RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#) Cite this: *RSC Med. Chem.*, 2020, **11**, 212

The effect of polar headgroups and spacer length on the DNA transfection of cholesterol-based cationic lipids†

Widchaya Radchatawedchakoon,^a ^{*,a} Chopaka Thongbamrer,^b Wuttiphong Konbamrung,^a Phakamas Khattawee,^b Uthai Sakee,^a Wanlapa Roobsoong,^c ^c Jetsumon Sattabongkot,^c Praneet Opanasopit^d and Boon-ek Yingyongnarongkul^b

This article is related to the effects of the headgroups and spacer length of cationic lipids on transfection efficiency. To develop highly potent cationic lipids, a series of divalent lysine-diamine conjugated cholesterol-based cationic lipids with three different headgroups (ammonium, trimethyl ammonium, and guanidinium) were synthesized. The newly synthesized cationic lipids (1–6)A formed cationic liposomes in the presence and absence of a zwitterionic helper lipid, DOPE (dioleoylphosphatidylethanolamine). A gel retardation assay showed that most of the prepared lipoplexes could retard DNA migration in the presence of DOPE. We attempted to modify the diverse cationic headgroups to improve the transfection efficiency. However, the lysine-1,3-diaminopropane-conjugated cholesterol-based lipid 4A, having divalent ammonium of unmodified lysine headgroup, exhibited high relative transfection efficiency in HEK293. When the transfection efficiency of 4A was formulated with DOPE (1:1 weight ratio), it produced the same range in comparison with that of a commercially available transfection agent, Lipofectamine™ 2000 (L2K). The lipid 4A was studied to optimize the conditions with respect to the lipid/DOPE and DNA/lipid ratios and the amount of DNA. The transfection efficiency of the highly potent lipid 4A was also studied to determine the transfection efficiency of HeLa, PC3, and HC-04 cell lines. This lipid also protected the DNA from a serum and had low toxicity. Lipoplexes 4A with DOPE had the particle size of around 300–600 nm and the zeta potential of around 0–45 mV. In summary, cationic liposomes 4A demonstrated a high performance as DNA carriers.

Received 24th September 2019,
Accepted 11th December 2019

DOI: 10.1039/c9md00459a

rsc.li/medchem

Introduction

Gene therapy is a strategy that uses genes to prevent diseases by inhibiting the expression of a protein or by inserting a gene to induce the expression of a specific protein.^{1–3} The transfection of a genetic material into the targeted cells will be an important *in vitro* biochemical technique for gene

therapy in the future.⁴ There are multiple applications of nucleic acid delivery. For example, it is used for treating inherited disorders and monogenic diseases (cystic fibrosis)⁵ as well as for conducting the *in vitro* transfection of fibromodulin, leading to better healing after surgical injuries;⁶ it is also used for achieving effective antitumor T-cell immunity.⁷ Although viral vectors are the most efficient carriers, their immunogenicity, toxicity and mutagenicity limit their applications.⁸ Therefore, nonviral or synthetic vectors are interesting and are being developed for applications in these areas. Different vehicles are being used as machines for the transfer of genetic materials into cells. One of the nonviral vectors is the cationic lipid.^{9–12} Cationic lipids can form liposomes in a buffer. The complexes between cationic liposomes and nucleic acids (lipoplexes) are commonly used as nucleic acid delivery systems. These lipoplexes can also encapsulate nucleic acids and generate adhesion to the negatively charged cell membrane. The nucleic acids enter the cells either by the process of endocytosis¹³ or by endosomal membrane destabilization.¹⁴ However, the exact

^a Creative Chemistry and Innovation Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Mahasarakham University, Maha Sarakham, 44150, Thailand.

E-mail: widchaya.r@msu.ac.th, widchaya@hotmail.com; Fax: +66 43 754246; Tel: +66 43 754246

^b Department of Chemistry and Center of Excellence for Innovation in Chemistry (PERCH-CIC), Faculty of Science, Ramkhamhaeng University, Bangkok, 10240, Thailand

^c Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand

^d Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, 73000, Thailand

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9md00459a

mechanism of gene transfection remains unclear. Helper lipids such as cholesterol, dioleoylphosphatidylethanolamine (DOPE) and phosphatidylcholine (PC) perform an assistant function and promote the endosomal release of antisense oligonucleotides.^{15,16} Lipoplexes are often prepared by adding a helper lipid in order to augment their stability and enhance the transfection efficiency.¹⁷

Normally, a cationic lipid comprises three basic parts: a positively charged headgroup, a linker, and one or two hydrophobic tail regions.¹⁸ These domains of cationic lipids can affect their transfection efficiency and toxicity.^{19–22} Cationic lipids can adopt various structural phases when suspended in an aqueous solution.^{23,24} In 1987, Felgner and co-workers first reported the usage of the *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) cationic lipid for transfecting DNA into mouse L cells.²⁵ One of the most popular cationic headgroups of cationic lipids is an amino acid. Several researchers have reported the efficient transfection efficiencies of cationic lipids with amino acid moieties. As for the headgroups, Park and colleagues also reported highly efficient cationic amphiphilic headgroups based on cholesterol directly linked to *L*-lysineamide and *L*-ornithineamide.²⁶ Gu and collaborators could improve the transfection efficiency in the presence of a serum by using synthesized amino acids based on cationic lipids: lysinated and arginated cholesterol.²⁷ Cao *et al.* and Zhang *et al.* reported that the *L*-lysine headgroup bearing lipids showed high pDNA binding affinity and also exhibited high transfection efficiency.^{28,29}

Guanidine headgroups have been added into cationic lipids and cationic transfection lipids as guanidinium headgroups are attractive nonviral gene delivery vectors. The Lehn group reported that the cholesterol derivatives that bore two guanidinium groups presented high transfection activity when formulated as liposomes with a helper lipid, DOPE.³⁰ Bradley and co-workers found that lipids having two guanidinium headgroups and one aliphatic tail had high transfection efficiency.³¹ Byk and colleagues reported the usage of cyclic guanidine headgroups as transfection agents.³² The methylation of the polar headgroups of cholesterol-based derivatives and transfection activities were also reported by Savva and co-workers.³³ DOTMA was the first cationic lipid with a quaternary ammonium headgroup.²⁵ In this regard, Stevens *et al.* reported that the quaternary ammonium headgroup of sophorolipids was proven to be a more efficient pDNA carrier.³⁴ Cao and co-workers further found that the synthesis of the modified headgroup of cholesterol-based lipids can be carried out by both methylation and ethylation reactions. The data indicated that among the synthesized lipids, gene transfer is gained by the methylated headgroup.³⁵

In addition, the transfection efficiency of the cationic lipids with imidazole headgroups is parallel to that of the commercially available 3β-[*N*-(*N,N'*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).³⁶ The cationic polymer with a highly branched trimethylphosphonium group showed high

transfection efficiencies and reduced cytotoxicity.³⁷ The trimethylarsonium cationic headgroup had a high potential for the extended study of nonviral vectors.³⁸ The pyridinium headgroups of cationic lipids with a different linker and hydrophobic tail were able to transfect NCI-H23 and had low cytotoxicity.³⁹

Previous reports demonstrated that the spacer length between the cationic headgroup and the hydrophobic tail influenced the transfection results.^{21,35,40,41} The transfection efficiency of divalent cationic lipids was higher with a shorter spacer between the cationic heads.⁴²

Herein, we focused on the effect of the headgroups and spacer length, synthetic methodology, DNA binding property, and transfection efficiency of the newly synthesized divalent lysine-diamine-conjugated cholesterol-based cationic lipids (1–6)A (Fig. 1). Various factors were involved: different cationic headgroups and spacer lengths and the appropriate ratio of the prepared liposomes. Their influence largely affected the results of transfection efficiencies. Moreover, we reported the transfection optimization conditions for achieving a highly efficient branch-shaped lipid. We also further characterized the highly efficient lipoplexes on the basis of cytotoxicity, particle size and zeta potential. Our data yielded the versatility and usefulness of the synthesized cationic lipids for gene transfer.

