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Gene transfer efficacies of serum-resistant amino acids-based cationic lipids: Dependence on headgroup, lipoplex stability and cellular uptake

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ABSTRACT

Serum is a major obstacle to efficient cationic liposome-mediated gene transfection. In this paper, three alkaline amino acids based cationic lipids including lysinylated cholesterol (lipid 1), histidinylated cholesterol (lipid 2) and argininylated cholesterol (lipid 3) were used as non-viral gene vectors. The physicochemical properties such as size, Zeta potential, stability and cellular uptake of the lipoplexes formed from lipids 1–3 as well as the transfection efficacies with or without serum were investigated. The results demonstrated that lipid 1 and lipid 3 showed good properties in lipoplex stability and cellular uptake. Interestingly, lipid 3-based liposome showed serum-enhanced effect on the gene transfection. The transfection efficiency of lipid 1 and lipid 3 was remarkably higher than that of lipid 2. Moreover, they exhibited 10–20-fold more efficaciously than the control, 1,2-dioleoyloxy-3-(trimethylammonio)-propane (DOTAP) liposome in serum-containing media. The data suggested the strong effect of the type of the headgroup on gene transfection. The lysine/arginine derivative cationic lipids could be promising nonviral vectors for gene delivery *in vivo*.

1. Introduction

Development of safe and efficient gene delivery systems is one of the most important requirements for gene therapy. Viral vectors including retroviruses, adenoviruses and adeno-associated viruses have shown high transfection efficiency and been used in many clinical trials, but their safety concerns persist (Marshall, 2002). In the past two decades, much attention has been devoted to nonviral vectors due to the advantages of non-immunogenicity, convenience of handling, and high delivery capacity of genetic materials (Kim et al., 2009; Mintzer and Simanek, 2009). Among nonviral vectors, cationic liposomes have been extensively studied for more than 20 years since the first lipofection was reported in 1987 (Felgner et al., 1987). Although some cationic lipids such as 1,2-dioleoyloxy-3-(trimethylammonio)-propane (DOTAP) (McLachlan et al., 1996; Porteous et al., 1997) and 3-[N-(dimethyl-aminoethane)carbamoyl]cholesterol (DC-Chol) (Hui et al., 1997) have been used for gene therapy in clinical trials, the major problem cationic liposomes faced is the low transfection efficiency.

Serum has been reported as a major barrier to cationic liposome-mediated gene transfection (Bhattacharya and Haldar, 1996; Yang and Huang, 1997). It dramatically impairs the transfection activity. Previous studies have indicated that such inhibitory effect may arise from the negatively charged proteins in serum (Audouy et al., 2000; Yang and Huang, 1997). Once plasma proteins were absorbed on the cationic liposome/DNA complexes (lipoplexes), the complexes aggregated to precipitate or disintegrated to release DNA, thus leading to the failure of cell transfection. Many strategies have been carried out to resolve this problem. For example, some serum-resistant cationic lipids such as BGTC (Lewis et al., 1996), SAINTs (Audouy et al., 2000), arg-chol (Sochanik et al., 2000), amino acids and alkaline cationic lipids (Obata et al., 2008) and COPA (Han et al., 2008) were synthesized. The addition of the third reagent, e.g. helper lipids (Bhattacharya and Haldar, 1996; Takahashi et al., 2005), polyamines (Vitiello et al., 1996), protamine (Faneca et al., 2004), PEGylated lipids (Ross and Hui, 1999; Kim et al., 2010) and albumins (Faneca et al., 2004; Simoes et al., 2000) could improve the transfection activity in the presence of serum via the enhanced protection of DNA. The increase of lipid/DNA charge ratio (Yang and Huang, 1997) or lipoplex size (Almofti et al., 2003; Esposito et al., 2006) can also work effectively to overcome the serum inhibition. In spite of these improvements, the overall progress is still slow. With the consideration of these strategies, we have investigated the structure–efficacy relationship between lipids and transfection. Furthermore, we have studied physicochemical properties of lipoplexes to better elucidate the precise mechanism that governs the transfection behavior in serum. In our previous studies, we have described the synthesis, characterization and transfection properties of a new series of alkaline amino acids-based cationic lipids including lysinylated cholesterol (lipid 1), histidinylated cholesterol (lipid 2) and argininylated cholesterol (lipid 3) (Li et al., 2008, 2009). The transfections of the three lipids to a variety of cell lines

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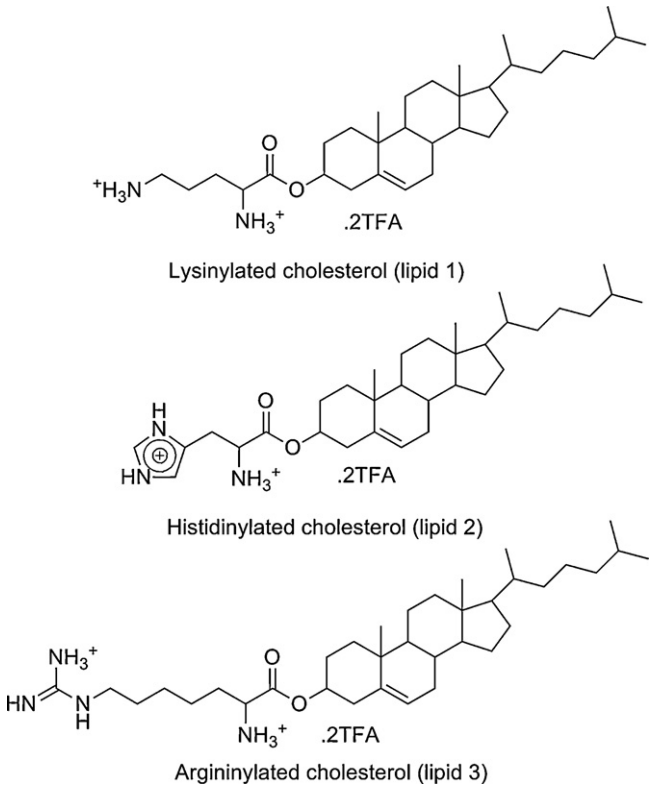

Fig. 1. Chemical structure of amino acid-based cationic lipids 1–3 used in this study.

in the absence of serum were studied and it was found that they showed much higher transfection efficiency compared with DOTAP liposome and low cytotoxicity on 293T cell line (Fig. 1).

