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Plasmid DNA-encapsulating liposomes: Effect of a spacer between the cationic head group and hydrophobic moieties of the lipids on gene expression efficiency

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ABSTRACT

We have synthesized a series of cationic amino acid-based lipids having a spacer between the cationic head group and hydrophobic moieties and examined the influence of the spacer on a liposome gene delivery system. As a comparable spacer, a hydrophobic spacer with a hydrocarbon chain composed of 0, 3, 5, 7, or 11 carbons, and a hydrophilic spacer with an oxyethylene chain (10 carbon and 3 oxygen molecules) were investigated. Plasmid DNA (pDNA)-encapsulating liposomes were prepared by mixing an ethanol solution of the lipids with an aqueous solution of pDNA. The zeta potentials and cellular uptake efficiency of the cationic liposomes containing each synthetic lipid were almost equivalent. However, the cationic lipids with the hydrophobic spacer were subject to fuse with biomembrane-mimicking liposomes. 1,5-Dihexadecyl-*N*-lysyl-*N*-heptyl-L-glutamate, having a seven carbon atom spacer, exhibited the highest fusogenic potential among the synthetic lipids. Increased fusion potential correlated with enhanced gene expression efficiency. By contrast, an oxyethylene chain spacer showed low gene expression efficiency. We conclude that a hydrophobic spacer between the cationic head group and hydrophobic moieties is a key component for improving pDNA delivery.

1. Introduction

Gene therapy has become an important alternative approach for the treatment of diseases that until recently were considered incurable. Viral vectors, such as adenovirus vectors, adeno-associated virus vectors or retrovirus vectors, have been utilized in clinical trials. However, some side effects including immunoresponse and infection have been reported [1,2]. Therefore, the development of non-viral vectors for practical gene therapy is keenly awaited.

Cationic liposomes are mostly utilized as a gene carrier for plasmid DNA (pDNA) [3–5], antisense oligonucleotides [6] or small interfering RNA (siRNA) [7,8]. For transfection of cultured cells, cationic liposomes generally form lipoplexes with nucleic acids by electrostatic interaction between the positively charged liposomes and the negatively charged nucleic acids. The resulting complex is then applied to the cells to bring about gene expression. Lipoplexes have been applied both *in vitro* and *in vivo* [9,10]. However, clinical studies using lipoplexes suggested that the DNA was subject to rapid degradation *via* biological defense systems such as endogenous DNase activity. The DNA degradation inevitably leads to a reduction of gene expression efficiency. Therefore, a system to prevent DNA degradation will be expected to significantly enhance gene expression efficiency for *in vivo* therapy. Encapsulation of the DNA within liposomes would be useful for practical gene therapy because the liposomal membrane would limit accessibility of DNase. However, due to its large mass molecular weight, pDNA is difficult to encapsulate within the internal phase of liposomes. Cationic compounds, such as poly-L-lysine (PLL), [11,12] and polyethyleneimine (PEI) [13], have been used to condense the DNA in order to facilitate encapsulation within liposomes. However, these cationic compounds sometimes display high levels of cytotoxicity. By contrast, Jeffs et al. reported the spontaneous free pDNA encapsulation within liposomes by optimizing ethanol concentration, buffer pH and ionic strength [14]. Indeed, polyethyleneglycol (PEG)-lipids and cationic lipids were utilized in the liposomal membrane for stabilization and for providing positively charged liposomes [15,16].

Cationic liposomes are usually composed of cationic lipids such as didecyldimethylammonium bromide (DDAB) [17,18], 1,2-dipleoyl-3-dimethylammoniumpropane (DODAP) [14] or *N*-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP) [19]. Moreover, various cationic lipid structures have been reported to improve lipoplex-mediated transfection. Indeed, we have reported the high utility of cationic lipids comprising a lysine or arginine head group as comparable transgenic reagents [20]. Moreover, the amino-acid based cationic lipids display very low levels of cytotoxicity

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compared with other, more expensive, commercially available lipid-based transgenic reagents. Therefore, we fully anticipate these amino-acid based cationic lipids to be promising liposomal components for the construction of efficient and safe gene carrier systems. However, the cationic lipids were only investigated in the lipoplex system so far. Therefore, we explored the most appropriate lipid structure for construction of pDNA-encapsulating liposomes.

