer length on the phase transition temperature of cationic assemblies (Table 1). We speculate that this difference in the

influence of spacers on the phase transition temperature is due to the structural difference of our amino-acid based cationic assemblies with the cationic gemini amphiphiles. In the amino acid-based cationic assemblies, hydrocarbon chain spacers are present between the hydrophilic lysine head group and the hydrophobic moiety. In this case, the orientation of the spacer is vertical with respect to the membrane. Hence, the spacers strongly influence the location of the cationic head group but not the molecular packing of cationic assemblies. By contrast, the spacers in cationic gemini amphiphiles act as a cross linker between the cationic head groups of two lipid molecules (monomers). The spacers, therefore, influence the lateral molecular packing in the membrane structure. Because of differences in the lipid conformation, the role of the spacer in molecular packing of cationic assemblies is likely to vary.

4.4. Transfection efficiency and cytotoxicity of cationic assemblies

The gene delivery efficiency of **2c** was found to be significantly higher than that of **2a** and **2b** (Fig. 3a) whereas, **3c** was found to be slightly superior to that of **3a** and **3b** (Fig. 3b) in the absence of serum. Vesicular structures have higher transfection efficiencies than that of micellar structures, which can be explained in terms of the packing factor P (Wang et al., 2007). When considering the binding to the negatively charged DNA, the value of P for a vesicle system ($P > 0.5$) is larger than that for a micelle system ($P < 0.5$). Thus, it is easier for a vesicle system to form nonlamellar structures, such as inverted hexagonal H_{II} or cubic phases, than a micelle system. These phases of low curvature have proven to be a controlling factor in lipid mediated DNA delivery due to the ease of DNA release when the lipoplexes interact with anionic lipids (Koynova et al., 2005).

The overall transfection efficiency of cationic lipids is severely compromised in the presence of serum proteins, which has been a major barrier to the clinical application of gene therapy (Bailey and Cullis, 1997). A previous report suggested that high density lipoprotein in serum can replace the phospholipid molecules of the liposomal membrane (Allen and Cleland, 1980) resulting in lower fusogenic ability to the cell membrane in the presence of FBS. Our results show that the transfection efficiency of Lipofectamine™ 2000, which contains DOPE, is reduced 4-fold in the presence of serum (10% FBS) and it was 9-fold and 4-fold less when compared to **2c** and **3c** (Fig. 3c–d), respectively.

The cytotoxicity of the amino acid-based cationic assemblies was investigated using the COS-7 cell line and compared with that of Lipofectamine™ 2000. Just over 40% of cells were found to be viable in the presence of each cationic assembly even at a concentration of 1.5 mg/mL (Fig. 4a and b). By contrast, all of the cells were dead in the presence of a much lower concentration (0.6 mg/mL) of Lipofectamine™ 2000. The high toxicity of Lipofectamine™ 2000 might be related to the presence of multivalent cationic compounds such as DOGS or DOSPA. After releasing plasmid DNA, these multivalent cationic compounds form aggregates with the cellular organelles leading to cellular death (Obata et al., 2008). However, amino acid-based cationic lipids are easily dissociated and metabolised. The ester bond in the linker region of the amino acid-based cationic lipids is readily hydrolysed in the cell, thereby lowering the level of cytotoxicity (Leventis and Silvius, 1990). The slightly higher cytotoxicity of **2b** over **2c** could be due to the higher hydrophilicity of the former (Eliyahu et al., 2005).

5. Conclusions

We have synthesized a series of cationic lipids with different ionization states in the hydrophilic head group and different hydrocarbon chain spacers between the hydrophilic head group and the hydrophobic moiety. The morphology of the cationic assemblies was influenced by both the ionization state of the head group and the length of the hydrocarbon chain spacer unit. Furthermore, the phase transition temperature of the cationic assemblies was influenced by the ionization state of the cationic head group, which, in turn, affected the corresponding transfection efficiency. Of all the synthetic cationic lipids, **2c** showed the highest transfection efficiency with very low associated cytotoxicity. Our data clearly show that the gene expression efficiency of cationic lipids is significantly influenced by the ionization state of the cationic head group. More specifically, the gene delivery efficiency in relation to ionization states of the hydrophilic head group is as follows: $-NH_3^+TFA^- > -NH_3^+Cl^- > -NH_2$. Taken together, the present findings reveal that the difference in the ionization states of the hydrophilic head group and in the hydrocarbon chain spacer length profoundly influences gene delivery efficiency of cationic assemblies. The strikingly high transfection efficiency and low cytotoxicity, even in the presence of serum, make our amino acid-based assemblies suitable for further investigation *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.10.044.

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