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Original Article

Headgroup modification of cholesterol-based cationic lipids: Synthesis, transfection efficiency evaluation, and serum compatibility

Chopaka Thongbamrer[1], Nattisa Niyomtham[2], Chayutra Chaiwut[3], Charoen Posa[3], Nuttapon Apiratikul[4], Ek Sangvichien[5], Praneet Opanasopit[6], Uthai Sakee[1], Boon-ek Yingyongnarongkul[3], and Widchaya Radchatawedchakoon[1*]

[1] *Center of Excellence for Innovation in Chemistry (PERCH-CIC), and Creative Chemistry and Innovation Research Unit, Department of Chemistry, Faculty of Science, Mahasarakham University, Kantharawichai, Maha Sarakham, 44150 Thailand*

[2] *Walailak University International College of Dentistry, Phaya Thai, Bangkok, 10400 Thailand*

[3] *Center of Excellence for Innovation in Chemistry (PERCH-CIC), Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bang Kapi, Bangkok, 10240 Thailand*

[4] *Department of Chemistry, Faculty of Science, Srinakharinwirot University, Wadhana, Bangkok, 10110 Thailand*

[5] *Department of Biology, Faculty of Science, Ramkhamhaeng University, Bang Kapi, Bangkok, 10240 Thailand*

[6] *Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Mueang, Nakhon Pathom, 73000 Thailand*

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Abstract

There were two objectives for this study. First, it focused on eight lipids having cholesterol as hydrophobic tails, carbamate linkers, and different polar headgroups. Second, it aimed to study the physicochemical properties of those eight lipids, including DNA binding, size, zeta potential, and the transfection efficiency. Cholesterol-1,2-diaminoethane and tetramethylguanidinium conjugated lipid showed the highest transfection efficacy into human embryonic kidney cells cells. The optimal formulation of this lipid was found to be 1:1 (weight/weight) for cationic lipid/DOPE, and 1:20 for DNA/liposome. GFP expression experiments further revealed that the liposome exhibited higher transfection efficiency under a 10−40% serum condition than Lipofectamine™ 2000.

Keywords: cationic lipid, cholesterol, serum compatibility, tetramethylguanidinium headgroup, transfection

*Corresponding author
Email address: widchaya.r@msu.ac.th; widchaya@hotmail.com

1. Introduction

Gene therapy has been widely known as a treatment for several diseases, such as cystic fibrosis (Conese *et al.*, 2011), hemophilia (Coutu *et al.*, 2011), and cancers (Han, Liu, Yang, Cui, & Xu, 2017; Sun *et al.*, 2012). Genetic materials such as pDNA, oligonucleotides, or siRNA, are introduced to the target cells by vectors to induce, enhance, or inhibit gene expression (Kim, Ryu, & Kim, 2014). The vectors can be classified into two major groups: viral and non-viral. The viral vectors include retrovirus, adenovirus, herpes simplex virus, adeno-associated virus, and lentivirus (Choudhury *et al.*, 2017; Merten & Gaillet, 2016). Although viral vectors have high transfection efficiency for both gene delivery and expression, they have several drawbacks, including toxicity, immune response, safety issues, insertional mutagenesis, low transgene size, and a lack of cell-specific targeting (Pandey & Sawant, 2016; Schmidt-Wolf & Schmidt-Wolf, 2003). Non-viral gene carriers, for example, lipids, polymers, and peptides, have multiple advantages over their viral counter-parts. Furthermore, problems associated with the immune response have not been found.

Cationic lipids are amphiphilic organic molecules. They contain three primary domains: a positive charge headgroup, a hydrophobic tail, and a linker, which are tethered to the polar and non-polar regions. Recent research showed that the hydrophobic tail was found to be a long chain fatty acid (Niyomtham, Apiratikul, Suksen, Opanasopit, & Yingyongnarongkul, 2015; Viola *et al.*, 2009) or cholesterol moiety (Gao *et al.*, 2010; Medvedeva *et al.*, 2009). There are several advantages of a cationic liposome, for instance, the ability to deliver both hydrophilic and hydrophobic drugs. Additionally, this liposome generates low toxicity and has no activation against the immune system. In this regard, the cationic liposome also delivers the bioactive compounds to the targeted site of action (Foldvari *et al.*, 2016; Nayerossadat, Maedeh, & Ali, 2012).

Serum is a major barrier for efficient cationic liposome-mediated gene delivery. Previous studies reported that cationic lipids bearing cholesterol-based lipoplexes were stable in the presence of serum (Ju *et al.*, 2016; Li *et al.*, 2011). Several reports on sterol-based cationic lipids indicated high transfection results and low toxicity (Bajaj, Mishra, Kondaiah, & Bhattacharya, 2008; Radchatawedchakoon, Watanapokasin, Krajarng, &Yingyongnarongkul, 2010). The polar headgroups cause condensation of the DNA by electrostatic attraction with the phosphate group. Many previous studies reported the effects of the headgroups of cationic lipids on transfection efficiency. The ammonium, quaternary ammonium, and guanidinium functionalities of cationic lipids have been reported (Gao & Hui, 2001; Kim *et al.*, 2009; Zhi *et al.*, 2013). Consequently, we became interested in studying the effect of the headgroup on gene transfer.