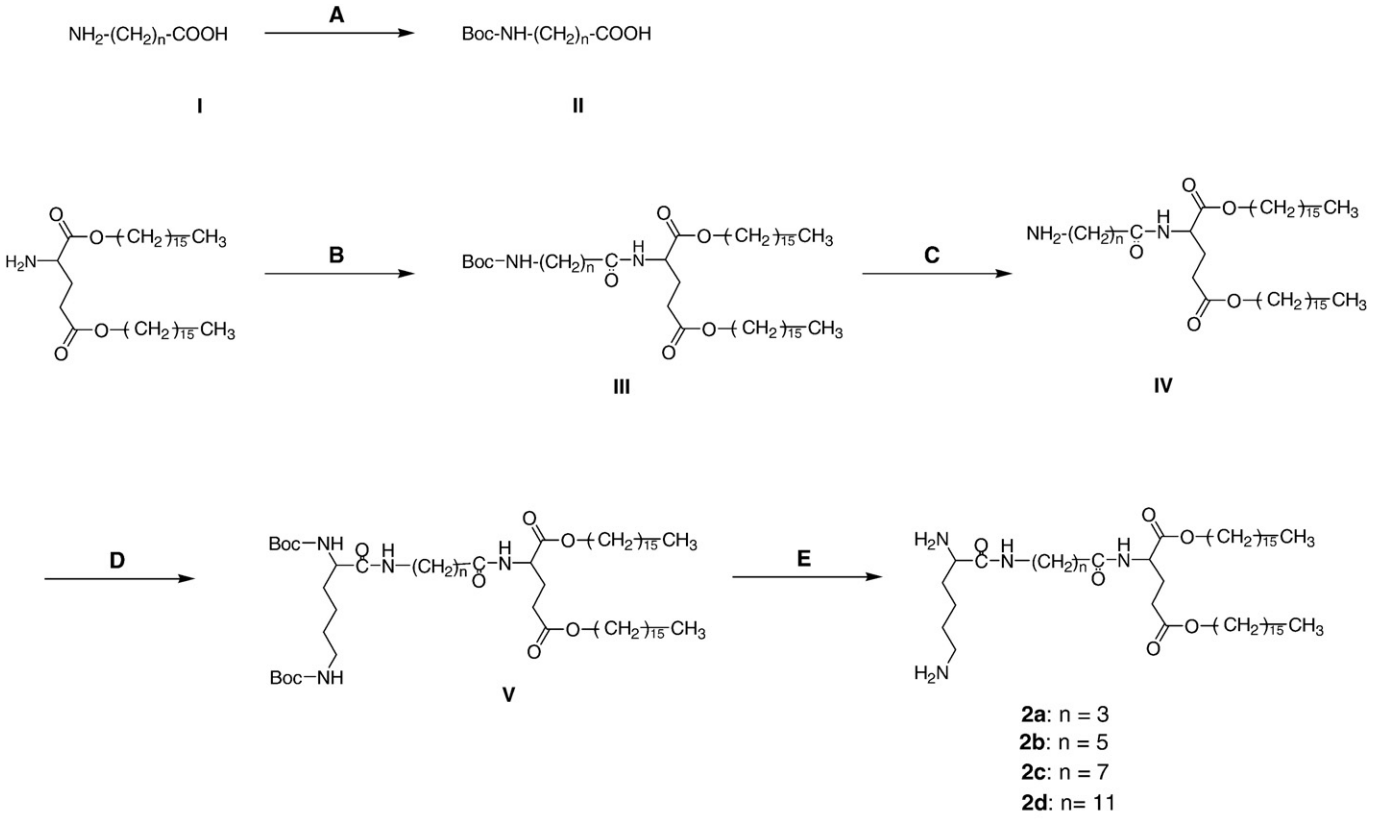
The pDNA-encapsulating liposomes generally fuse to endosomal membranes by electrostatic interaction between the cationic liposomal membrane and the biomembrane after cellular endocytosis [21–23]. For efficient transfection, pDNA-encapsulating cationic liposomes are required to then release the encapsulated pDNA into the cytoplasm. Thus, the fusion process is the most important factor in determining gene expression efficiency [24,25]. In order to improve the fusogenic potential of the cationic liposomes with the endosomal membrane, we concentrated on the spacer unit between the cationic head group and hydrophobic moieties of the lipids oriented on the liposomal membrane. This is because the hydrophobic component of the lipid is preferably fused to the cellular membrane. Few reports suggested that the hydrophilic or hydrophobic spacers of the cationic lipids influenced gene expression, although they were evaluated only in the lipoplex system [26–28]. We investigated the influence of the spacer unit in order to improve intracellular pDNA delivery using the pDNA-encapsulating liposomes *in vitro*.