In this paper, our aim is to investigate the transfection efficiencies and serum compatibility of lipids 1–3 in a variety of cell lines as well as major factors affecting transfection efficacies in the media with serum. The results have shown that the transfection performance of lipids 1–3 in the presence of serum strongly depend on the lipid structure (headgroups), stability of lipoplexes and the extent of cellular uptake.

2. Materials and methods

2.1. Materials

Lipids 1–3 were synthesized from lysine, histidine and arginine after esterification with cholesterol (Li et al., 2008). 1,2-Dioleoyloxy-3-(trimethylammonio)-propane (DOTAP) liposome was purchased from Roche Life Technologies (Basel, Switzerland). 1,2-Dioleoyl-sn-glycero-3-phosphaethanolamine (DOPE) and Lissamine rhodamine B-labeled DOPE (Rh-DOPE) were purchased from Avanti Polar Lipids (Alabaster, USA). YOYO-1 was obtained from Molecular Probe (Eugene, OR). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI-1640) medium, penicillin/streptomycin, and fetal bovine serum (FCS) were obtained from Gibco (Lawrenceville, GA), whilst pGL3 and pORF-LacZ plasmids, and luciferase assay kits were purchased from Promega (Madison, WI, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Qiagen Giga Endo-free plasmid purification kit was purchased from Qiagen (Chatsworth, CA). 293T human embryonic kidney cell lines, NIH3T3 mouse fibroblast cell lines and Hela human cervical cancer cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All other reagents were purchased from local commercial suppliers with analytical grade and used without further purification.

2.2. Plasmids isolation and labeling

The luciferase and lacZ reporter genes were used to characterize transfection efficiency in this study. The plasmids pGL3 and pORF-LacZ were isolated from *Escherichia coli* using Qiagen Giga Endo-free plasmid purification kit according to the manufacturer's protocol. For confocal laser scanning microscopy (CLSM), plasmid DNA was stained with YOYO-1. The labeling reaction was carried out with a molar ratio of 1 dye molecule per 320 base pairs at room temperature in the dark (Breunig et al., 2005).

2.3. Preparation of liposome and lipoplex

For the preparation of lipoplexes used for transfection, the cationic lipid and DOPE were dissolved in a mixed solvent (chloroform:methanol = 3:1, v/v) at an equimolar ratio, after which the solvent was removed under reduced pressure. The lipid film was placed under high vacuum for several hours to remove the last trace of the solvent. Then, the dry film was hydrated in sterile Millipore water at a final concentration of 0.4 mM. After 5 min of vortexing and bath sonication, the turbid suspension was cleared by probe sonication. Cationic liposome/DNA complexes were prepared at 4, 8, 12 and 16 nmol of lipid per μg of DNA (i.e. +/− charge ratios of 2.7, 5.3, 8.0 and 10.7). The (+/−) charge ratio was theoretically calculated as mole ratio of lipid 1 (two charges per molecule), lipid 2 (two charges per molecule) or lipid 3 (two charges per molecule) to nucleotide residue (average MW 330). The resulting lipoplexes were incubated for 15 min at room temperature and diluted with the culture medium to the final volume of 0.1 mL before use.

DOTAP/DNA lipoplexes were prepared according to the supplier's protocol. Briefly, 6 μL DOTAP liposome (contain DOTAP 6 μg) were mixed with 1 μg of DNA (100 ng/mL). Upon complexation for 15 min at room temperature, lipoplex solution was increased to 0.1 mL by adding the culture medium.

2.4. Effect of serum on size and Zeta potential

The average size and the Zeta potential of lipoplexes were measured by the method of dynamic light scattering (DLS) using Zetasizer NS (Malvern Instruments, USA) at 25 °C. 50 μL of lipoplex solution (containing 1 μg of pGL3) were prepared at desirable (+/−) charge ratios ranging from 2.7 to 10.7. After 15 min incubation, lipoplex solutions were diluted to final volume of 1 mL before measurement. For measurement of size and Zeta potential in the serum-containing medium, 5 μL of FBS was added into the lipoplex solutions (50 μL) and followed by 5 min of incubation. Then the yielding lipoplex solutions were diluted to final volume of 1 mL for measurement. Measured data were presented as the average value of 5 runs.

2.5. Interaction of lipoplexes with heparin

The experiment was conducted by a slightly modified method with Takahashi's report (Takahashi et al., 2005). In brief, the lipoplexes at (+/−) charge ratio of 5.3 were prepared by mixing plasmid DNA solution (5 μL, 0.1 μg/μL) and liposome (10 μL, 0.4 mM). After 15 min-incubation at room temperature, the lipoplexes were added to a given amount (0–40 μg) of heparin dissolved in PBS buffer (5 μL) and incubated for 30 min at room temperature. The samples were electrophoresed on 0.8% agarose gel in TBE buffer at 80 V for 30 min. GoldView was used as dye and the stained bands were visualized using an AlphaImager EP Fluorescent and Visible Light Gel Imaging System (NatureGene, USA).

2.6. Confocal laser scanning microscopy analysis

NIH3T3 cells were seeded at a density of 1×10^4 cells/well onto the 6-well plate with a sterile cover glass (22 mm × 26 mm) in each well and incubated for 24 h. For transfection in the absence of serum, the medium was exchange with serum-free medium. As for transfection in the presence of serum, the medium was exchanged with serum-containing medium. Complexes of liposomes containing Rh-DOPE (1%, molar ratio in liposome formulation) and YOYO-1 labeled pGL3 at a given concentration were added to each well. After 0.5 or 3.5 h, 5 μL of Hoechst 33258 (1 mg/mL) was added into the medium to stain the nucleus for 30 min. Then the cells on the cover glass were washed for 3 times with PBS buffer, fixed with 4% paraformaldehyde (dissolved with PBS buffer) for 15 min and then observed under the confocal laser scanning microscope TCP SP5 (Leica, Germany).