In this work, we synthesized cationic lipids which consisted of cholesterol as the hydrophobic tail, carbamate linker, and different polar headgroups (i.e. amine, trimethyl-ammonium, guanidinium, and the novel tetramethyl-guanidinium) to study transfection efficiency (Figure 1). The synthesized lipids were evaluated for their transfection efficiency into human embryonic kidney cells (HEK293), serum compatibility, and cytotoxicity.

2. Materials and Methods

2.1 Chemicals and materials

IR spectra were recorded on a Perkin-Elmer FT-IR 400 spectrometer with attenuated total reflection while nuclear magnetic resonance spectra were recorded on Bruker AVANCE 400 and Ascend^{TM} 400 spectrometers, which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All coupling constants (*J* values) were measured in Hertz. Electrospray ionization mass spectra were recorded with a Finnigan LCQ mass spectrometer. High-resolution electro-spray ionisation mass spectrometry of pure compounds was obtained using a Bruker micrOTOF-II mass spectrometer. Reagents were purchased from commercial suppliers. The starting materials of cholesteryl chloroformate, *N*,*N*'-diisopropylethylamine, and 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea were purchased from Acros Organics. Methyl iodide and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Sigma-Aldrich.

2.2 DNA binding affinities

Gel electrophoresis was used to study the DNA binding ability of DNA/lipid complexes. The ability to retard DNA migration was examined at DNA/lipid ratios of 1:5, 1:10, and 1:20 through 1.0% agarose gel electrophoresis. Ethidium bromide was added to the agarose plate. The complexes were prepared by adding liposome into DNA (0.1 μg). Loading dry reagent was then added to the lipoplexes. The solutions (10 μL) were loaded into the gel, and the electrophoresis was performed in TBE buffer (Tris-borate-EDTA) under +100 V for 30 min.

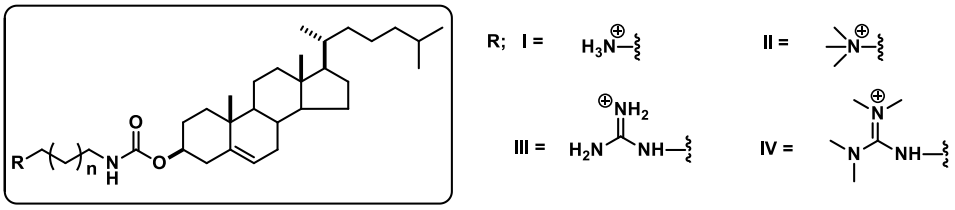


Figure 1. Structures of different polarhead cholesterol-based cationic lipids.

2.3 Liposome preparation

A thin film was prepared from the stock solution of cationic lipid and dioleoylphosphatidylethanolamine (DOPE) (1μg/μL in CH_2Cl_2 or EtOH). DOPE (250 μL) and cationic lipid (250 μL) were mixed at 1:1 weight ratio in an Eppendorf tube. The solvent was evaporated to dryness by a stream of nitrogen and then stored in high vacuum for 4−8 h. The thin film was dissolved with PBS buffer, vortexed for 30 sec and sonicated for 20 min at room temperature.

2.4 Transfection procedure

Plasmid DNA (pCH110; Promega) containing the β-galactosidase reporter gene under the control of the SV40 promoter was grown using the standard techniques and purified by column chromatography (NucleoBond® Xtra Maxi EF). UV adsorption measured the concentration of plasmid DNA at 260 nm. The A_{260}/A_{280} ratios were between 1.80−2.00. HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100% units/mL), streptomycin (100 μg/mL) and L-glutamine (4 mM) at 37 °C under 5% CO_2. The cells were seeded up to 1×10^4 cells/well in a 96-well plate to give 50−70% confluence for use the next day. The old medium was removed and washed with PBS and replaced with 100 μL of fresh serum-free DMEM medium. The lipoplexes were prepared by adding the liposome (1 μg/μL) into DNA (0.1 μg/μL) and the mixture was diluted with PBS buffer to the 10 μL of total volume. The lipoplexes were added into the cells and incubated at 37 °C under 5% CO_2 for 48 h. For the Lipofectamine™ 2000 transfection, the procedure was performed by following the manufacturer's instructions. After 48 h, the old medium was removed and the Z buffer (100 μL) and sodium dodecyl sulfate (SDS) agent (50 μL) were added into the cells. After 15 min, *o*-nitrophenyl-β-d-galactopyranoside (ONPG) solution (10 mg/mL; 100 μL) was added and the cells were then incubated for 4 h before measuring the absorbance at 405 nm. In order to evaluate the transfection efficiency, pEGFP-C2 plasmid DNA encoding green fluorescent protein (GFP) was employed. After incubating at 37 °C under 5% CO_2, the cells were measured by fluorescent microscopy.