Results and discussion

Synthesis of designed cationic lipids

The synthetic routes for a series of novel cationic lipids with modified headgroups of *N*-terminal α-amino groups and different spacer arms are shown in Scheme 1. Amphiphiles consist of natural lipids and amino acids that can be metabolized in the cells.⁴³ The hydrophobic cholesteryl part of cationic lipids is connected by a spacer with different lengths of either two or three carbons and by a carbamate bond to different headgroups. Starting from cholesteryl chloroformate 7, diaminoalkanes reacted *via* a very high dilution method. The resulting products coupled with *N*-α-Fmoc-*N*-ε-Boc-*L*-lysine (Fmoc-Lys(Boc)-OH) by using *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluoro phosphate (HBTU) as a coupling agent.⁴⁴ Then, Fmoc, which protected the amino groups of the compounds

Fig. 1 General structure of the synthesized lipids (1–6)A.

Scheme 1 Synthesis of modified headgroups of *N*-terminal α -amino groups of lysine-diamine conjugated cholesterol-based cationic lipids (1–6)A. Reaction conditions: (a) 1,2-diaminoethane or 1,3-diaminopropane, CH_2Cl_2 , rt, 4 h; (b) *N*- α -Fmoc-*N*- ϵ -Boc-L-lysine, HBTU, DIEA, CH_2Cl_2 , acetonitrile, rt, 3 h; (c) 20% piperidine, CH_2Cl_2 , 2 h; (d) 20% TFA/ CH_2Cl_2 , 2 h; (e) CH_3I , DIEA, CH_2Cl_2 ; (f) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, DIEA, CH_2Cl_2 ; HBTU = *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; DIEA = diisopropylethylamine; TFA = trifluoroacetic acid; Boc = *tert*-butoxycarbonyl.

10–11, was subsequently removed by treating with 20% piperidine in dichloromethane to give free primary amines of the compounds 12–13. In this regard, Lehn and co-workers reported the guanylation of amine in guanidinium-cholesterol cationic lipids by reacting with 1*H*-pyrazole-1-carboxamide hydrochloride in the presence of diisopropylethylamine.³⁰ We selected 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea for guanylation.⁴¹ To modify the headgroup of the synthesized cationic lipids, the compounds 12–13 were reacted with methyl iodide and 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in basic conditions to afford the compounds 14–17. The Boc protecting group of the synthesized compounds was treated with a solution of 20% trifluoroacetic acid in dichloromethane to give the cationic lipids (1–6)A. The amino groups were stored as the salts of trifluoroacetic acid. The structures of the isolated products were confirmed by spectroscopic evidence.

Interaction of synthesized cationic lipids with DNA

To approve the formation of complexes, the DNA binding affinities of the synthesized cholesterol-based cationic lipids were investigated by gel retardation assays at various ratios.⁴⁵ The gel electrophoresis results are shown in Fig. 2. Because of the negative charge on the phosphate group in DNA molecules, the nucleic acid migrates to the positive pole during electrophoresis. The migration of free DNA bands gradually decreased when the amount of cholesterol-based cationic lipids increased. For DNA condensation, cationic liposomes were prepared as the sample by taking cationic

liposomes (1–6)A with DOPE at a weight ratio of cationic lipid:DOPE as 1:1 and without DOPE at the weight ratios of DNA:cationic lipid as 1:10, 1:20 and 1:40. The lipids 2A and 5A without DOPE and 1A, 2A, 4A and 5A with DOPE could completely retard DNA at all weight ratios, while the other lipids with and without DOPE showed lower DNA binding affinity and complete DNA retardation at all weight ratios. The results also indicated that the liposomes completely inhibited the electrophoretic mobility of plasmid DNA when lipids 1A and 4A were mixed with the helper lipid (DOPE). The results also suggested that the helper lipid was considered as the influencing factor for DNA binding

Fig. 2 Electrophoretic gel retardation assays of lipoplexes at weight ratios of DNA:cationic lipid as 1:10, 1:20 and 1:40 (control: naked DNA). (a) Lipoplexes (1–6)A without DOPE; (b) lipoplexes (1–6)A with DOPE at a weight ratio of cationic lipid:DOPE at 1:1. The samples were electrophoresed on 1% agarose gel in TBE buffer at 100 mV for 30 min.

affinities. The lipids **1A** and **4A** with divalent ammonium headgroups showed highly efficient DNA retardation while preparing the liposomes, which had the helper lipid DOPE. The divalent ammonium headgroups of the synthesized lipids might form efficient non-covalent chemical bonds with DNA.

The trimethylammonium headgroup of the lipids **2A** and **5A** exhibited a high potential for DNA binding efficiency both with and without DOPE. The guanidinium headgroups of lipids **3A** and **6A** did not show the retardation of the DNA movement.

In vitro gene transfection

In order to find out the effective cationic lipids, all the synthesized lipids (**1–6A**) were tested in human embryonic kidney cells (HEK293). The optimal weight ratios of the DNA : cationic liposome from 1 : 10 to 1 : 40 of the lipoplexes were estimated by using the gel retardation experiment. The band

of DNA with different DNA : lipid ratios, displayed in Fig. 2, showed that the range of ratios did not exhibit the tendency of DNA binding. Therefore, we chose a moderate ratio of 1 : 20 (DNA : cationic lipid) for transfection screening. The transfection efficiencies of the cationic liposomes were examined using the weight ratio of lipid : DOPE as 1 : 1 and the weight ratio of DNA : lipid as 1 : 20. In Fig. 3a, the results of the transfection screening of all lipids are shown. The commercial transfection agent Lipofectamine™ 2000 (L2k) was chosen as the control. DNA (pCH110-encoding β -galactosidase)/cationic liposome complexes were prepared. β -Galactosidase was investigated by adding the solution of *o*-nitrophenyl-galactopyranoside (ONPG). The intensity of absorbance at 405 nm was compared and converted with that for the positive control Lipofectamine™ 2000 (L2k). The transfection results of all the synthesized lipids indicated that high transfection efficiencies were exhibited by the lipids **4A**, **1A**, **5A**, and **2A** with DOPE. The helper lipid (DOPE) is one of the factors influencing the transfection efficiency. A

Fig. 3 Relative transfection efficiency of cationic lipids (a) with DOPE ratio of 1 : 1 and without DOPE; the corresponding lipoplexes were prepared from pCH110-encoding β -galactosidase (0.1 μ g per well) and the lipids in the ratio of 1 : 20 by weight; (b) with the different ratios of lipid : DOPE at 3 : 1, 2 : 1, 1 : 1, 1 : 2 and 1 : 3, and 0.1 μ g of DNA per well in HEK293 cells; (c) with the ratios of DNA : cationic lipid at 1 : 5, 1 : 10, 1 : 20, 1 : 30, 1 : 40 and 1 : 50, 0.1 μ g of DNA per well in HEK293 cells, 3 : 1 by weight for lipid **1A** : DOPE and 1 : 1 by weight for lipid **4A** : DOPE; (d) with different amounts of DNA as 0.1, 0.2 and 0.4 μ g of DNA per well, 3 : 1 by weight for lipid **1A** : DOPE, 1 : 1 by weight for lipid **4A** : DOPE, 1 : 50 by weight for DNA : lipid **1A** and 1 : 10 by weight for DNA : lipid **4A**. Commercially available Lipofectamine™ 2000 (L2k) was used as the reference (100%) for the transfection (data not shown).

high transfection efficiency was achieved with the cationic liposome **4A** having three carbons in the spacer. Varying the distance between the cationic headgroup and the cholesteryl hydrophobic tail affected the transfection screening efficiency with the helper lipid DOPE in the order **4A** > **1A** > **5A** > **2A** > **3A** = **6A** (Fig. 3a). In the study by Cao *et al.*,³⁵ they also observed similar results with cholesterol-based lipids; their study yielded that three carbons of spacer length of 1,3-diaminopropane part influenced transfection activity. Among the six synthesized cationic lipids (**1–6A**), the lipid **4A** was found to be more active than others in the transfection screening efficiency. The modified headgroups of the parent lipids **1A** and **4A** showed a significant decrease in the transfection activity. The results additionally indicated that the quaternary ammonium lipids exhibited low transfection efficiency because there was no buffering function in these headgroups.¹⁹ Consequently, we studied the effect of various ratios of lipid:DOPE and DNA:lipid and amounts of DNA on the transfection of liposomes. As can be seen from Fig. 3b, the lipids exhibiting high transfection are subjected to evaluation in various amounts of lipid:DOPE at the ratios of 3:1, 2:1, 1:1, 1:2 and 1:3. The higher transfection efficiency of the liposomes of **4A** was obtained at a weight ratio of 1:1 and for the liposomes of **1A**, it was obtained at a weight ratio of 3:1.