2.7. Quantitative and qualitative assay of transfection in vitro

NIH3T3, Hela, and 293T cells were seeded at a density of 5×10^4 cells/well in a 24-well plate in DMEM (NIH3T3 and Hela cells) or RPMI-1640 medium (293T cells) containing 10% FCS and grown to reach 70–80% confluence prior to transfection. For transfection in the absence of serum, the medium of each well was exchanged for fresh serum-free medium. For transfection in the presence of serum, the medium of each well was not changed at this time. Subsequently, the cells were treated with lipoplexes (containing 1 μg of pGL3 or pORF-LacZ) at different (+/−) charge ratios for 4 h at 37 °C. The medium was then completely refreshed with the completed culture media, and cells were cultured for another 48 h.

Luciferase gene expression was quantitatively measured by a luciferase assay kit according to supplier's instruction using Lmax II 384 luminometer (Molecular Devices Co., USA). Proteins in each sample were determined by BCA protein assay kit. Luciferase activity was normalized by the protein content, and expressed as the relative luminescence units per mg of protein (RLU/mg protein). All experiments were performed in triplicate.

For qualitative assay, β-galactosidase gene expression was examined by the method of X-gal stain. After X-gal staining, cells were observed under IX71 inverted microscope (Olympus Co., Japan).

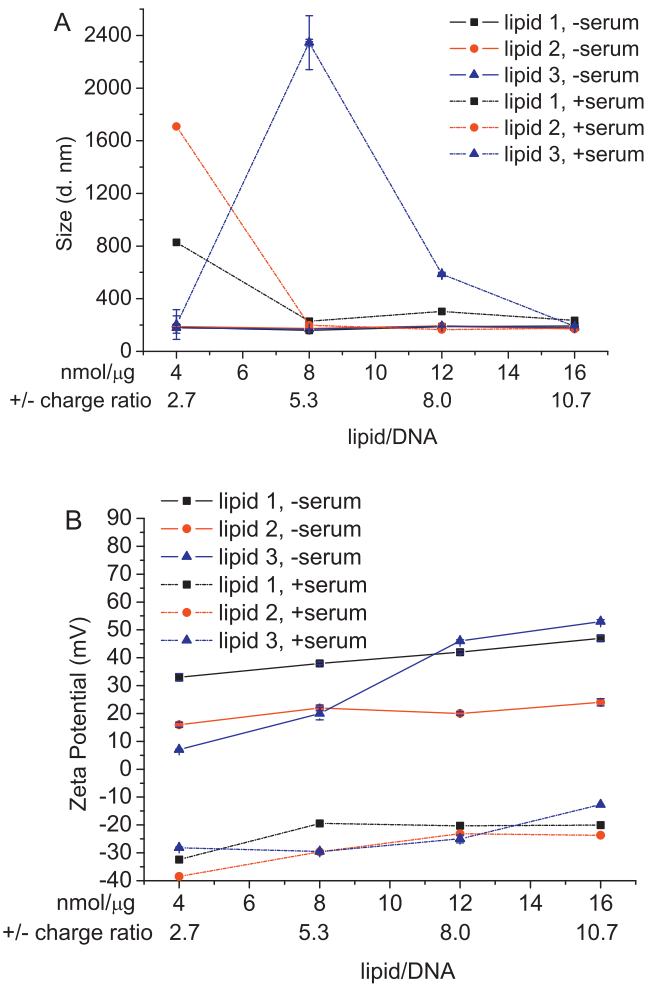

Fig. 2. Particle size (A) and Zeta potential (B) of lipoplexes 1–3 in the absence and the presence of serum. Liposome solution and DNA solution (containing 1 μg of pGL3) were gently mixed and incubated for 15 min. The yielding 50 μL of lipoplexes was diluted to 1 mL before measurement in the absence of serum. For measurement of size and Zeta potential in the serum-containing medium, 5 μL of FBS was added into the lipoplex solutions (50 μL) and followed by 5 min of incubation. Then the resultant lipoplex solution was diluted to final volume of 1 mL for measurement.

3. Results

3.1. Average size and Zeta-potential measurement of lipoplexes

As illustrated in Fig. 2A, in the serum-free medium, the particle size of all lipoplexes formed with 1–3 were between 180 ± 20 nm when the (+/−) ratio ranged from 2.7 to 10.7, showing little effect of molecular structure of the lipids. The mean diameters showed clear increase at a low lipid/DNA ratio after incubation with 10% serum for 5 min. Over the same charge ratio range, the diameters increased to 200–300 nm. At the lowest (+/−) charge ratio of 2.7, the sizes were much greater. When serum was added, the average size was 828 ± 51 nm for lipoplex 1 and 1710 ± 66 nm for lipoplex 2. In contrast, the average size of lipoplex 3 peaked at 2345 ± 171 nm at the (+/−) charge ratio of 5.3. As the charge ratio increased, the average diameters also decreased to 200–300 nm. These results revealed that as sufficient amount of lipids was added, the average lipoplex sizes tended to about 200 nm. The presence of serum could cause some further increase in lipoplex size at low lipid/DNA ratio.