2.5 Transfection cytotoxicity

HEK293 cells were seeded in a 96-well plate. The cells were incubated for 24 h at 37 °C under 5% CO_2. This step followed the same procedure as the transfection experiment. After incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) dissolved in PBS was added to each well to reach the final concentration of 5 μg/μL. The cells were incubated again for 4 h at 37 °C under 5% CO_2. The absorbance was measured at 550 nm on the microplate reader.

2.6 Serum stability of deoxyoligonucleotides (ODN)

At this step, examining the serum stability assay was in line with the previous procedures. The lipoplexes of the lipid **4a** were incubated with fetal bovine serum at 37 °C for 1, 2, 4, 6, 12, and 24 h. Serum enzyme was inactivated by heating at 70 °C for 15 min. Then, gel electrophoresis was performed under +100 V for 30 min on 1% agarose gel, which contained ethidium bromide for visualization.

2.7 Size and zeta potential measurements

Photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments Ltd. Malvern, UK) was employed to analyze the particle size and surface charge of DNA/liposome complexes. The prepared complexes were diluted with distilled water and then filtered through a 0.22 μm membrane before performing the measurements in five replicates at room temperature.

3. Results and Discussion

3.1 Synthesis

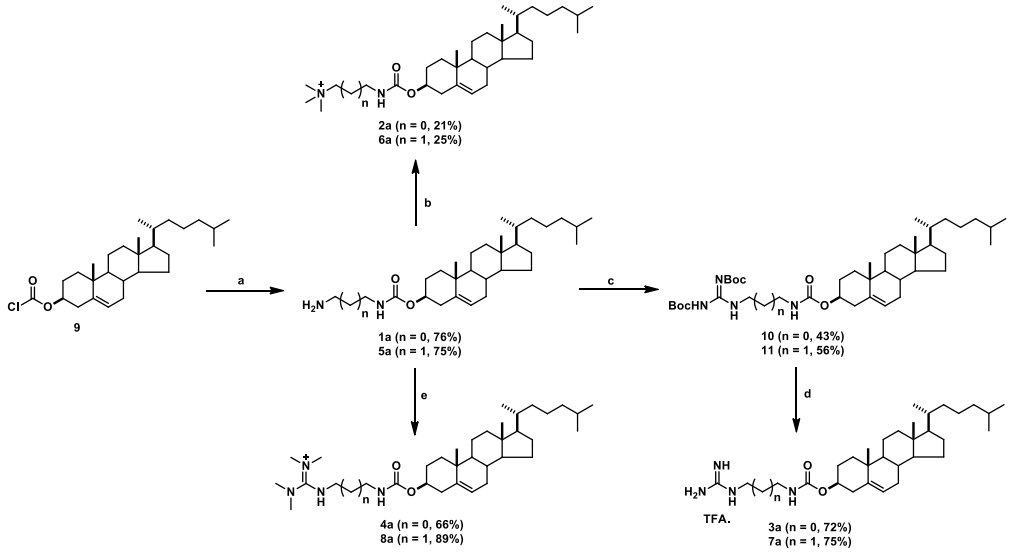
In this study, we synthesized both new, [**4a**, (**7**–**8**)**a**] and known, [(**1**–**3**)**a**, (**5**–**6**)**a**] cholesterol-based cationic lipids with different spacers and hydrophilic headgroups via a carbamate type linker (Scheme 1). It was found that cholesteryl chloroformate (**9**) reacted with 1,2-diaminoethane or 1,3-diaminopropane in highly diluted conditions to afford lipids **1a** or **5a**, respectively. Lipids **1a** and **5a** were used as starting compounds to synthesize cationic lipids **2a**, **3a**, **6a**, and **7a** through the previously reported method (Radchata wedchakoon *et al.*, 2010). According to a previous study by Valeur & Bradley (2009), lipids **1a** and **5a** reacted with HBTU to afford lipids **4a** and **8a**, respectively. In this regard, spectroscopic techniques confirmed the structures of the desired lipids.

3.1.1 Spectroscopic data of active lipid 3β-[(2-((*N′*,*N′*,*N″*,*N″* tetramethyl)guanidinyl) ethyl)carbamoyl]cholesterol (4a)