According to the transfection results for the HEK293 cells shown in Fig. 3c, the optimal ratios of the amount of DOPE were selected for the next experiment by varying the amounts of DNA:lipid. Here, the transfection efficiencies of the cationic liposomes **4A** and **1A** were considered, including the amount of the lipid:DOPE ratios of 1:1 and 3:1. The liposome **4A** gave high transfection efficiency at a DNA:lipid weight ratio of 1:10. The optimal ratios shown in Fig. 3(a–c) were taken to clarify the transfection of DNA. The results showed that the transfection efficiency of the liposome **4A** was the highest with a DNA amount of 0.2 µg. After the optimization of the cationic lipid:DOPE ratio, it can be seen from Fig. 3(a–d) that the cationic lipid with the highest transfection efficiency is the liposome **4A** at the weight ratios of lipid:DOPE = 1:1 and DNA:lipid = 1:10 and the amount of DNA = 0.2 µg.

Scherman and collaborators reported that serum compatibility is a major difficulty with cationic liposome-mediated gene transfection.⁴⁶ A serum significantly reduces the transfection efficiency of liposomal formulations because negatively charged serum proteins can compete with the cationic liposome–DNA complexes.⁴⁷ As can be seen from Fig. 3, the selected liposome **4A** was found to be one of the choices in the serum-free transfections in the HEK293 cells. As shown in Fig. 4, we carried out transfection experiments in the presence of 10–40% serum with the liposome **4A** at optimal ratios by using the ONPG substrate. The results are normalized in the function of the transfection results obtained with Lipofectamine™ (L2k). However, the transfection efficiency of the liposome **4A** slightly increased to produce a decent result and revealed above 90% of transfection ability in the presence of the serum. Lipid **4A**

Fig. 4 Relative transfection efficiency of cationic lipid **4A** in the presence of 10, 20, 30, and 40% of serum, 0.2 µg of DNA per well, 1:1 by weight for lipid **4A**:DOPE, and 1:10 by weight for DNA:lipid **4A**. Commercially available Lipofectamine™ 2000 (L2k) was used as the reference (100%) for transfection (data not shown).

exhibited transfection activity relatively equivalent to that for L2k in the presence of the serum.

The liposome **4A** was also evaluated for transfection efficiency into human cervical adenocarcinoma (HeLa), human prostate adenocarcinoma (PC3) and human hepatocyte (HC-04) cell lines, employing the commercially available transfection agent Lipofectamine™ 2000 (L2k) as a positive control. The concentration of transfected cells was visualized by using a green fluorescent protein with the

Fig. 5 Expression of pEGFP-C2 gene into HEK293, HeLa, PC3 and HC-04 cells by cationic liposome **4A** at optimized ratios (lipid/DOPE weight ratio of 1:1, DNA/lipid weight ratio of 1:10 and amount of DNA per well, 0.2 µg). The optimized ratios at different percentages of serum (0, 10, 20, and 40% FBS) were investigated. Lipofectamine™ 2000 (L2k) was used as a positive control.

HEK293, HeLa, PC3 and HC-04 cell lines. After incubation with the optimized conditions of lipoplexes **4A**, the internalization of pEGFP-C2 was measured by a fluorescence microscope. We present the images of the transfected cells using four different percentages of the serum and four types of cell lines (Fig. 5). The results indicated that the liposome **4A** had highly potent transfection efficiency in the presence of the serum with different cell lines, especially with the HEK293, HeLa and PC3 cells.

A flow cytometry assay was then used to confirm the transfection of pEGFP-C2. The percentage of the GFP cells and the mean fluorescence intensity (MFI)^{48,49} of lipoplexes **4A** at different percentages of the serum and cell lines are shown in Fig. 6. The GFP cell experiment showed that a high potential is observed for the liposome **4A** at the optimized transfection conditions. The percentage of the GFP cells and MFI of liposome **4A** with HEK293 at 40% FBS were obtained as $47 \pm 6\%$ and $(5.3 \pm 0.6) \times 10^6$, respectively. The results of MFI were consistent with the GFP expression, as shown in Fig. 5. When compared with Lipofectamine™ 2000 (L2k), the liposome **4A** showed higher transfection efficiency at 40% of serum with the HEK293 cell lines. The results exhibited that the efficiency of liposome **4A** in HeLa is lower than that in HEK293. The results suggested that the divalent ammonium lysine headgroup of lipid **4A** could enhance the ability of cationic liposomes for the transfection study, which is probably due to the balance between the synthesized lipid and surface charge density. The lipids are composed of lysine moieties that are

considered to be capable of interacting electrostatically with the phosphate groups of pDNA.²⁶

Protection of DNA from serum

The results of testing the exonucleolytic degradation of a deoxyoligonucleotide (ODN) by a serum based on a previous report⁵⁰ can be seen in Fig. 7. Free or encapsulated ODN was incubated at 30 °C for several times. ODN encapsulation showed that the DNA in 10% serum was 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 could retard the migration of DNA. The results indicated that the lipoplex (with and without DOPE) encapsulated ODN in 10% serum (Fig. 7(a–d)). Moreover, 40% serum also showed approximately similar ODN encapsulation efficiency (Fig. 7(e–h)).

Transfection cytotoxicity

The *in vitro* cytotoxicity of lipids was evaluated by a cell viability assay (MTT assay).⁵¹ The cytotoxicity of the selected cationic liposomes with a relatively higher transfection efficiency was assayed. We evaluated the cytotoxicity of lipoplexes **4A** at the lipid:DOPE weight ratio of 1:1, DNA:lipid weight ratio of 1:10 and amount of DNA per well of 0.2 µg in the HEK293, HeLa, PC3 and HC-04 cells (Fig. 8). Cell viability tests showed that the selected cationic liposomes **4A** exhibited less cytotoxicity (more than 85% cell viability) in all tested cell lines. The transfection cytotoxicity data of the liposome **4A** were consistent with the

Fig. 6 GFP cell (%) and mean fluorescence intensity (MFI) of lipid **4A** with optimized conditions in a) HEK293, b) HeLa, c) PC3 and d) HC-04 cell lines quantified by flow cytometry analysis. Lipofectamine™ 2000 (L2k) was used as the positive control. Data represent mean $\pm$ SD ($n = 3$).

Fig. 7 Electrophoretic gel retardation assays with and without DOPE at 10% and 40% serum, serum stability of pDNA at different times. (a) Free DNA in 10% and 0% serum, (b) Lipofectamine™ 2000 (L2k) in 10% serum with and without 0.5% SDS, (c) lipid 4A in 10% serum with and without 0.5% SDS, (d) lipid 4A : DOPE (1:1) in 10% serum with and without 0.5% SDS, (e) free DNA in 40% and 0% serum, (f) Lipofectamine™ 2000 (L2k) in 40% serum with and without 0.5% SDS, (g) lipid 4A in 40% serum with and without 0.5% SDS, and (h) lipid 4A : DOPE (1:1) in 40% serum with and without 0.5% SDS.