In this paper, we synthesized a series of cationic lipids having a hydrophilic or hydrophobic spacer to evaluate the effect of the spacer on gene expression efficiency using the pDNA-encapsulating liposomes. We constructed either a hydrocarbon chain of various lengths or an oxyethylene chain as hydrophobic or hydrophilic spacer units for the cationic lipids, respectively. The cationic liposomes were then examined in terms of zeta potential, cellular uptake efficiency using COS-1 cells, fusogenic potential to biomembranes, resistance to DNase and gene expression efficiency. The appropriate spacer type and length were determined by comparison with a non-spacer type lipid; 1,5-dihexadecyl *N*-lysyl-L-glutamate.

2. Materials and methods

2.1. Materials

The following reagents were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan): 4-aminobutyric acid, 6-aminohexanoic acid, and 12-aminolauric acid. *p*-toluenesulfonic acid monohydrate (*p*-Tos), benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP), tetradecyl alcohol and hexadecyl alcohol. Octadecyl alcohol was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). 8-Aminocaprylic acid was purchased from Sigma-Aldrich (St. Louis, MO). 1-*N*-triphenylmethyl-4,7,10-trioxa-1,13-tridecanediamine-bis[2-(3-aminopropoxy)ethyl] ether (Trt-diamino-$(PEG)_2$-NH_2) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). N_{α},N_{ε}-Di-*tert*-butoxycarbonyl-L-lysine hydroxysuccinimide ester (Boc-Lys(Boc)-OSu) and di-*tert*-butyl dicarbonate were obtained from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All organic reagents employed in the syntheses of the cationic lipids were purchased from Kanto Chemical (Tokyo, Japan). Dioleoylphosphatidylcholine (DOPC) and cholesterol were purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan) and utilized without further purification. Poly(ethylene glycol)-1,5-octadecyl-L-glutamate (PEG: Mw5000, PEG-Glu2C_{18}) [29] and 1,5-dihexadecyl *N*-lysyl-L-glutamate [20] were synthesized in our laboratory.



[a]Reaction conditions: (A) $(Boc)_2O$/TEA/methanol, 60°C; (B) Boc-NH-$(CH_2)_n$-COOH/BOP/TEA/DCM; (C) TFA, 4°C; (D) Boc-Lys(Boc)-OSu/TEA/DCM; (E) TFA, 4°C.

Scheme 1. Syntheses of the cationic lipids having a hydrocarbon-type spacer.

2.2. Syntheses

2.2.1. Syntheses of cationic lipids **2a**–**2d** containing a hydrocarbon chain spacer

We constructed hydrocarbon chain spacers with different carbon lengths from an ammonioalkanoic acid as shown in Scheme 1. 4-Aminobutyric acid (5 g, 48.5 mmol), 6-aminohexanoic acid (5 g, 38.2 mmol), 8-aminooctanoic acid (5 g, 31.4 mmol), or 12-aminolauric acid (5 g, 23.3 mmol) was dissolved in 100 mL methanol, and then triethylamine, and 1.1 amount of di-*t*-butyl dicarbonate ($(Boc)_2O$) to the ammonioalkanoic acid group was added to the solution, respectively. After stirring for 12 h at 60 °C, the solvent was evaporated. Ethyl acetate (100 mL) was added to the remaining sticky liquid. The ethyl acetate solution was treated with a 0.2-*N* hydrochloride solution (100 mL×2) and washed with distilled water (100 mL×2). After the solution was evaporated, *N*-butoxycarbonyl-aminobutyric acid, *N*-butoxycarbonyl-aminohexanoic acid, *N*-butoxycarbonyl-aminooctanoic acid and *N*-butoxycarbonyl-aminolauric acid (Compound II in Scheme 1) was recrystallized from hexane (100 mL) at 4 °C to obtain a white powder with yields of 71%, 72%, 35%, and 84%, respectively.