Yield: 66%; IR: ν_{max} 3403, 3360, 2939, 2867, 2848, 1693, 1620, 1582, 1522, 1404, 1237, 1225, 1011, 832, 776 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 10 drops of CD_3OD): δ 0.57 (s, 3H, CH_3-18), 0.76 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27), 0.82 (d, J = 6.4 Hz, 3H, CH_3-21), 0.90 (s, 3H, CH_3-19), 0.88−2.20 (m, 30H, protons in cholesteryl skeleton), 2.88 (s, 10H, (−NHC<u>H</u>$_2$C<u>H</u>$_2$NHC(N(C<u>H</u>$_3$)$_2$)), 3.35 (s, 6H, −NHC<u>H</u>$_2$ C<u>H</u>$_2$NHC(N$^+$(C<u>H</u>$_3$)$_2$)), 4.32 (br s, 1H, H-3-Chol), 5.26 (br s, 1H, H-6-Chol), 6.82 (s, 1H, N=CN<u>H</u>CH$_2$CH$_2$NHCO$_2$Chol), 7.87 (s, 1H, N<u>H</u>CO$_2$Chol); ^{13}C NMR (100 MHz, $CDCl_3$ + 10 drops of CD_3OD): 11.8, 18.6, 19.2, 20.9, 22.5, 22.7, 23.7, 24.2, 27.9, 28.0, 28.1, 31.7, 31.8, 35.7, 36.1, 36.5, 36.9, 39.6, 40.0, 42.2, 42.6, 49.9, 56.0, 56.6, 74.5, 122.4, 139.7 (carbons in cholesteryl skeleton), 38.5 (C=N$^+$(<u>C</u>H$_3$)$_2$), 39.7 (N(<u>C</u>H$_3$)$_2$), 39.4 (−NH<u>C</u>H$_2$CH$_2$NHCO$_2$Chol), 40.0 (−NHCH$_2$<u>C</u>H$_2$NHCO$_2$ Chol), 157.2 (C=O carbamoyl), 161.9 (C=NH); MS (ESI$^+$): *m/z* 572.4 ([M]$^+$, 100%); HRMS (ESI-TOF) *m/z*: calcd. for $C_{35}H_{63}N_4O_2$: 571.4945; found 571.4916.

3.2 DNA binding affinity

Cationic liposomes are formulated from either cationic lipids alone or more frequently with a combination of cationic lipids and neutral lipids such as DOPE (Mochizuki *et al.*, 2013). In this study, cationic liposomes were prepared from individual lipids (**1**−**8**)**a** and with DOPE at a 1:1 weight



Scheme 1. Reagents and conditions: (a) 1,2-Diaminoethane (ethylenediamine) or 1,3-diaminopropane (1 equiv), CH_2Cl_2, 24 h; (b) CH_3I (4 equiv), DIEA, DMF, 12 h; (c) 1,3-Bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (1.2 equiv), DIEA, DMF, 12 h; (d) 20% TFA in CH_2Cl_2 , 2 h; (e) *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (1.2 equiv), DIEA, DMF, 12 h.

ratio. Lipoplexes were formed by addition of DNA to these cationic liposomes. The DNA binding abilities were studied by gel retardation assay. Most of the synthesized lipids did not bind to the DNA (Figure 2). Cationic liposomes **4a**/DOPE, **6a**/DOPE, and **8a**/DOPE containing tetramethyguanidinium and trimethylammonium as the polarheads could form the complex with DNA.

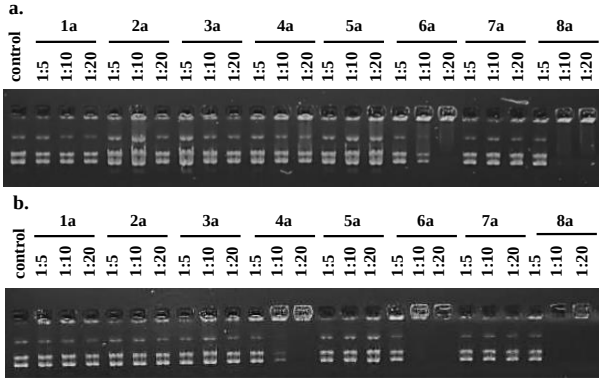


Figure 2. Electrophoretic gel retardation assays of lipoplex at DNA:lipid weight ratios of 1:5, 1:10, and 1:20. (a) Lipoplexes (**1**-**8**)**a** without DOPE (b) Lipoplexes (**1**-**8**)**a** with DOPE (weight ratio of lipid:DOPE at 1:1). The samples were electrophoresed on 1% agarose gel in TBE buffer at 100 mV for 30 min.

3.3 Transfection screening

Cationic lipids were examined for transfection activity against HEK293 cells. ONPG was used as a substrate to determine the gene expression of β-galactosidase (Radchata wedchakoon, Krajarng, Niyomtham, Watanapokasin, & Yingyongnarongkul, 2011; Wirtz, Galle, & Neurath, 1999). The screening condition used 0.1 µg DNA/well and 1:20 (w/w) DNA/lipids with and without the helper lipid (DOPE). The ratio of liposome and DOPE was used at a weight ratio of 1:1. The relative transfection efficiency is shown in Figure 3a. The lipids **4a** and **6a** with DOPE exhibited higher transfection efficiency than the other lipids (compared with Lipofectamine^{TM} 2000, 100%). The lipids **4a** and **6a** were selected for the additional transfection optimization by varying the lipid/DOPE ratios. Then, the selected lipids further optimized the DNA/amount of cationic lipids ratios and the amounts of DNA per well.