Fig. 8 Percentages of cell viability indicates the cytotoxicity in HEK293, HeLa, PC3 and HC-04 cells. The ratios of lipid 4A : DOPE weight ratio of 1:1, DNA : lipid 4A weight ratio of 1:10 and amount of DNA per well 0.2 μ g. Cell metabolic activity was determined by MTT assay. L2k = Lipofectamine™ 2000.

transfection results except with the HC-04 cell line. The synthesized lipid 4A with three carbons in the spacer exhibited low cytotoxicity, which was consistent with a previous result.³⁵ Lipid 4A comprised amide and carbamate bonds that could be degraded in the cells.²⁶

Characterization of liposome : DNA complexes (lipoplexes): particle size, polydispersity index (PDI) and zeta potential

The physicochemical properties of the lipid : DNA nanoparticles were measured by a dynamic light scattering

Table 1 Particle size, polydispersity index (PDI) and zeta potential of cationic liposomes **4A** and lipoplexes **4A** at different weight ratios with and without DOPE. The data are represented as the mean $\pm$ SD of five measurements

Lipid 4A : DOPE	DNA:lipid 4A (w/w)	Particle size (nm)	PDI	Zeta potential (mV)
1:0	0:2	635.12 $\pm$ 81.09	0.44 $\pm$ 0.13	36.62 $\pm$ 1.06
	1:2	1027.62 $\pm$ 117.36	0.52 $\pm$ 0.09	3.10 $\pm$ 2.72
	1:5	1374.60 $\pm$ 109.40	0.49 $\pm$ 0.07	24.48 $\pm$ 1.59
	1:10	862.32 $\pm$ 94.30	0.44 $\pm$ 0.04	35.28 $\pm$ 1.71
	1:20	433.72 $\pm$ 30.98	0.62 $\pm$ 0.13	24.54 $\pm$ 7.89
1:1	1:30	494.10 $\pm$ 28.33	0.57 $\pm$ 0.20	43.24 $\pm$ 1.49
	0:2	551.46 $\pm$ 133.81	0.58 $\pm$ 0.15	35.42 $\pm$ 2.42
	1:2	377.26 $\pm$ 23.13	0.27 $\pm$ 0.02	-3.66 $\pm$ (-1.06)
	1:5	462.22 $\pm$ 123.34	0.50 $\pm$ 0.04	11.10 $\pm$ 5.19
	1:10	593.88 $\pm$ 41.70	0.37 $\pm$ 0.07	41.82 $\pm$ 0.73
1:1	1:20	400.36 $\pm$ 8.55	0.39 $\pm$ 0.06	44.30 $\pm$ 1.02
	1:30	286.28 $\pm$ 12.10	0.42 $\pm$ 0.07	46.04 $\pm$ 1.52

assay (DLS) at various weight ratios.⁵² As can be seen from Table 1, the particle sizes of liposomes and lipoplexes (from 286.3 to 593.8 nm (with DOPE) and from 433.7 to 1374.6 nm (without DOPE)), prepared from the lipid **4A**, show mean $\pm$ standard deviation of five measurements. The observation revealed that the co-assembly (DOPE) played a role in condensing lipoplex complexes.⁵³ Zeta potential is an indicator of the surface charges on the lipid:DNA nanoparticles. The electrostatic interaction between a positively charged surface and anionic cell surfaces facilitates cellular transfection. As shown in Table 1, the zeta-potential values of the lipid **4A**:DNA lipoplexes with DOPE ranging from -3 mV to +46 mV increase along with the increase in weight ratios. The trend of the zeta potential values sharply increased with a charge reverse to positive (-3, 11 and +41 mV at the lipid:DNA weight ratios of 2, 5 and 10, respectively). This trend indicated DNA condensation by the cationic lipid. For the lipid:DNA weight ratios of 10, 20 and 30, the values were all found to be slightly increased: +41, +44 and +46 mV, respectively; this suggested that full DNA condensation was achieved. The broad range of the zeta potential was found because its measurement employed a wide range of concentrations from 1:2 to 1:30 (DNA:lipid; w/w). The high positive zeta potential is electrically stabilized nanoparticle-dispersions.⁵⁴ The data indicated that the average particle size of the complexes without a helper lipid (DOPE) was higher than that with DOPE. In the optimized conditions of **4A**, DNA:lipid at 1:10 (w/w) with DOPE showed a PDI value of 0.37 $\pm$ 0.07. This PDI value exhibited a homogenous population of lipid vesicles.⁵⁵

Conclusions

We designed a new series of lysine-diamine conjugated cholesterol-based cationic lipids. The methylene spacing emerged to be a crucial factor in encouraging efficient DNA delivery. The branch-shaped lipid 3 β -[N-(3-(N-lysine)-amide)-

aminopropyl]carbamoyl]cholesterol (**4A**), which bore a headgroup based on the lysine amino acid with two charges in the protonated form, showed superior transfection efficiencies into the HEK293 cell line. Thus, it was evident that the introduction of the modified headgroups of N-terminal α -amino groups to the parent lipids **1A** and **4A** resulted in decreased transfection efficiency. The optimized conditions for the lipid **4A** were a lipid/DOPE weight ratio of 1:1, DNA:lipid ratio of 1:10 and the amount of DNA of 0.2 μ g per well. Liposome **4A** exhibited high transfection efficiency, which exceeded that of the commercially available transfection agent Lipofectamine™ 2000 (L2k) in the presence of a serum. The gel retardation assay of the lipoplexes **4A** in the presence of a helper lipid (DOPE) generated consistent transfection results. The GFP cell percentage and MFI of lipoplexes **4A** indicated good delivering ability. The particle size and zeta potential of lipoplexes **4A** with DOPE were around 300–600 nm and 0–45 mV, respectively. The results suggested that our synthesized lipid **4A** will perhaps be a promising nonviral vector for gene or drug delivery and low toxicity.

Experimental section

Materials and method

IR spectra were recorded on a Perkin-Elmer FT-IR 400 spectrometer (ATR). NMR spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 MHz for ¹H and 100 MHz for ¹³C. All coupling constants (*J* values) were measured in hertz. All NMR data of synthesized compounds are summarized in the supplementary information. ES mass spectra were recorded with a Finnigan LCQ mass spectrometer. HR-ESI-MS of the separated compounds was obtained using a Bruker micrOTOF-II mass spectrometer. Reagents were purchased from commercial suppliers. The starting materials for 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'*-tetramethyluroniumhexafluorophosphate (HBTU) were purchased from Sigma-Aldrich.

Preparation of compounds 8–9

Cholesteryl chloroformate (**7**) (3.01 g (6.71 mmol) or 4.30 g (9.59 mmol)) was dissolved in dichloromethane (CH₂Cl₂) (200 mL) and then poured into a 250 mL separatory funnel. 1,2-Diaminoethane or 1,3-diaminopropane (4.5 mL (67.1 mmol) and 8.1 mL (95.9 mmol), respectively) was dissolved in CH₂Cl₂ (250 mL) in a 500 mL Erlenmeyer flask. Then, each solution of cholesteryl chloroformate was individually and slowly added to the solution of 1,2-diaminoethane or 1,3-diaminopropane. The resulting mixture was stirred at room temperature for 24 h. The mixture was then washed with water (200 mL). The organic phase was combined, dried over anhydrous sodium sulphate (anh. Na₂SO₄), filtered, and concentrated to afford a white amorphous solid. The

resulting crude product was further purified by column chromatography on a silica gel with a gradient system of CH_2Cl_2 to CH_2Cl_2 /methanol (MeOH) (85:15), which afforded the products **8** or **9**.