For the construction of **2a** (1,5-dihexadecyl-*N*-lysyl-*N*-trityl-L-glutamate), **2b** (1,5-dihexadecyl-*N*-lysyl-*N*-pentyl-L-glutamate), **2c** (1,5-dihexadecyl-*N*-lysyl-*N*-heptyl-L-glutamate), and **2d** (1,5-dihexadecyl-*N*-lysyl-*N*-undecyl-L-glutamate), the amino-group protected *N*-butoxycarbonyl-aminobutyric acid (0.751 g, 3.7 mmol), *N*-butoxycarbonyl-aminohexanoic acid (0.855 g, 3.7 mmol), *N*-butoxycarbonyl-aminooctanoic acid (0.958 g, 3.7 mmol) or *N*-butoxycarbonyl-aminolauric acid (1.17 g, 3.7 mmol) was dissolved in dichloromethane (100 mL), respectively. Then, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 4.07 mmol) was added to the solution to activate the carboxyl group. After stirring with 1,5-dihexadecy glutamate for 12 h at room temperature, the solution was treated with a sodium carbonate solution (100 mL×2) and washed with distilled water (100 mL×2). After the solution was evaporated, 1,5-dihexadecyl-*N*-butoxycarbonyl-*N*-trityl-L-glutamate, 1,5-dihexadecyl-*N*-butoxycarbonyl-*N*-pentyl-L-glutamate, 1,5-dihexadecyl-*N*-butoxycarbonyl-*N*-heptyl-L-glutamate, or 1,5-dihexadecyl-*N*-butoxycarbonyl-*N*-undecyl-L-glutamate (Compound III in Scheme 1) was recrystallized from methanol (100 mL) at 4 °C to obtain a white powder. The powder was dissolved in chloroform (10 mL), and then trifluoroacetate (10 mL) was added to the solution to remove the Boc group. After incubation for 2 h at 4 °C, the solution was treated with a sodium carbonate solution (100 mL×2) and washed with distilled water (100 mL×2). After the chloroform solution was evaporated, 1,5-dihexadecyl-*N*-trityl-L-glutamate, 1,5-dihexadecyl-*N*-pentyl-L-glutamate, 1,5-dihexadecyl-*N*-heptyl-L-glutamate, or 1,5-dihexadecyl-*N*-undecyl-L-glutamate (Compound IV in Scheme 1) was obtained as a white powder with yields of 58%, 48%, 56%, or 54% respectively.

1,5-dihexadecyl-*N*-trityl-L-glutamate (1 g, 1.47 mmol), 1,5-dihexadecyl-*N*-pentyl-L-glutamate (1 g, 1.42 mol), 1,5-dihexadecyl-*N*-heptyl-L-glutamate (1 g, 1.37 mmol), or 1,5-dihexadecyl-*N*-undecyl-L-glutamate (1 g, 1.29 mmol) was added to dichloromethane (100 mL), and then trimethylamine (1.51 mmol) and Boc-Lys(Boc)-OSu (1.5 mmol) were added to each solution. After stirring for 12 h, the solution was treated with a sodium carbonate solution (100 mL×2) and washed with distilled water (100 mL×2). When the chloroform solution was evaporated, **2a**, **2b**, **2c** or **2d** was obtained after deprotection of Boc group with TFA with yields of 57%, 35%, 45% or 39%, respectively.

2a: Rf: 0.1 ($CHCl_3/CH_3OH/H_2O$ (65/25/4)), ^{1}H-NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, CH_2CH_3); 1.20–1.34 (br, 54H, CH_2), 1.42–1.52 (m, 2H, $NHCH_2CH_2CH_2CO$), 1.54–1.71 (m, 6H, $OCOCH_2CH_2$, $NH_2CH_2CH_2$) 1.76–1.86 (m, 2H, $NH_2CH(CO)CH_2$), 1.98–2.13 (m, 2H, $CONHCHCH_2$), 2.28 (t, 2H, $NHCOCH_2$), 2.35–2.47 (m, 2H, CH_2COO), 2.92 (t, 2H, NH_2CH_2), 3.25–3.30 (m, 2H, $CONHCH_2$),3.43–3.47 (m, 1H, NH_2CH), 4.05–4.12 (m, 4H, $COOCH_2$), 4.50–4.57 (m, 1H, CONHCH), 7.36(d, 1H, CHNH), 7.82(t, 1H, CH_2NH). MS(ESI): $(M+H)^+$ calcd. for $C_{47}H_{92}N_4O_6$, 809.7; found, 809.9.