3.4 Transfection optimization: cationic lipid/DOPE ratios, DNA/amount of cationic lipids ratios and the amount of DNA per well

DOPE is a well-known helper-lipid. DOPE is a fusogenic lipid for destabilizing the bilayer in a liposome (Mochizuki *et al.,* 2013). Normally, cationic lipids are mixed with DOPE to promote the conversion of lamellar lipoplex phase into a hexagonal phase which is known to improve the transfection efficiency. In this experiment, the liposomes were formulated from different weight ratios of cationic lipids:DOPE at 3:1, 2:1, 1:1, 1:2, and 1:3. The lipoplexes were prepared by mixing the DNA and liposomes at a DNA concentration of 0.1 µg/well (1:20 w/w, DNA/lipid). The appropriate weight ratios of lipid **4a**/DOPE were 2:1 and 1:1. For the lipid **6a**/DOPE, the ratios were 3:1 and 1:1 (Figure 3b). The cationic lipids **4a** (lipid/DOPE ratio at 1:1) and **6a** (lipid/DOPE ratio at 1:1) showed maximum transfection efficiency. In addition, the lower transfection efficiency was found at the ratios of 1:2 and 1:3 for all selected cationic lipids. As a result, the amount of DOPE influenced gene delivery. The highly efficient lipids **4a** and **6a** were subjected in the next experiment to vary the amount of liposome.

The liposomes from the lipids **4a** and **6a** at a 1:1 weight ratio of lipid/DOPE were used to vary the amount of cationic liposome. The DNA/amount of cationic liposome

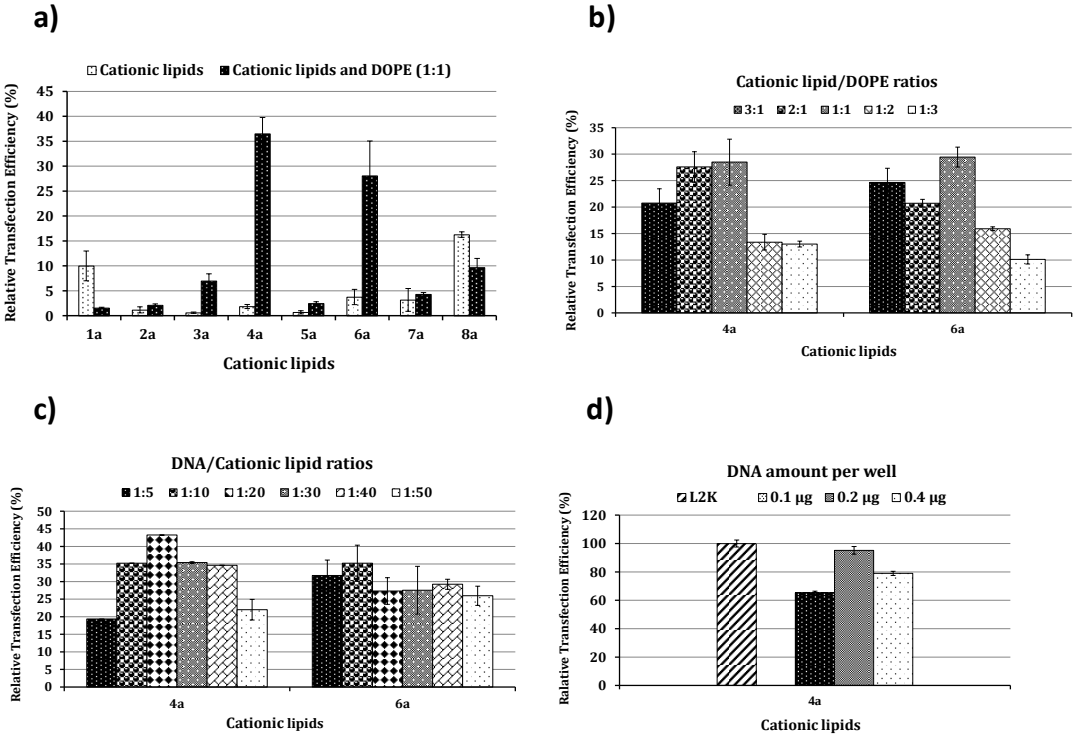


Figure 3. a) Relative transfection screening efficiency of cationic lipids with DOPE ratio of 1:1 and without DOPE the corresponding lipoplexes were prepared from pCH110-encoding β-galactosidase (0.1 µg/well) and the lipids in the ratio of 1:20 by weight. b) Relative transfection efficiency of cationic lipids **4a** and **6a** with the different ratios of lipid:DOPE at 3:1, 2:1, 1:1, 1:2, and 1:3, and 0.1 µg of DNA/well in HEK293 cells. c) Relative transfection efficiency of cationic lipids **4a** and **6a** containing the appropriate amount of DOPE from Figure 3b with the ratios of DNA:cationic lipid at 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50, and 0.1 µg of DNA/well in HEK293 cells. Commercially available Lipofectamine™ 2000 was used as the reference (100%) for the transfection (data not shown). d) Relative transfection efficiency of amount of DNA for gene transfer. The optimal amount of DOPE (Figure 3b) and DNA/cationic lipids ratios (Figure 3c) were selected to use with different amounts of DNA at 0.1, 0.2, and 0.4 µg of DNA/well.

ratios at 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50 (w/w) assayed the transfection activity (0.1 µg of DNA/well). We found that lipids **4a** and **6a** collectively revealed the highest transfection efficiency at the DNA/amount of cationic lipid ratios of 1:20 and 1:10, respectively (Figure 3c). The results indicated that low transfection efficiency was observed as the number of cationic lipids increased.