Fig. 2B demonstrates that in the absence of serum, the Zeta potential of lipoplexes 1–3 was positive values and the value increased with the (+/−) charge ratio increasing. In most cases, lipoplex 2 showed the lowest Zeta potential among the three lipoplexes at the same charge ratio, due to the weak alkaline of imidazole. The result revealed the significant effect of lipid structure on Zeta potential. In the presence of serum, however, lipoplexes 1–3 displayed negative Zeta potential at all (+/−) charge ratios studied, showing that the negatively charged components in serum were absorbed onto the surface of lipoplex particles. Lipoplexes 1–3 exhibited the lowest negative Zeta potentials at the (+/−) charge ratio of 2.7, 2.7 and 5.3, respectively. Subsequent increase in the charge ratio led to the increase in Zeta potential. Lipoplexes 1–3 showed similar Zeta potential with serum, which was independent of lipid structure. The results suggested that the strong effect of serum on Zeta potentials, but the exact influence was dependent on charge ratio.

3.2. Interaction between lipoplexes and heparin

The stability of lipoplexes 1–3 formulations after their exposure to negatively charged heparin was examined by agarose gel electrophoresis (Fig. 3). It was observed that a band corresponding to free pDNA appeared with the increase of heparin addition into the lipoplexes, suggesting that these lipoplexes were destabilized and the plasmid DNA was released. Lipoplex 3 could resist the disassociation from a large amount of heparin (>40 μg); lipoplex 1 could

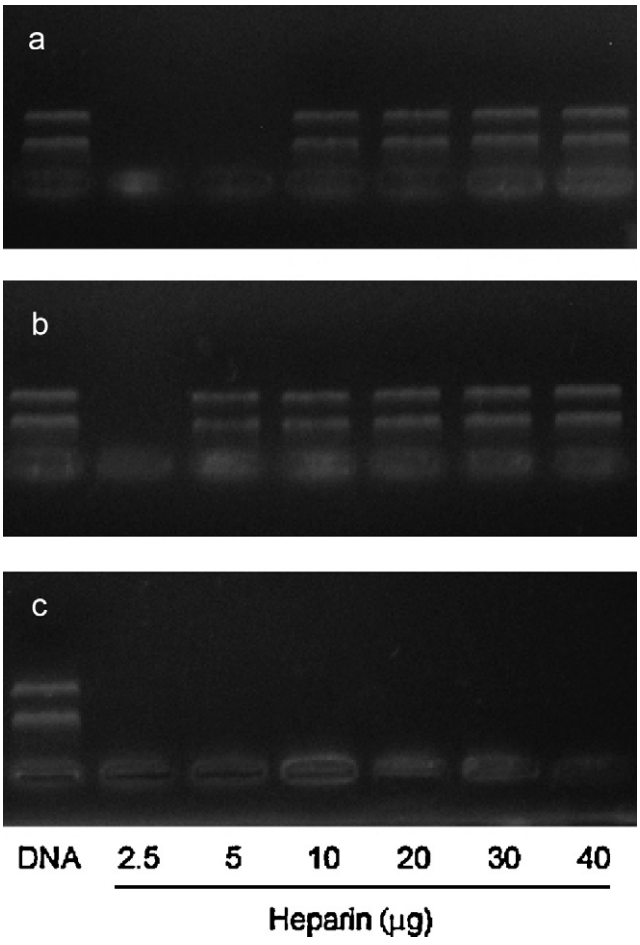

Fig. 3. Agarose gel electrophoresis of lipoplex 1 (A) lipoplex 2 (B) and lipoplex 3 (C) with (+/−) charge ratio of 5.3 in the presence of varying amounts of heparin. Lipoplexes 1–3 with (+/−) charge ratio of 5.3 were prepared by mixing plasmid DNA solution (5 μL, 0.1 μg/μL) and liposome (10 μL, 0.4 mM). After 15 min-incubation at room temperature, the lipoplexes were added to a given amount (0–40 μg) of heparin dissolved in PBS (5 μL) and incubated for 30 min at room temperature. The samples were electrophoresed on 0.8% agarose gel in TBE buffer at 80 V for 30 min.

resist a moderate amount of heparin (5 μg). In contrast, lipoplex 2 was readily disintegrated by the least amount of heparin (2.5 μg).

3.3. Cellular uptake and intercellular trafficking

Cellular uptake and intracellular trafficking of the lipoplexes 1–3 were observed by confocal microscopy. YOYO-1 for pDNA (green) and rhodamine-DOPE (red) for liposome were used as the fluorescence probe. Co-localization of pDNA and liposomes yielded yellow spots. Fig. 4 shows the overlap image of confocal laser scanning microscopy and the phase contrast images of NIH3T3 cells treated with lipid/DNA complexes. After 1 h transfection, weak intracellular fluorescence was observed in the cytosols of the cells treated with lipoplexes 1–3 and the fluorescence density subsequently increased with increasing transfection time. The co-incubation time of lipoplexes and cells for CLSM and transfection study was thus fixed at 4 h. In addition, the intracellular fluorescence signal in all cases appeared as stable signals in the cytosols indicating that effective endocytosis was the main pathway of internalization for the lipoplexes (Elouahabi and Ruysschaert, 2005). For lipoplex 1 and lipoplex 2, the serum did not obviously affect the cellular uptake and distribution. Intriguingly, lipoplex 3 exhibited serum-enhanced cellular uptake. A similar amount of lipoplexes was introduced into cells after the treatment of lipoplex 1 and lipoplex 3. It was found that a less amount of lipoplex 2 was internalized by cells.

3.4. Transfection in vitro

3.4.1. Luciferase gene transfection

Our previous studies have reported the synthesis of lipids 1–3 and the transfection of lipoplexes 1–3 (Li et al., 2008, 2009). It was found that lipoplexes 1–3 at a (+/−) charge ratio of 5.3 exhibited superior transfection activity in the serum-free medium. In this study, we firstly investigated the effect of serum concentration on transfection efficiency of lipoplexes 1–3 in 293T cells (Fig. 5). For lipoplex 1, the transfection efficiencies in the presence of 10% and 20% serum were as good as that in the absence of serum. High concentration of serum (from 30% to 40%) caused 68% and 60% reductions of luciferase activity, respectively. In contrast, the presence of even 10% of serum abolished transfection activity of lipoplex 2. For lipoplex 3, a large reduction of transfection efficiency was caused by the presence of 10% serum. No significant activity change was observed with the increase of serum concentration from 20% to 40%. These results indicated that the effect of the serum concentration on transfection activity of lipoplexes 1–3 was dependent on the lipid structure and sensitive to the presence of serum at (+/−) charge ratio of 5.3.