2b: Rf: 0.1 ($CHCl_3/CH_3OH/H_2O$ (65/25/4)), ^{1}H-NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, CH_2CH_3); 1.20–1.35 (br, 58H, CH_2), 1.40–1.51 (br, 4H, $CONHCH_2CH_2$, $NH_2CH_2CH_2$) 1.52–1.68 (m, 6H, $OCOCH_2CH_2$, $NH_2CH(CO)CH_2$), 1.81–1.99 (m, 2H, $CONHCHCH_2$), 2.13–2.25 (br, 2H, $NHCOCH_2$), 2.33–2.41 (m, 2H, CH_2COO), 2.90–2.98 (br, 2H, NH_2CH_2), 3.16–3.25 (br, 2H, $CONHCH_2$),3.75–3.90 (m, 1H, NH_2CH), 4.02–4.13 (m, 4H, $COOCH_2$), 4.53–4.56 (m, 1H, CONHCH), 7.09(d, 1H, CHNH), 8.19(t, 1H, CH_2NH). MS(ESI): $(M+H)^+$ calcd. for $C_{49}H_{96}N_4O_6$, 837.7; found, 837.8.

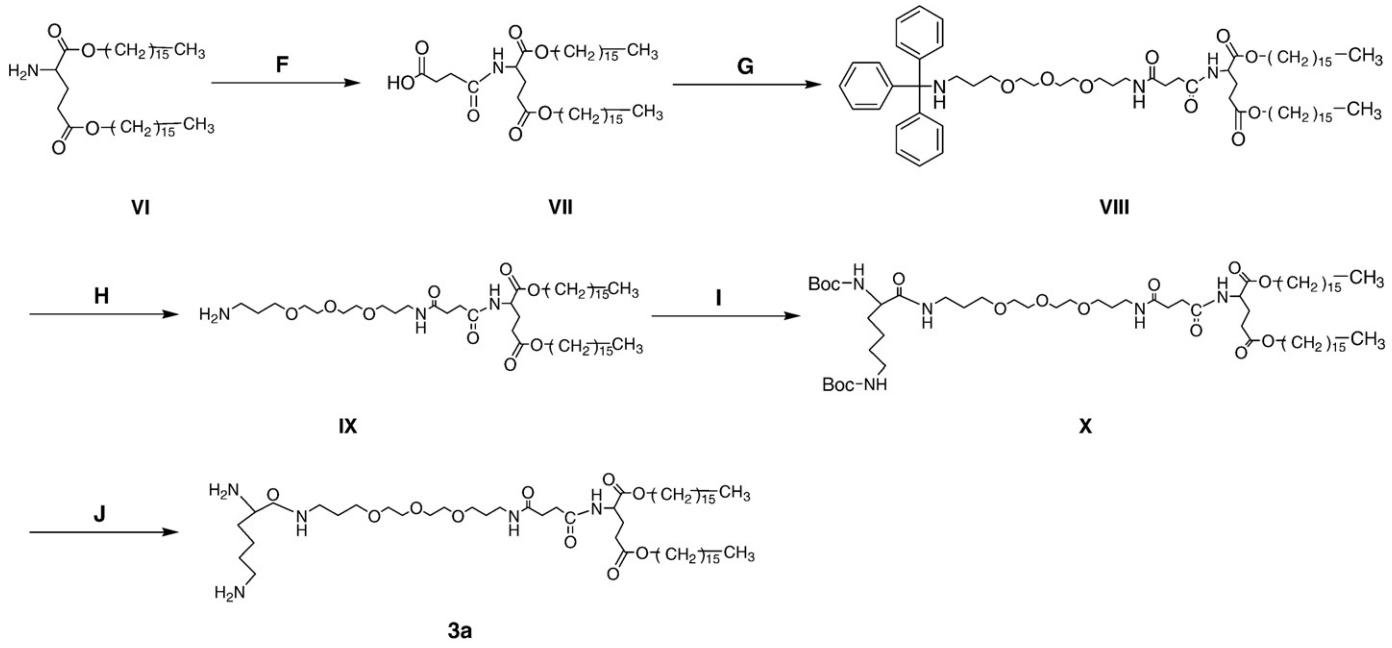
2c: Rf: 0.1 ($CHCl_3/CH_3OH/H_2O$ (65/25/4)), ^{1}H-NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, CH_2CH_3); 1.14–1.34 (br, 60H, CH_2), 1.40–1.52 (br, 2H, $NHCOCH_2CH_2$) 1.54–1.70 (m, 8H, $OCOCH_2CH_2$, $NH_2CH_2CH_2$, $CONHCH_2CH_2$), 1.75–1.82(m, 2H, $NH_2CH(CO)CH_2$), 1.92–2.24 (m, 2H, $CONHCHCH_2$), 2.21 (t, 2H, $NHCOCH_2$), 2.30–2.46 (m, 2H, CH_2COO), 2.85 (t, 2H, NH_2CH_2), 3.16–3.24 (m, 2H, $CONHCH_2$), 3.34–3.76 (m, 1H, NH_2CH), 4.02–4.15 (m, 4H, $COOCH_2$), 4.57–4.61 (m, 1H, CONHCH), 6.47(d, 1H, CHNH), 7.52(t, 1H, CH_2NH). MS(ESI): $(M+H)^+$ calcd. for $C_{51}H_{100}N_4O_6$, 865.8; found, 866.1.