3 β -[N-(2-Aminoethyl)carbamoyl]cholesterol (8). (2.64 g, 5.58 mmol, yield 83%). IR: ν_{max} 3336, 2940, 2890, 2867, 2851, 1714, 1695, 1551, 1468, 1382, 1374, 1365, 1275, 1247, 1193, 1139, 1076, 1016 cm^{-1} ; ESI (positive): m/z 473.6 ($[\text{M} + \text{H}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{30}\text{H}_{53}\text{N}_2\text{O}_2$ $[\text{M} + \text{H}]^+$; 473.4101 found 473.4103.

3 β -[N-(3-Aminopropyl)carbamoyl]cholesterol (9). (4.30 g, 8.82 mmol, yield 92%). IR: ν_{max} 3355, 3209, 2963, 2935, 2868, 1696, 1593, 1569, 1555, 1465, 1456, 1435, 1375, 1331, 1315, 1267, 1198, 1172, 1137, 1118, 1080, 1037, 1020 cm^{-1} ; ESI (positive): m/z 487.9 ($[\text{M} + \text{H}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{31}\text{H}_{55}\text{N}_2\text{O}_2$ $[\text{M} + \text{H}]^+$; 487.4258 found 487.4318.

Preparation of compounds 10–11

The solutions of *N*-alpha-Fmoc-*N*-epsilon-Boc-L-lysine (Fmoc-Lys-OH) (1.64 g (3.49 mmol) or 0.700 g (1.50 mmol)) in 100 mL of CH_2Cl_2 and *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (1.60 g (4.23 mmol) or 0.76 g (1.99 mmol)) in 5 mL of acetonitrile were stirred. After being stirred at room temperature for 5 min, the cocktails were added to the solution of compound **8** (1.10 g (2.32 mmol)) or compound **9** (0.480 g (0.985 mmol)) in CH_2Cl_2 and 0.50 mL of diisopropylethylamine (DIEA). The reaction mixtures were stirred at room temperature for 3 h. The reactions were monitored by the TLC technique. The crude products were extracted by CH_2Cl_2 . The organic layer was combined, dried over anhydrous Na_2SO_4 , and concentrated under reduced pressure. The crude mixtures were purified by silica gel column chromatography with CH_2Cl_2 /MeOH (19:1) to afford the compounds **10** or **11**.

3 β -[N-(2-(*N'*-(Fmoc-lysine(Boc)amide))aminoethyl)carbamoyl]cholesterol (10). (1.84 g, 1.99 mmol, yield 86%). IR: ν_{max} 3347, 3320, 3300, 2941, 2866, 1694, 1682, 1655, 1531, 1450, 1365, 1309, 1245, 1169, 1029 cm^{-1} ; ESI (positive): m/z 946.1 ($[\text{M} + \text{Na}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{56}\text{H}_{82}\text{N}_4\text{NaO}_7$ $[\text{M} + \text{Na}]^+$; 945.6075 found 945.6088.

3 β -[N-(3-(*N'*-(Fmoc-lysine(Boc)amide))aminopropyl)carbamoyl]cholesterol (11). (0.770 g, 0.822 mmol, yield 83%). IR: ν_{max} 3312, 2938, 2866, 1683, 1646, 1531, 1449, 1390, 1381, 1364, 1262, 1247, 1234, 1167, 1137, 1087, 1019 cm^{-1} ; ESI (positive): m/z 960.1 ($[\text{M} + \text{Na}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{57}\text{H}_{84}\text{N}_4\text{NaO}_7$ $[\text{M} + \text{Na}]^+$; 959.6242 found 959.6247.

Preparation of compounds 12–13

A solution of 20% piperidine (1 mL) was added to a solution of compound **10** (0.300 g, (0.325 mmol)) or **11** (0.300 g, (0.320 mmol)) in 4.0 mL of CH_2Cl_2 . The reaction mixture was stirred at room temperature for 2 h and monitored by TLC. The reaction mixture was diluted by CH_2Cl_2 and was washed by water for 3 times. Then, the organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The

desired compounds were purified by silica gel column chromatography with CH_2Cl_2 /MeOH (19:1) to afford the compounds **12** or **13**.

3 β -[N-(2-(*N'*-(Lysine(Boc)amide))aminoethyl)carbamoyl]cholesterol (12). (0.193 g, 0.275 mmol, yield 85%). IR: ν_{max} 3324, 2937, 2867, 1690, 1525, 1459, 1365, 1333, 1251, 1167, 1030, 1012 cm^{-1} ; ESI (positive): m/z 701.9 ($[\text{M} + \text{H}]^+$ 100%) and m/z 1403.2 ($[2\text{M} + \text{H}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{41}\text{H}_{73}\text{N}_4\text{O}_5$ $[\text{M} + \text{H}]^+$; 701.5575 found 701.5598.

3 β -[N-(3-(*N'*-(Lysine(Boc)amide))aminopropyl)carbamoyl]cholesterol (13). (0.219 g, 0.306 mmol, yield 96%). IR: ν_{max} 3321, 3073, 2937, 2867, 1684, 1524, 1460, 1441, 1391, 1380, 1365, 1330, 1249, 1168, 1138, 1085, 1052, 1029, 1011 cm^{-1} ; ESI (positive): m/z 716.1 ($[\text{M} + \text{H}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{42}\text{H}_{75}\text{N}_4\text{O}_5$ $[\text{M} + \text{H}]^+$; 715.5731 found 715.5753.

Preparation of compounds 14–15

Compound **12** (0.110 g (0.157 mmol)) or **13** (0.220 g (0.307 mmol)) was dissolved in DMF and added to a round bottom flask. To the solution, methyl iodide (500 μL (0.0240 mmol) or 300 μL (1.83 mmol), respectively) and 0.50 mL of diisopropylethylamine (DIEA) were added. The reaction mixtures were stirred at room temperature for 24 h and tested by TLC. The mixtures were washed by water in CH_2Cl_2 for several times. The organic phase was evaporated under reduced pressure and purified by silica gel column chromatography with CH_2Cl_2 :MeOH (1.7:0.3), affording the products **14** or **15**.

3 β -[N-(2-(*N'*-(*N''*,*N''*,*N''*)-Trimethylammonium-lysine(Boc)-amide))aminoethyl)carbamoyl]cholesterol (14). (0.114 g, 0.153 mmol, yield 97%). IR: ν_{max} 3327, 3058, 2938, 2867, 1682, 1517, 1460, 1365, 1332, 1248, 1166, 1135, 1107, 1030, 1014 cm^{-1} ; ESI (positive): m/z 745.1 ($[\text{M} + \text{H}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{44}\text{H}_{79}\text{N}_4\text{O}_5$ $[\text{M}]^+$; 743.6044 found 743.6069.

3 β -[N-(3-(*N'*-(*N''*,*N''*,*N''*)-Trimethylammonium-lysine(Boc)-amide))aminopropyl)carbamoyl]cholesterol (15). (0.219 g, 0.289 mmol, yield 94%). IR: ν_{max} 3420, 3331, 2938, 2868, 1782, 1680, 1626, 1517, 1460, 1405, 1391, 1380, 1366, 1247, 1167, 1086, 1066, 1030, 1015 cm^{-1} ; ESI (positive): m/z 758.2 ($[\text{M}]^+$ 100%); HRMS (ESI): m/z calcd. For $\text{C}_{45}\text{H}_{81}\text{N}_4\text{O}_5$ $[\text{M}]^+$; 757.6201 found 757.6207.

Preparation of compounds 16–17

Compound **12** (0.100 g (0.143 mmol)) or **13** (0.130 g (0.182 mmol)) was dissolved in DMF. 1,3-Bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (0.0830 g (0.290 mmol) and 0.110 g (0.390 mmol) for **12** and **13**, respectively) was added to a solution of **12** or **13**. Then, 3–4 drops of DIEA were added. The final solution was stirred at room temperature for 12 h. The mixture was then washed with water (100 mL). The organic phase was combined, dried over anhydrous Na_2SO_4 , filtered, and concentrated to afford a white amorphous solid. The crude solid was further purified by column chromatography on silica gel with a system of 2% MeOH in CH_2Cl_2 , which afforded the products **16** or **17**.