As we know, the [lipid]/[DNA] ratio is an important influencing factor for transfection efficiency with serum or without serum. The optimum [lipid]/[DNA] ratio of the lipoplex for transfection in the presence of serum might differ from that optimized in the absence of serum. So in order to investigate the relationship between the cationic lipid structure and the serum-sensitive property, the highest transfection efficacies of lipoplexes 1–3 at their optimum complexation ratio with or without serum were compared. DOTAP, a commercially available liposome, was used as the positive control. As shown in Fig. 6A, in the absence of serum, DOTAP/DNA lipoplex gave luciferase signals (5.7×10^7 RLU/mg protein) on 293T cells. The transfection efficiencies of lipoplexes 1–3 at their optimum ratios were about 2.8-, 3.5- and 3.5-fold higher than that of DOTAP, respectively. In the presence of 10% serum, the transgene expression of DOTAP decreased sharply by approximately 82%. Similarly, the highest transfection efficiency of lipoplex 2 (at +/− charge ratio of 8.0) was lowered dramatically (75% reduction) with serum comparing to that (at +/− charge ratio of 5.3) without serum. Nevertheless, lipoplex 2 was still 3-fold more effective than DOTAP. Unlike lipoplex 2, the maximal transfection efficiencies of lipoplex 1 and lipoplex 3 were not inhibited by serum. They exhibited about 20 times higher transfection activity comparing to DOTAP/DNA lipoplex. In NIH3T3 cells (Fig. 6B), the peak lipofection activity of lipoplex 1 and lipoplex 2 were decreased by 35% and 30% in the presence of serum compared to that in the absence of serum. Conversely, the maximal efficiency of lipoplex 3 in the present study was enhanced by 21% in serum. The transfection efficiencies of lipoplexes 1–3 were about 8-, 3- and 10-fold higher than that of DOTAP in the presence of serum. In Hela cells, the maximal transfection activity of lipoplex 1 slightly reduced when serum was added. Nevertheless, the presence of serum caused the peak transfection efficiency of lipoplex 2 and lipoplex 3 to increase 40% and 875%, respectively (Fig. 6C). The lipofection efficiency of lipoplex 1 and lipoplex 3 was higher than that of DOTAP with or without serum in the culture media. However, the transfection activity of lipoplex 2 was clearly lower than that of DOTAP and the other two cationic lipids.

3.4.2. β-Galactosidase gene transfection

β-Galactosidase gene transfection mediated by lipoplex 1 and lipoplex 3 were carried out in 293T and Hela cell lines. As shown in Fig. 7, lipoplex 1 and lipoplex 3 transfected more cells than DOTAP