When we achieved the optimal ratios of cationic lipid/DOPE (Figure 3b) and the amount of cationic lipid (Figure 3c), the amount of DNA was varied at various ratios in the next experiment. Additionally, the amounts of DNA used were 0.1, 0.2, and 0.4 µg/well. It was also found that the lipid **4a** exhibited the highest transfection activity at 0.2 µg of DNA/well (Figure 3d).

3.5 Effect of serum

To visualize and compare the transfection efficiency of the lipid **4a** with the commercial agent, the transfection experiments were performed on HEK293 cells by using GFP as the reporter gene. In the absence of serum, some reports revealed that cationic liposomes with high transfection activity lost their efficiency when transfected in the presence of serum (Ghosh, Visweswariah, & Bhattacharya, 2000; Yingyongnarongkul, Radchatawedchakoon, Krajarng, Watana pokasin, & Suksamrarn, 2009). Therefore, the transfection ability of the lipid **4a** under serum containing conditions was also studied. The transfection efficiency of the lipid **4a** was compared with Lipofectamine™ 2000 by observing the transfected cells under a fluorescence microscope (Figure 4) and the fluorescence cells were counted per square centimeter (cells/cm^2) (Figure 5). The lipid **4a** (6280±26 cells/cm^2) had a slightly lower *in vitro* transfection efficiency than the Lipofectamine™ 2000 (8140±98 cells/cm^2) under the serum-free condition (Figures 4 and 5). The transfection efficiency of the lipid **4a** under the serum condition was further investigated. The highest transfection efficiency of the lipid **4a** (6840±15 cells/cm^2) at 10% serum was higher than the Lipofectamine™ 2000 (4760±32 cells/cm^2). The efficiency slightly decreased when the experiments were performed under 20% and 40% serum conditions. The transfection efficiency of Lipofectamine™ 2000 was dramatically diminished when performed under serum conditions. Lipofectamine™ 2000 lost half of its transfection efficiency under the 20% serum condition (Figure 5). From these experiments, we found that the lipid **4a** could protect the DNA from the serum.

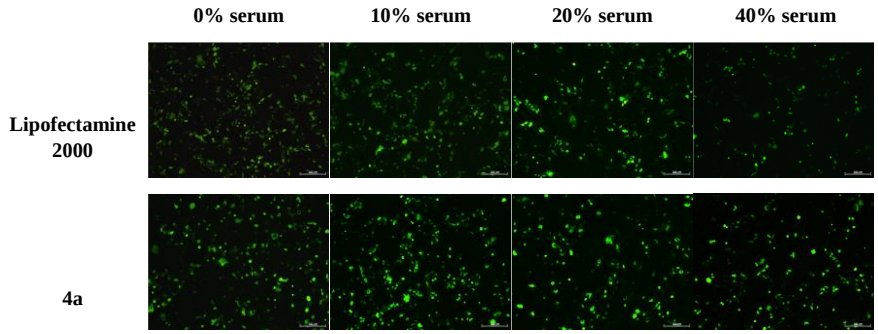


Figure 4. After 48 h, the expression of gene encoding for GFP transferred into HEK293 cells by **4a**/DOPE = 1/1 at ratio of 1:20 (DNA/lipid) and Lipofectamine™ 2000 under 0%, 10%, 20%, and 40% serum conditions was observed under fluorescence microscopy.

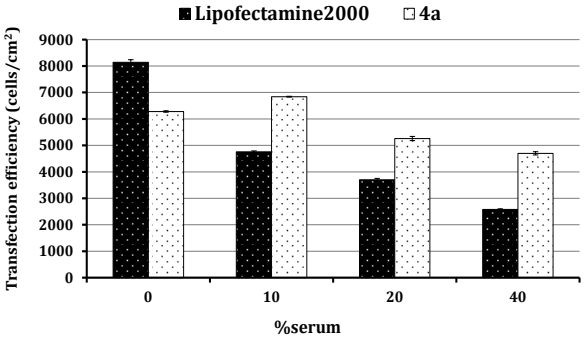


Figure 5. Transfection efficiency of DNA/lipid weight ratio 1:20 in HEK293 cells.

3.6 Protection of DNA from serum

The exonucleolytic degradation of ODN by serum was tested on the basis of previous reports (Obata, Saito, Takeda, & Takeoka, 2009; Semple *et al.*, 2001). Free or encapsulated ODN was incubated at 30 °C several times. ODN encapsulation showed that the DNA in 10% serum became completely degraded. When the lipoplex **4a** was treated in the presence of 0.5% SDS it could not bind DNA. In contrast, the lipoplex **4a** in the absence of 0.5% SDS retarded the migration of DNA. The results indicated that the lipoplex could be encapsulated by ODN in 10% serum. Using Lipofectamine™ 2000 as the positive control provided the same result (Figure 6). Moreover, the 40% serum also demonstrated the approximated ODN encapsulation efficiency (Figure 7).