2d: Rf: 0.1 ($CHCl_3/CH_3OH/H_2O$ (65/25/4)), ^{1}H-NMR ($CDCl_3$, 500 MHz, δ ppm): 0.88 (t, 6H, CH_2CH_3); 1.14–1.34 (br, 68H, CH_2), 1.40–1.52 (br, 2H, $NHCOCH_2CH_2$) 1.52–1.71 (m, 8H, $OCOCH_2CH_2$, $NH_2CH_2CH_2$, $CONHCH_2CH_2$), 1.78–1.86(m, 2H, $NH_2CH(CO)CH_2$), 1.94–2.24 (m, 2H, $CONHCHCH_2$), 2.21 (t, 2H, $NHCOCH_2$), 2.30–2.46 (m, 2H, CH_2COO), 2.80 (t, 2H, NH_2CH_2), 3.10–3.24 (m, 2H, $CONHCH_2$), 3.33–3.40 (m, 1H, NH_2CH), 4.02–4.14 (m, 4H, $COOCH_2$), 4.58–4.62 (m, 1H, CONHCH), 7.60(d, 1H, CHNH), 8.21(t, 1H, CH_2NH). MS(ESI): $(M+H)^+$ calcd. for $C_{55}H_{108}N_4O_6$, 1026.8; found, 1026.9.

2.2.2. Synthesis of the cationic lipid **3a** containing an oxyethylene-type spacer

1,5-Dihexadecyl-L-glutamate (3.5 g, 5.87 mmol) was dissolved in a solution (50 mL) of dichloromethane/tetrahydrofuran (1/1), and then succinic anhydride (0.88 g, 8.81 mmol) was added to the solution. After stirring for 12 h at room temperature, the solution was dropped into 500 mL of acetone and cooled to 4 °C. After filtration through a glass filter, the 1,5-dihexadecyl-*N*-succinic-L-glutamate was obtained in a yield of 85%. 1,5-Dihexadecyl-*N*-succinic-L-glutamate (1.66 g, 2.39 mmol) and BOP reagent (1.12 g, 2.82 mmol) were dissolved in dichloroform (100 mL), and then 1-*N*-triphenylmethyl-4,7,10-trioxa-1,13-tridecanediamine-bis[2-(3-aminopropoxy)ethyl] ether (trt-$(PEG)_2$-NH_2, 1 g, 2.16 mmol) was added to the solution. After stirring for 12 h at room temperature, chloroform was evaporated, and the residue was purified by column chromatography (silica gel; $CHCl_3$/MeOH = 5/1 (v/v)) resulting in Compound VIII (Scheme 2). After deprotection of the Boc-group with TFA as described above, Compound IX was obtained. Compound IX (500 mg, 0.56 mmol), Boc-Lys(Boc)-OSu (280 mg, 0.62 mmol) and triethylamine (0.62 mmol) were added to dichloromethane (50 mL), and the solution was stirred for 12 h at room temperature. After evaporation, the residue was purified by column chromatography (silica gel; $CHCl_3$/MeOH = 20/1 (v/v)) resulting in Compound X. After deprotection of the Boc-group, **3a** was obtained as a white powder with a yield of 39%.