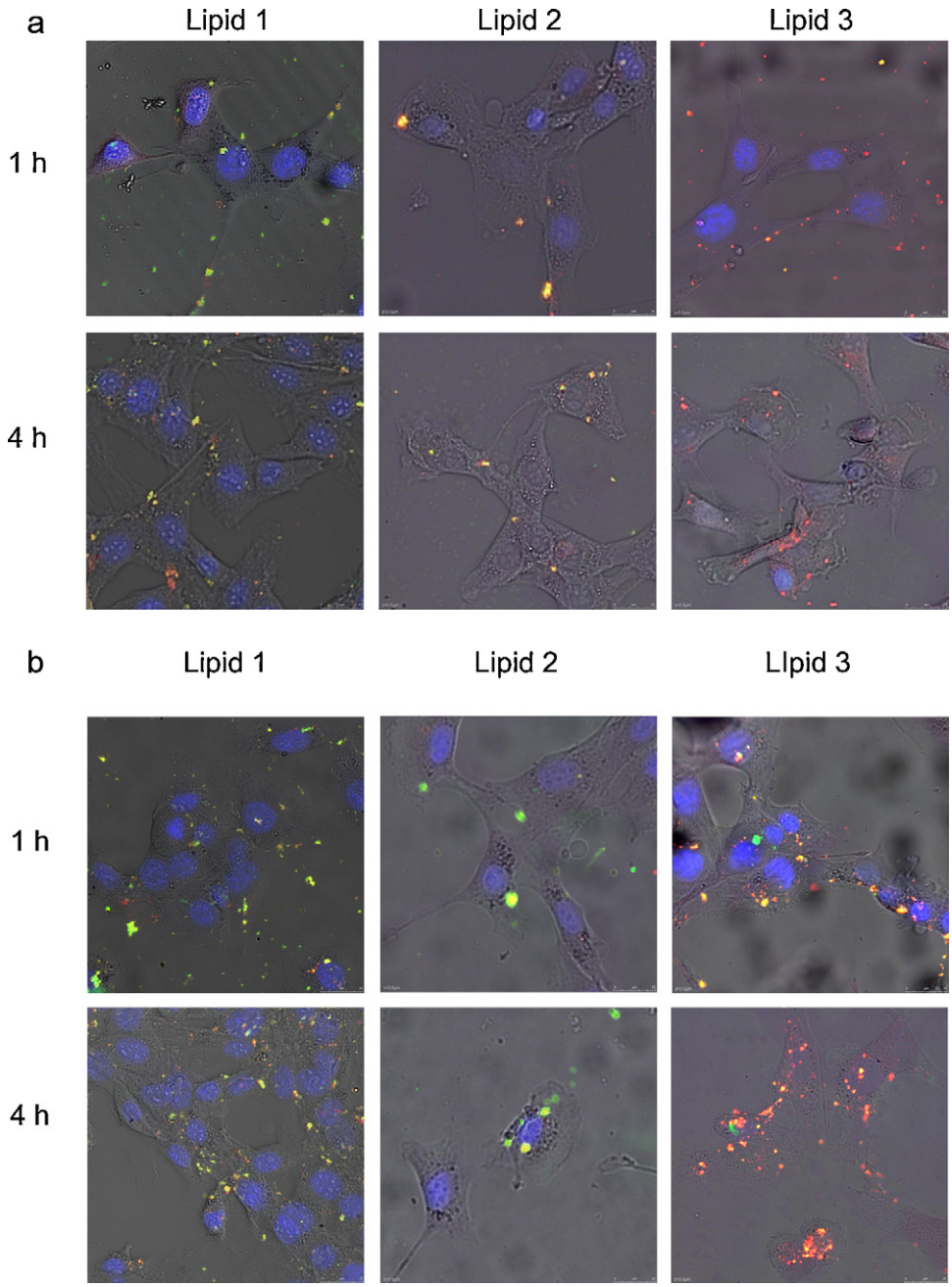

Fig. 4. The cellular uptake and intracellular trafficking of plasmid DNA in NIH3T3 cells. The cells were transfected with cationic liposome/DNA (pDNA 1 μg/well, (+/−) charge = 5.3) in serum free (A) or serum-containing (B) medium as described in Section 2.6. After treatment for 0.5 or 3.5 h, Hoechst 33258 was added into the medium to stain the nucleus for 30 min. Then the cells were fixed with PFA (paraformaldehyde) for 15 min. The transfected cells were studied under a confocal microscopy. The plasmid DNA labeled by YOYO-1 was seen as green in (A) and (B). Liposome containing 1% rhodamine-DOPE in molar ratio is seen as red. The cell nucleus stained with Hoechst 33258 is blue. All images were overlaps of fluorescent and phase contrast images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

did. Moreover, little cytotoxicity of lipoplex 1 and lipoplex 3 was observed even at the (+/−) charge ratio of 10.7.

4. Discussions

Among all the barriers in transfection *in vivo*, serum is well known for its inhibitory effect. It is worth noting that, however, the argininylated lipid-based liposome showed serum-enhanced gene transfection in the study. Furthermore, lysinylated and argininylated lipids were approximately 10–20 times more effective than DOTAP liposome in the presence of serum in all tested cell lines. The results revealed that the transfection efficiency and the serum resistance of lipoplexes were mainly depended on the structure of cationic lipids. In order to understand the structure–efficacy relationship better, the relevant physicochemical and biological properties of lipoplexes 1–3 were investigated.

Among different physicochemical parameters of lipoplexes, size, Zeta potential and lipoplex stability have been reported to be important factors of transfection efficiency. Our data indicated that lipoplex stability (Fig. 3) was crucial for gene delivery. Heparin experiment suggested that lipid 3 could sufficiently protect DNA against release by heparin prior to the cellular uptake. The high stability of lipoplex 3, due to the formation of strong charge interaction between the guanidinium group on lipid 3 and the phos-

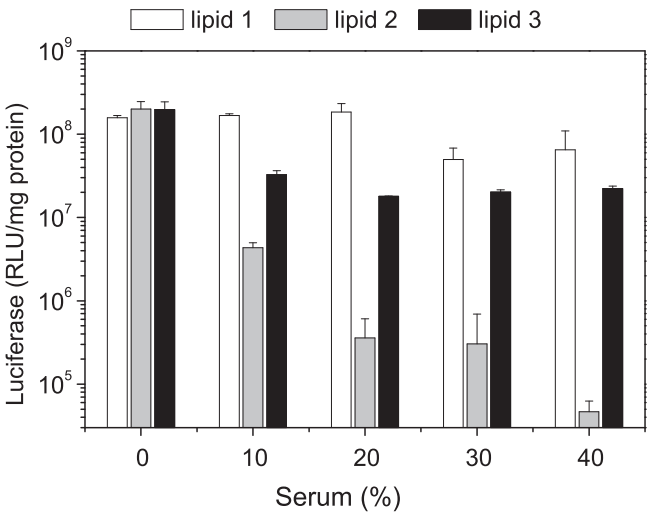

Fig. 5. Gene transfection of lipoplexes 1–3 in media with 10–40% serum or without serum. 293T cells were incubated with lipoplexes 1–3 containing 1.0 μg of pGl3 at lipid/DNA (+/−) charge ratio of 5.3 for 4 h in media with or without serum. Luciferase gene expression was measured by a luciferase assay kit. Proteins in each sample were determined by BCA protein assay kit. Luciferase activity was means ± SD of three experiments ($n = 3$).

phate moiety on DNA (Rothbard et al., 2005; Vigneron et al., 1996), was a reasonable explanation of its high efficiency in serum. As for lipoplex 1, the stability originated from the similar interaction between amino group on lipid 1 and DNA (Mishra et al., 2008; Rothbard et al., 2005). It also exhibited high efficiency in gene transfection in serum. These findings demonstrated that lipoplex 1 and lipoplex 3 may fall into the category of modest stability promoted by Stern et al. (2010) recently. In contrast, lipoplex 2 was rather instable in the heparin-containing media. As a result, DNA was most likely to be released and the complexation became ineffective. Therefore, the instability of lipoplex 2, mainly due to the weak interaction between imidazole ring and DNA, was responsible for poor transfection efficiency in serum. Similarly, the good correlation between lipoplex stability and transfection in serum were also reported by Sochanik group (Sochanik et al., 2000) and Takahashi group (Takahashi et al., 2005). Nonviral vectors condensing DNA with larger size (>700 nm) were reported to be more effective than smaller ones (<250 nm) in the presence of serum *in vitro*, since the large size possibly facilitated size-dependent cellular uptake via a switch from a chathrin-dependent to caveolae-mediated entry pathway (Caracciolo et al., 2010), protecting DNA against the degradation of DNase I (Garcia et al., 2007), and increasing serum resistance (Almofti et al., 2003). However, the similar activities of lipoplexes with the different sizes indicated that the sizes were unlikely to be responsible for different transfection efficiencies observed from lipoplexes 1–3 (Fig. 2A). Similar results for cationic lipids such as SAINTs (Audouy et al., 2000), COPA (Han et al., 2008), have been reported previously. No obvious correlation between Zeta potential and transfection activity was observed, either (Fig. 2B). The morphology of lipoplex assemblies such as a tube, vesicles or fiber was reported to affect their gene delivery performance (Obata et al., 2008). The cationic assemblies formed from lysine or arginine based lipids with two symmetric alkyl chains gave unilamellar vesicles (100 nm), whereas the morphology of histidine-type lipids was tube-like. The poor transfection efficiency of histidine-type lipids was probably due to the tube-like assem-