3 β -[N-(2-(N'-(N''-(N'''-diBoc(guanidinyl-lysine(Boc-amide))))aminoethyl)carbamoyl]cholesterol (16). (0.0970 g, 0.103 mmol, yield 72%). IR: $\nu_{\max}$ 3325, 2937, 2867, 1714, 1691, 1638, 1590, 1513, 1454, 1412, 1390, 1381, 1366, 1333, 1303, 1244, 1152, 1029, 1011 cm^{-1} ; ESI (positive): m/z 966.1 ($[M + Na]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{52}H_{90}N_6NaO_9$ $[M + Na]^+$; 965.6661 found 965.6665.

3 β -[N-(3-(N'-(N''-(N'''-diBoc(guanidinyl-lysine(Boc-amide))))aminopropyl)carbamoyl]cholesterol (17). (0.133 g, 0.136 mmol, yield 75%). IR: $\nu_{\max}$ 3178, 3080, 2947, 1666, 1520, 1435, 1366, 1252, 1177, 1123, 1057 cm^{-1} ; HRMS (ESI): m/z calcd. For $C_{53}H_{92}N_6NaO_9$ $[M + Na]^+$; 979.6817 found 979.6844.

Preparation of lipids 1A and 4A

Compound **12** (0.100 g (0.143 mmol)) or **13** (0.0740 g (0.103 mmol)) was dissolved in 5 mL of CH_2Cl_2 (2 mL) and 0.5 mL of 20% TFA in CH_2Cl_2 was added into the solution. The mixture was stirred at room temperature for 2 h followed by the flow of nitrogen gas. The crude products were further purified by column chromatography on silica gel with a system of 15% MeOH in CH_2Cl_2 . Here, the purification afforded the product **1A** or **4A**.

3 β -[N-(2-(N'-Lysinamide)aminoethyl)carbamoyl]cholesterol (1A). (0.0800 g, 0.133 mmol, yield 93%). IR: $\nu_{\max}$ 3287, 3075, 2941, 2868, 1668, 1530, 1466, 1435, 1380, 1366, 1333, 1262, 1198, 1183, 1131, 1029, 1015 cm^{-1} ; ESI (positive): m/z 602.5 ($[M]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{36}H_{65}N_4O_3$ $[M]^+$; 601.5051 found 601.5050.

3 β -[N-(3-(N'-Lysinamide)aminopropyl)carbamoyl]cholesterol (4A). (0.0500 g, 0.0812 mmol, yield 79%, purity 93% (see ESI[†])). IR: $\nu_{\max}$ 3315, 2935, 2866, 1693, 1647, 1533, 1464, 1439, 1379, 1366, 1329, 1251, 1136, 1084, 1032, 1012 cm^{-1} ; ESI (positive): m/z 616.2 ($[M + H]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{37}H_{67}N_4O_3$ $[M + H]^+$; 615.5207 found 615.5225.

Preparation of lipids 2A and 5A

Compound **14** (0.0300 g (0.0403 mmol)) or **15** (0.0330 g (0.0436 mmol)) was dissolved in 5 mL of CH_2Cl_2 (2 mL). Then, 0.5 mL of 20% TFA in CH_2Cl_2 was added into the solution. The mixture was stirred at room temperature for 2 h followed by the flow of nitrogen gas. The crude products were then purified by column chromatography on silica gel with a system of 15% MeOH in CH_2Cl_2 . The purification afforded the products **2A** or **5A**.

3 β -[N-(2-(N'-(N'',N''',-Trimethylammonium-lysineamide))-aminoethyl)carbamoyl]cholesterol (2A). (0.0230 g, 0.0357 mmol, yield 89%). IR: $\nu_{\max}$ 3372, 3283, 3061, 2943, 2868, 1672, 1534, 1491, 1466, 1440, 1381, 1365, 1332, 1254, 1200, 1179, 1129, 1029, 1015 cm^{-1} ; ESI (positive): m/z 645.2 ($[M + H]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{39}H_{71}N_4O_3$ $[M]^+$; 643.5520 found 643.5539.

3 β -[N-(3-(N'-(N'',N''',-trimethylammonium-lysineamide))-amino propyl)carbamoyl]cholesterol (5A). (0.0220 g, 0.0335

mmol, yield 77%). IR: $\nu_{\max}$ 3364, 3063, 2941, 2866, 1673, 1557, 1540, 1532, 1467, 1457, 1442, 1418, 1384, 1264, 1254, 1198, 1179, 1174, 1166, 1133, 1124, 1118, 1034 cm^{-1} ; ESI (positive): m/z 658.2 ($[M]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{40}H_{73}N_4O_3$ $[M]^+$; 657.5677 found 657.5688.

Preparation of lipids 3A and 6A

Compound **16** (0.0300 g (0.0318 mmol)) or **17** (0.0100 g (0.0102 mmol)) was dissolved in 5 mL of CH_2Cl_2 (2 mL) and 0.5 mL of 20% TFA in CH_2Cl_2 was added into the solution. The mixture was stirred at room temperature for 2 h followed by the flow of nitrogen gas. The crude products were then purified by column chromatography on silica gel with a system of 15% MeOH in CH_2Cl_2 . The purification afforded the products **3A** or **6A**.

3 β -[N-(2-(N'-(N''-Guanidinyl-lysineamide))aminoethyl)-carbamoyl]cholesterol (3A). (0.0170 g, 0.0264 mmol, yield 83%). IR: $\nu_{\max}$ 3334, 2945, 2868, 1662, 1536, 1466, 1435, 1382, 1366, 1334, 1319, 1286, 1270, 1249, 1199, 1183, 1020 cm^{-1} ; ESI (positive): m/z 644.0 ($[M + H]^+$ 100%); HRMS (ESI): m/z calcd. For $C_{37}H_{67}N_6O_3$ $[M + H]^+$; 643.5269 found 643.5315.

3 β -[N-(3-(N'-(N''-guanidinyl-lysineamide))aminopropyl)-carbamoyl]cholesterol (6A). (0.005 g, 0.008 mmol, yield 78%). IR: $\nu_{\max}$ 3300, 2938, 2865, 1673, 1528, 1465, 1440, 1378, 1365, 1259, 1202, 1133, 1033, cm^{-1} ; HRMS (ESI): m/z calcd. For $C_{38}H_{69}N_6O_3$ $[M + H]^+$; 657.5425 found 657.5417.

DNA binding affinities

Gel electrophoresis was used to study the DNA binding ability of the DNA:lipid complexes. The ability to retard DNA migration was examined at the DNA:lipid ratios of 1:5, 1:10 and 1:20 through 1.0% agarose gel electrophoresis. The complexes were prepared by adding liposomes into the DNA (0.1 μg). Loading dye reagent was then added to the lipoplexes. The solutions (10 μL) were loaded into the gel and electrophoresis was performed in TAE buffer under +100 V for 30 min. DNA bands were visualized by ethidium bromide staining.

Liposome preparation

A thin film was prepared from the stock solution of cationic lipid and DOPE (1 μg μL^{-1} in CH_2Cl_2 or EtOH). DOPE (250 μL) and cationic lipid (250 μL) were mixed in an Eppendorf tube and the solvent was evaporated to dryness by a stream of nitrogen and then under a high vacuum for 4–8 h. The thin film was suspended with PBS buffer, vortexed for 30 seconds and sonicated for 20 min at room temperature.

Transfection procedure

Plasmid DNA (pCH110; Promega), used in the transfection experiments shown in Fig. 2 and 3 and containing the β -galactosidase reporter gene under the control of the SV40 promoter, was grown using standard techniques and purified by column chromatography (NucleoBond® Xtra Maxi EF).