3.7 Cytotoxicity

The cytotoxicity of a cationic vector is related to the chemical structure and the nature of the vector. The toxicity of the transfection agent must be taken into account before development for an *in vivo* application. The cytotoxicity of the cationic liposome **4a** was investigated by MTT assay on HEK293 cells. The liposome **4a** was not toxic to the tested cells (Figure 8). Ultimately, the non-toxic liposome could potentially reach the preclinical and clinical trials in the future.

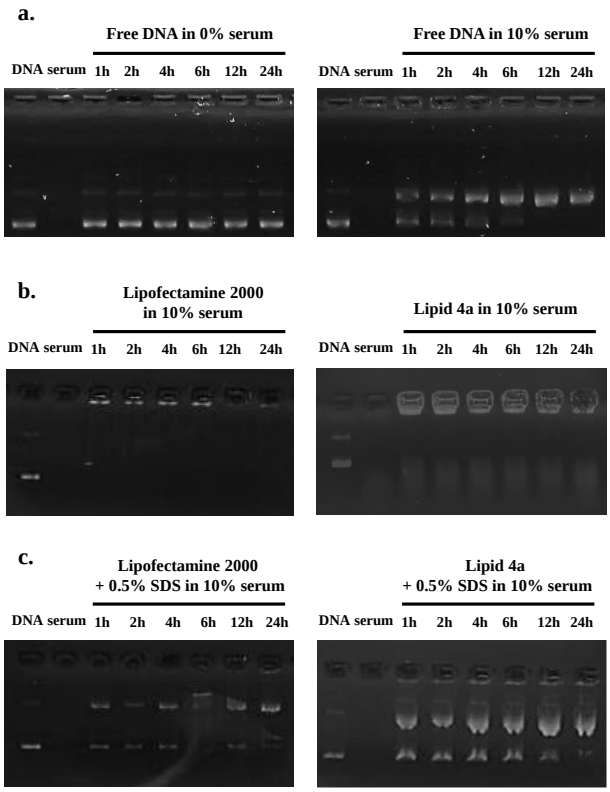


Figure 6. At 10% serum, serum stability of pDNA at different times. (a) Both DNA in serum and pure DNA, (b) Lipofectamine™ 2000 and lipid **4a** in 10% serum and (c) Lipofectamine™ 2000 and lipid **4a** in 10% serum that contained 0.5% SDS.

3.8 Particle size and zeta potential measurements

Particle size and zeta potential of the lipoplexes were analyzed by dynamic light scattering assay. The results indicated that the largest particle size was obtained when the amount of lipid was in the DNA/liposome ratio of 1:10 (Figure 9a). The lipoplexes **4a** had a particle size of approximately 286−770 nm. It was proved that the average particle size of the prepared liposomes in that range was

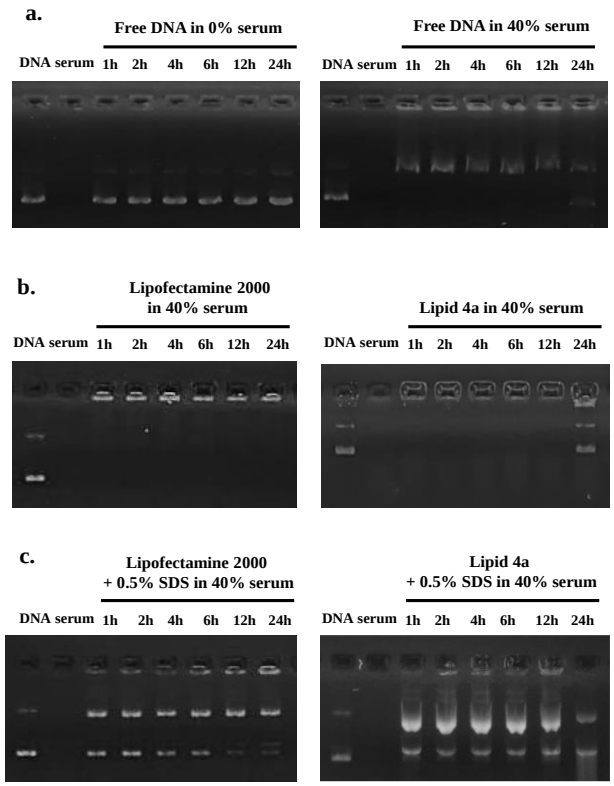


Figure 7. At 40% serum, serum stability of pDNA at different times. (a) Both DNA in serum and pure DNA, (b) Lipofectamine™ 2000 and lipid **4a** in 40% serum and (c) Lipofectamine™ 2000 and lipid **4a** in 40% serum that contained 0.5% SDS.