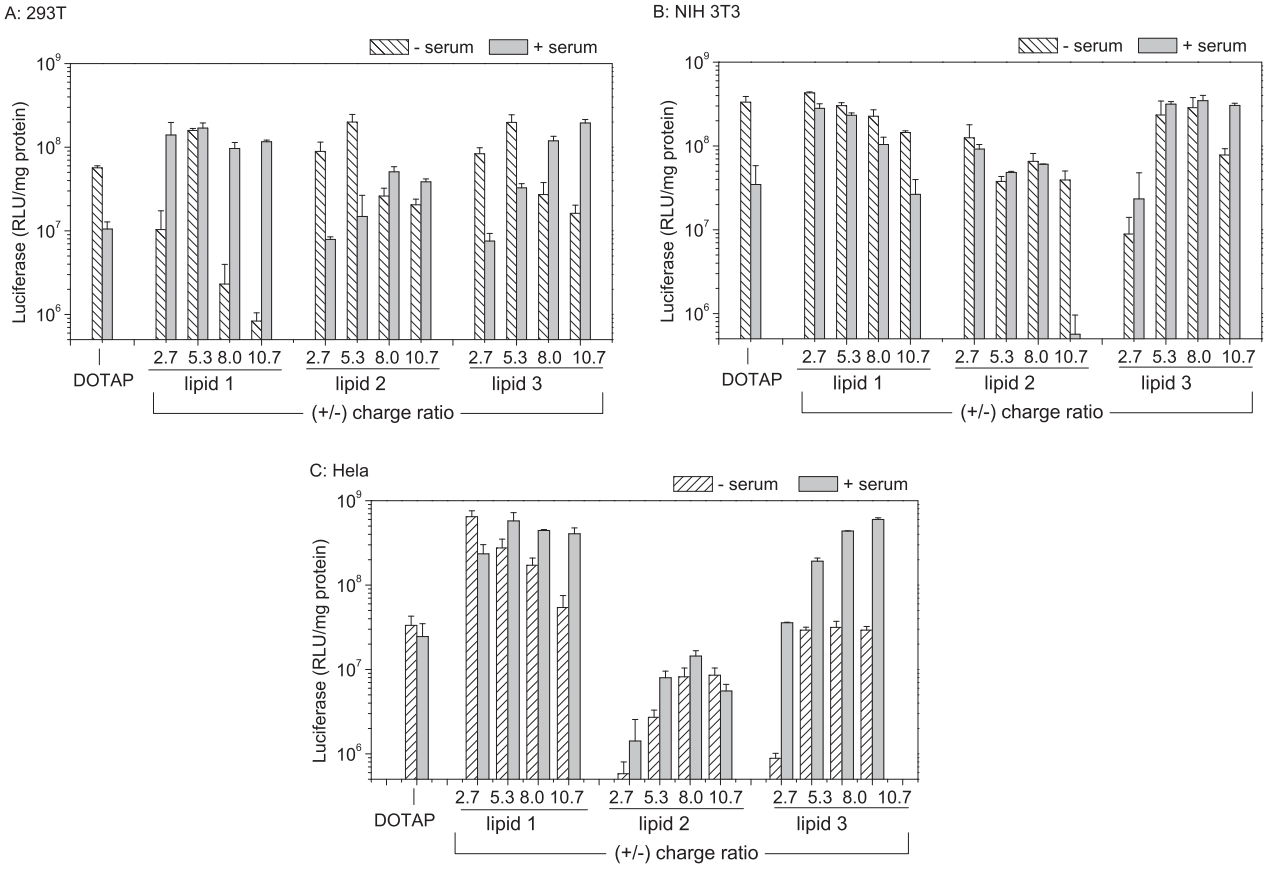

Fig. 6. Gene transfection of lipoplexes 1–3 in 293T cells (A) NIH3T3 cells (B) and Hela cells (C) in media with or without serum. Cells were incubated with lipoplexes 1–3 at varying lipid/DNA ratio or DOTAP/DNA complex containing 1.0 μg of pGl3 for 4 h in media with or without serum. Luciferase gene expression was measured by a luciferase assay kit. Proteins in each sample were determined by BCA protein assay kit. Luciferase activity was means ± SD of three experiments ($n = 3$).