The concentration of plasmid was measured by UV adsorption at 260 nm. The A_{260}/A_{280} ratios were between 1.80 and 2.00.

Human embryonic kidney 293 (HEK293) and human cervical adenocarcinoma (HeLa) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Grand Island, NY). Human prostate adenocarcinoma (PC3) was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium. Human hepatocyte cell line (HC-04) was maintained in essential medium (MEM)/Ham's F12 50/50 mix (Gibco). All media were supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (Gibco). Cells were incubated in a 37 °C humidified 5% CO₂ atmosphere. The cells were passaged with 0.25% trypsin-EDTA (Gibco) while reaching 90% confluence. Only cultures with at least 95% cell viability were used in the experiments. The cells were seeded on to 96 well plates (1 × 10⁴ cells per well).

Lipoplexes were prepared by adding the liposome (1 µg µL⁻¹) into DNA (0.1 µg µL⁻¹). The mixture was diluted with PBS buffer to the total volume of about 10 µL. The lipoplexes were added into the cells and incubated at 37 °C under 5% CO₂ for 48 h. For the Lipofectamine™ 2000 (L2k) transfection, the procedure was conducted by following the manufacturer's instructions. Lipofectamine™ 2000 (L2k)/DNA complex was set at the weight ratio of 2.5:1. After 48 h, the old medium was removed. The Z-buffer (100 µL) and SDS agent (50 µL) were added into the well. After 15 min, the β-galactosidase activity per well was determined by adding 100 µL of solution (*o*-nitrophenyl-galactosidase; ONPG, 0.2 M sodium phosphate, and 2 mM magnesium chloride) to the lysate in a 96-well plate. Then, the cells were incubated for 4 h before measuring the absorbance at 405 nm. The absorbance of *o*-nitrophenol was converted to percentage of relative transfection by comparing with the positive control L2k.

In order to evaluate the transfection efficiency, as shown in Fig. 5, the pEGFP-C2 plasmid DNA, encoding green fluorescent protein (GFP) was employed. For fluorescence microscopy images, the cells were transfected at 37 °C for 48 h under 5% CO₂. The medium was removed and washed twice with 1× PBS buffer and fixed with 100 µL of 4% paraformaldehyde (dissolved in 2× PBS buffer) for 1 h at room temperature. After incubation, 100 µL of 1× PBS buffer was added and then stored at 4 °C. The concentration of transfected cells was measured by fluorescence microscopy; IX73 (Olympus). All the transfection experiments were carried out in triplicate.

The GFP cell of the active lipid 4A-pEGFP-C2 complex was analyzed by flow cytometry. Briefly, the cells were seeded in 96 well plates (1 × 10⁴ cells per well) and grown for 24 h. The cells were incubated with the active lipid 4A-pEGFP-C2 complexes from the optimized transfection conditions in media for 48 h at 37 °C. The medium of each well was removed and washed twice with 100 µL 1× PBS buffer. Then, 25 µL of 0.25% trypsin-EDTA was added. The mixture was incubated for 1 min. After incubation, 200 µL of 1× PBS

buffer was added to inhibit the trypsin activity. The cells were transferred to an Eppendorf tube and centrifuged at 1000 × *g* at 25 °C for 5 min. After removing the supernatant, 200 µL of 1× PBS buffer was added and then centrifuged for 5 min. The supernatant was removed and the residue was dissolved in 25 µL of 2× PBS buffer followed by the addition of 4% paraformaldehyde; PFA (75 µL) and 0.05% glutaraldehyde (GDH). The cells were centrifuged and washed twice with 1× PBS buffer. Finally, the cell pellets were stored in 100 µL of 1× PBS buffer at 4 °C. The mean fluorescence intensity and percentage of cell transfection were analyzed by using a BD Accuri™ C6 flow cytometer.

Serum stability of ODN

A serum stability assay was performed according to previously reported procedures.³⁶ The lipoplexes of the lipid 4A were incubated with fetal bovine serum at 37 °C for 1, 2, 4, 6, 12 and 24 h. The serum enzyme was inactivated by heating at 70 °C for 15 min. Gel electrophoresis was performed under +100 V for 30 min on a 1% agarose gel containing ethidium bromide for visualization.

Transfection cytotoxicity

The cells were seeded on a 96 well plate. The cells were incubated for 24 h at 37 °C under 5% CO₂. This followed the same procedure as that for the transfection experiment. After the 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 3 mg mL⁻¹. The cells were incubated again for 2 h at 37 °C under 5% CO₂. The absorbance was measured at 520 nm on a microplate reader.

Particle size, polydispersity index (PDI) and zeta potential measurements

Photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instrument Ltd. Malvern, UK) was used to analyze the particle size, polydispersity index (PDI) and surface charge of DNA/liposome complexes at different weight ratios with and without DOPE. The prepared complexes were diluted with distilled water and then, measurements were obtained in five replicates at room temperature.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This project was financially supported by Mahasarakham University, fiscal year 2017. CT acknowledges The Science Achievement Scholarship of Thailand (SAST) for the scholarship. Partial support was from the Center of Excellence for Innovation in Chemistry (PERCH-CIC). Ministry of Higher Education, Science, Research and Innovation is gratefully

acknowledged. We would like to thank the Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, and Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University for the support in facilities. It would not have been possible without their help.