suitable for gene delivery (Li *et al.*, 2012; Paecharoenchai *et al.*, 2012). The zeta potential of liposomes is an indirect measurement of the surface charge (Zhao *et al.*, 2014). The surface charge of the complexes depends on the ratio of the condensing agent to DNA and also the nature of the agent. The zeta potential was approximately 34–35 mV (Figure 9b). As the weight of liposome increased, the zeta potential tended to grow from negative (around −12 and −7 mV at the ratios of 1:2 and 1:5, respectively) to positive values (around +0.12, +35, and +34 mV at the ratios of 1:10, 1:20, and 1:30, respectively). Since the highest zeta potential was observed at

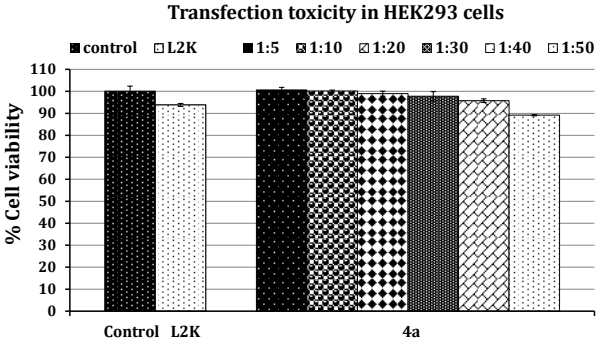


Figure 8. Percentage of cells viability indicates the cytotoxicity in HEK293 cells. The DNA/lipid ratios were at 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50. Cell metabolic activity was determined by MTT assay.

the weight ratio of 1:20, it firmly affirmed the full DNA condensation with liposome to form lipoplexes. Takeuchi *et al.* (1996) synthesized and measured the zeta potential of eight derivatives of cholesterol. The zeta potential values of these lipoplexes were found to be between +9.0 and +33.6 mV. The lipoplexes at the highest value (+33.6 mV) showed the highest transfection efficiency. In our investigation, the zeta potential of the lipid **4a** was +35.1 mV which, accordingly, showed the highest transfection efficiency. Hence, it can be concluded that the zeta potential was relatively consistent with the value of transfection activity. The result suggested that the zeta potential of a cationic liposome is one of the important factors to affect gene transfection by a cationic liposome. Turek *et al.* (2000) confirmed that the large-sized lipoplexes over 700 nm diameter induced efficient transfection in the presence of serum. However, we found that the cationic lipid **4a** showed higher transfection efficiency than the commercial reagent under serum conditions (Figure 5) even though the size of the lipoplexes was less than 700 nm (Figure 9a).

4. Conclusions

In summary, cationic lipids used for this study containing cholesterol as hydrophobic tails were successfully synthesized. The headgroups including trimethylammonium, guanidinium, and tetramethylguanidinium and a carbamate linker were employed. The novel cationic lipid **4a** with the

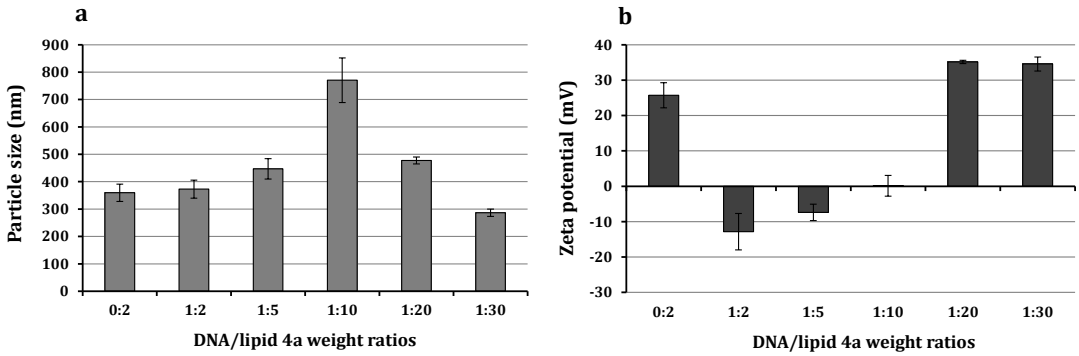


Figure 9. (a) Mean particle size and (b) zeta potential of the lipoplexes **4a** under various DNA/liposome **4a** weight ratios. Each value represents the mean±standard deviation of five measurements.

tetramethylguanidinium headgroup showed the highest transfection efficiency in the presence of the helper lipid, DOPE. However, cholesterol-based cationic lipids with the trimethylammonium headgroup also showed sufficient transfection efficiency. The optimal conditions of the cationic lipid **4a**, which presented the highest transfection efficiency into HEK293 cells, were comprised of lipid/DOPE at a weight ratio of 1:1, DNA/liposome ratio of 1:20, and the amount of DNA at 0.2 μg/well. The cationic lipid **4a** also exhibited better transfection efficiency than the positive control (Lipofectamine™ 2000) in the presence of serum. These optimal conditions could be applied as a non-viral transfection vector for further preclinical and clinical trials. The reasons why tetramethylguanidinium headgroup was the most potent in transfection are still not fully understood, but it probably relates to the high affinity of DNA binding. It may be due to the lipids with cholesterol and they could be formulated to the corresponding liposomes more easily.

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