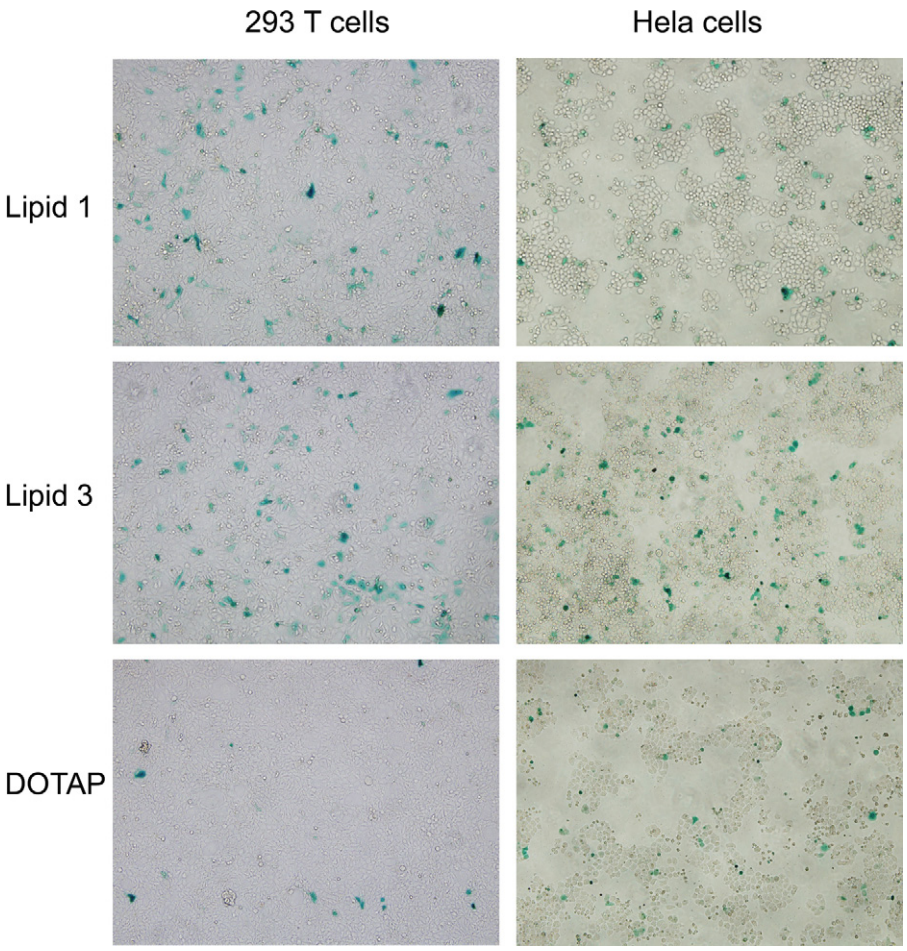

Fig. 7. Gene transfection of lipoplex 1 and lipoplex 3 in Hela cells (the left group) and 293T cells (the right group) in the presence of serum. Cells were incubated with lipoplex 1, lipoplex 3 or DOTAP/DNA complex containing 1.0 μg of pGl3 for 4 h, respectively, in media with serum. β-Galactosidase gene expression was examined by the method of X-gal stain. After X-gal staining, cells were observed under a microscope.

blies. However, all of our synthetic cationic lipids/DNA complexes gave vesicles (data not shown), so morphology was not a crucial factor determining transfection activity of amino acids based cholesterol derivates.

The interaction of lipoplexes with cell membrane surface and the subsequent internalization into cells after their exposure to serum were regarded as the limiting steps causing low transfection efficiencies (Audouy et al., 2000; Yang and Huang, 1997). CLSM results demonstrated that the amount of lipoplex 1 and lipoplex 3 internalized by NIH3T3 cells were similar, but were much greater than that of lipoplex 2. Surprisingly, lipoplex 1 and lipoplex 2 showed serum-insensitive cellular uptake. The internalization of lipoplex 3, however, was enhanced by the presence of serum. We found that the transfection activity correlated well with the cellular uptake. Then an obviously important question was how to elucidate the mechanism underlying the serum-insensitive and serum-enhanced cellular uptake. We hypothesized that lipoplexes 1–3 after absorption of plasma protein was possibly like a strawberry, on which black dots represented lipoplexes which were not shielded by serum proteins and the rest red part represented the serum protein coating. So the binding of the "strawberry"-like particles to the cell surface and their internalization inside the cell might be controlled by multiple factors, including the electrostatic interaction between "black dots on strawberry" and the cell surface. Guanidinium in lipid 3 could form bidentate hydrogen bonds that interact simultaneously with phosphate moieties on multiple lipid headgroups (Rothbard et al., 2005). According to a recently proposed hydrogen bonding-dependent mechanism of arg-rich transporters entry within cells, bidentate hydrogen bond between guanidinium and phospholipids on cell membranes most likely resulted in a negative Gaussian (saddle-splay) membrane curvature and final pore formation in cell membranes (Mishra et al., 2008). Lipoplexes could readily enter into cells through the pore, which was an important mechanism underlying high cellular uptake of lipoplex 3. Weak hydrogen bond between the imidazole on lipid 2 and phospholipids in cell membrane, mainly due to the stereo specific blockade of imidazole group, may lead to the poor cellular uptake. Another factor that could not exclude was the ligand–receptor interaction. Cholesterol can presumably bind to certain ligand components of serum, facilitating cellular delivery by ligand–receptor-mediated endocytosis (Kim et al., 2006; Soutschek et al., 2004). Through conjugated with cholesterol (Soutschek et al., 2004), siRNA achieved successful tissue delivery *in vivo* and showed improved pharmacokinetic properties as compared to unconjugated siRNAs. The noncovalent complexation of a synthetic siRNA with Chol-oligoarginine efficiently delivered siRNA into cells *in vitro* and *in vivo* (Kim et al., 2006). In addition, the positive charge of lipoplexes can be neutralized by serum, which might be related to the inhibitory effect of serum. However, rather than leading to a reduction of the amount of lipoplexes 1–3 internalized by cells, the cellular uptake displayed a similar or increased tendency. Taken together, these studies suggest that different interactions may work together to contribute.

In summary, we constructed gene non-viral vectors with synthetic amino acids based cationic lipids. In particular, cationic

lipids having lysine or arginine as a headgroup and cholesterol as hydrophobic domain were shown to display greater gene transfer efficiencies than that of standard DOTAP liposome in the presence of serum. Their transfection activities were determined by simultaneous lipoplex stability and good cellular uptake. The two properties of lipoplex 1 and lipoplex 3 were most likely arose from the structure of the cationic lipids which provided compact packing of DNA and increased the permeability of lipoplexes through cell membranes. Controlling the lipoplex stability and cellular uptake through designing the headgroup of cationic lipids may therefore be a successful strategy for gene delivery systems.

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