References

- 1 A. Mhashilkar, S. Chada, J. A. Roth and R. Ramesh, *Biotechnol. Adv.*, 2001, **19**, 279–297.
- 2 G. M. Rubanyi, *Mol. Aspects Med.*, 2001, **22**, 113–142.
- 3 B. Draghici and M. A. Ilies, *J. Med. Chem.*, 2015, **58**, 4091–4130.
- 4 A. Pensado, B. Seijo and A. Sanchez, *Expert Opin. Drug Delivery*, 2014, **11**, 1721–1731.
- 5 V. Prakash, M. Moore and R. J. Yáñez-Muñoz, *Mol. Ther.*, 2016, **24**, 465–474.
- 6 A. Delalande, M.-P. Gosselin, A. Suwalski, W. Guilmann, C. Leduc, M. Berchel, P.-A. Jaffrès, P. Baril, P. Midoux and C. Pichon, *Nanomedicine*, 2015, **11**, 1735–1744.
- 7 K. Van Der Jeught, S. De Koker, L. Bialkowski, C. Heirman, P. T. Joe, F. Perche, S. Maenhout, S. Bevers, K. Broos, K. Deswarte, V. Malard, H. Hammad, P. Baril, T. Benvegna, P.-A. Jaffrès, S. A. A. Kooijmans, R. Schiffelers, S. Lienenklaus, P. Midoux, C. Pichon, K. Breckpot and K. Thielemans, *ACS Nano*, 2018, **12**, 9815–9829.
- 8 J. L. Howarth, Y. B. Lee and J. B. Uney, *Cell Biol. Toxicol.*, 2010, **26**, 1–20.
- 9 A. D. Miller, *Angew. Chem., Int. Ed.*, 1998, **37**, 1768–1785.
- 10 S. Simões, A. Filipe, H. Faneca, M. Mano, N. Penacho, N. Düzgünes and M. P. Lima, *Expert Opin. Drug Delivery*, 2005, **2**, 237–254.
- 11 A. Gigante, M. Li, S. Junghänel, C. Hirschhäuser, S. Knauer and C. Schmuck, *Med. Chem. Commun.*, 2019, **10**, 1692–1718.
- 12 M. Damen, A. J. J. Groenen, S. F. M. van Dongen, R. J. M. Nolte, B. J. Scholte and M. C. Feiters, *Med. Chem. Commun.*, 2018, **9**, 1404–1425.
- 13 Y. Xu and F. C. Szoka, *Biochemistry*, 1996, **35**, 5616–5623.
- 14 C. Loney, M. Vandenbranden and J.-M. Ruyschaert, *Prog. Lipid Res.*, 2008, **47**, 340–347.
- 15 D. Hirsch-Lerner, M. Zhang, H. Eliyahu, M. E. Ferrari, C. J. Wheeler and Y. Barenholz, *Biochim. Biophys. Acta*, 2005, **1714**, 71–84.
- 16 S. Mochizuki, N. Kanegae, K. Nishina, Y. Kamikawa, K. Koiwai, H. Masunaga and K. Sakurai, *Biochim. Biophys. Acta*, 2013, **1828**, 412–418.
- 17 X. Cheng and R. J. Lee, *Adv. Drug Delivery Rev.*, 2016, **99**, 129–137.
- 18 S. Bhattacharya and A. Bajaj, *Chem. Commun.*, 2009, 4632–4656.
- 19 D. Zhi, S. Zhang, S. Cui, Y. Zhao, Y. Wang and D. Zhao, *Bioconjugate Chem.*, 2013, **24**, 487–519.
- 20 M. Castro, D. Griffiths, A. Patel, N. Patrick, C. Kitson and M. Ladlow, *Org. Biomol. Chem.*, 2004, **2**, 2814–2820.
- 21 W. Radchatawedchakoon, A. Krajarng, N. Niyomtham, R. Watanapokasin and B. Yingyongnarongkul, *Chem. – Eur. J.*, 2011, **17**, 3287–3295.
- 22 H. W. Yang, J. W. Yi, E.-K. Bang, E. M. Jeon and B. H. Kim, *Org. Biomol. Chem.*, 2011, **9**, 291–296.
- 23 I. S. Zuhorn, V. Oberle, W. H. Visser, J. B. F. N. Engberts, U. Bakowsky, E. Polushkin and D. Hoekstra, *Biophys. J.*, 2002, **83**, 2096–2108.
- 24 J. O. Rädler, I. Koltover, A. Jamieson, T. Salditt and C. R. Safinya, *Langmuir*, 1998, **14**, 4272–4283.
- 25 P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413–7417.
- 26 J. S. Choi, E. J. Lee, H. S. Jang and J. S. Park, *Bioconjugate Chem.*, 2001, **12**, 108–113.
- 27 L. Li, H. Song, K. Luo, B. He, Y. Nie, Y. Yang, Y. Wu and Z. Gu, *Int. J. Pharm.*, 2011, **408**, 183–190.
- 28 R. Sheng, T. Luo, H. Li, J. Sun, Z. Wang and A. Cao, *Colloids Surf., B*, 2014, **116**, 32–40.
- 29 J. Ju, M.-L. Huan, N. Wan, H. Qiu, S.-Y. Zhou and B.-L. Zhang, *Int. J. Mol. Sci.*, 2015, **16**, 5666–5681.
- 30 J.-P. Vigneron, N. Oudrhiri, M. Fauquet, L. Vergely, J.-C. Bradley, M. Basseville, P. Lehn and J.-M. Lehn, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 9682–9686.
- 31 B. Yingyongnarongkul, M. Howarth, T. Elliott and M. Bradley, *Chem. – Eur. J.*, 2004, **10**, 463–473.
- 32 M. Frederic, D. Scherman and G. Byk, *Tetrahedron Lett.*, 2000, **41**, 675–679.
- 33 M. D. Kearns, A.-M. Donkor and M. Savva, *Mol. Pharmaceutics*, 2008, **5**, 128–139.
- 34 E. I. P. Delbeke, O. Lozach, T. Le Gall, M. Berchel, T. Montier, P.-A. Jaffrès, K. M. Van Geem and C. V. Stevens, *Org. Biomol. Chem.*, 2016, **14**, 3744–3751.
- 35 P. Reynier, D. Briane, R. Coudert, G. Fadda, N. Bouchemal, P. Bissieres, E. Taillandier and A. Cao, *J. Drug Targeting*, 2004, **12**, 25–38.
- 36 J. Ju, M.-L. Huan, N. Wan, H. Qiu, S.-Y. Zhou and B.-L. Zhang, *Int. J. Mol. Sci.*, 2015, **16**, 5666–5681.
- 37 A. B. Cook, R. Peltier, T. R. Barlow, J. Tanaka, J. A. Burns and S. Perrier, *J. Interdiscip. Nanomed.*, 2018, **3**, 164–174.
- 38 A. Fraix, T. Montier, T. L. Gall, C. M. Sevrain, N. Carmoy, M. F. Lindberg, P. Lehn and P.-A. Jaffrès, *Org. Biomol. Chem.*, 2012, **10**, 2051–2058.
- 39 M. A. Ilies, W. A. Seitz, I. Ghiviriga, B. H. Johnson, A. Miller, E. B. Thompson and A. T. Balaban, *J. Med. Chem.*, 2004, **47**, 3744–3754.
- 40 B. Yingyongnarongkul, W. Radchatawedchakoon, A. Krajarng, R. Watanapokasin and A. Suksamrarn, *Bioorg. Med. Chem.*, 2009, **17**, 176–188.
- 41 W. Radchatawedchakoon, R. Watanapokasin, A. Krajarng and B. Yingyongnarongkul, *Bioorg. Med. Chem.*, 2010, **18**, 330–342.
- 42 M. Muñoz-Úbeda, S. K. Misra, A. L. Barrán-Berdón, S. Datta, C. Aicart-Ramos, P. Castro-Hartmann, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *Biomacromolecules*, 2012, **13**, 3926–3937.

- 43 R. P. Shank and G. L. Campbell, *J. Neurosci.*, 1984, **4**, 58–69.
- 44 E. Valeur and M. Bradley, *Chem. Soc. Rev.*, 2009, **38**, 606–631.
- 45 S. J. Eastman, C. Siegel, J. Tousignant, A. E. Smith, S. H. Cheng and R. K. Scheule, *Biochim. Biophys. Acta*, 1997, **1325**, 41–62.
- 46 V. Escriou, C. Ciolina, F. Lacroix, G. Byk, D. Scherman and P. Wils, *Biochim. Biophys. Acta*, 1998, **1368**, 276–288.
- 47 B. Maiti, M. Kamra, A. A. Karande and S. Bhattacharya, *Org. Biomol. Chem.*, 2018, **16**, 1983–1993.
- 48 Q. Liu, R.-C. Su, W.-J. Yi, L.-T. Zheng, S.-S. Lu and Z.-G. Zhao, *Eur. J. Med. Chem.*, 2017, **129**, 1–11.
- 49 S. Pengnam, P. Patrojanasophon, T. Rojanarata, T. Ngawhirunpat, B. Yingyongnarongkul, W. Radchatawedchakoon and P. Opanasopit, *J. Drug Delivery Sci. Technol.*, 2019, **52**, 325–333.
- 50 N. Niyomtham, N. Apiratikul, K. Suksen, P. Opanasopit and B. Yingyongnarongkul, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 496–503.
- 51 N. Gharaati-Far, M. R. Tohidkia, A. Dehnad and Y. Omid, *Artif. Cells, Nanomed., Biotechnol.*, 2018, **46**, 1001–1008.
- 52 O. Paecharoenchai, N. Niyomtham, A. Apirakaramwong, T. Ngawhirunpat, T. Rojanarata, B. Yingyongnarongkul and P. Opanasopit, *AAPS PharmSciTech*, 2012, **13**, 1302–1308.
- 53 R. Sheng, X. Zhuang, Z. Wang, A. Cao, K. Lin and J. X. X. Zhu, *Nanomaterials*, 2016, **6**, 69.
- 54 S. Bhattacharjee, *J. Controlled Release*, 2016, **235**, 337–351.
- 55 M. Danaei, M. Dehghankhold, S. Ataei, F. Hasanzadeh Davarani, R. Javanmard, A. Dokhani, S. Khorasani and M. R. Mozafari, *Pharmaceutics*, 2018, **10**, E57.