3a: Rf: 0.08 ($CHCl_3/CH_3OH/H_2O$ (65/25/4)), ^{1}H-NMR ($CDCl_3$/MeOD = 4/1, 500 MHz, δ ppm): 0.88 (t, 6H, CH_2CH_3); 1.14–1.34 (br, 52H, CH_2), 1.46–1.52 (br, 2H, $CHCH_2CH_2$) 1.58–1.66 (m, 4H, $OCOCH_2CH_2$), 1.69–1.81(m, 6H, $NH_2CH(CO)CH_2$, $CONHCH_2CH_2$), 1.82–1.89 (m, 2H, $NH_2CH_2CH_2$), 1.93–2.19 (m, 2H, $CONHCHCH_2$), 2.33–2.41 (m, 2H, CH_2COO), 2.45–2.55 (m, 2H, $NHCOCH_2$), 2.96 (t, 2H, NH_2CH_2), 3.25–3.34 (m, 4H, $CONHCH_2$),3.50–3.65 (br, 12H, OCH_2), 3.88–3.92 (m, 1H, NH_2CH), 4.02–4.15 (m, 4H, $COOCH_2$), 4.46–4.51 (m, 1H, CONHCH), 6.27(d, 1H, CHNH), 7.35(t, 1H, CH_2NH). MS(ESI): $(M+H)^+$ calcd. for $C_{51}H_{100}N_4O_6$, 922.1; found, 921.8.



[a]Reaction conditions: (F) succinic anhydride/DCM:THF (5:5); (G) trt-$(PEG)_2$-NH_2/BOP/TEA/DCM; (H) TFA; (I) Boc-Lys(Boc)-OSu/TEA/DCM; (J) TFA

Scheme 2. Syntheses of the cationic lipid having an oligoxyethylene-type spacer.

All the synthetic compounds were further analyzed with a high-pressure liquid chromatography system (Shimadzu Co., Kyoto, Japan; mobile phase of 50% isopropanol and 0.1% trifluoroacetic acid at 0.5 mL/min) using linear gradients starting from 50% isopropanol to 100% in 10 min on a TSK-GEL ODS-80Ts reversed phase column (TOSOH, Tokyo, Japan) to confirm the purity.

2.3. Liposome preparation

pDNA-encapsulating liposomes were prepared by spontaneous entrapment of pDNA upon electrostatic interaction with ethanol-destabilized cationic liposomes [14]. The plasmid vector phRL-TK (Promega, Madison, WI) encoding luciferase was used for all experiments. Propagation of the vector was performed by heat shock transformation into *Escherichia coli* JM109 and growth in LB medium supplemented with 50 μg/mL ampicillin. The pDNA was isolated using a QIAGEN Plasmid Mega kit (Qiagen, Germantown, MD). In order to prepare pDNA-encapsulating liposomes, a pDNA solution was diluted with 20 mM phosphate buffer (pH 5.0) to achieve a pDNA concentration of 0.1 mg/mL. The dried mixed lipid powder (Table 1) prepared by freeze-drying from *t*-butyl alcohol was dissolved in ethanol, and then the solution was diluted with distilled water to achieve an ethanol concentration of 90 vol.%. Equal volumes (1 mL) of the pDNA solution and the resulting lipid solution were mixed and then incubated at 37 °C to prepare a liposomal dispersion. After 1 h incubation, the solution was washed with 20 mM HEPES buffer (pH 7.4) by ultracentrifugation (100,000×*g*, 30 min, 4 °C) and dispersion twice. Then, anion exchange chromatography using DEAE-Sepharose CL-6B (Sigma-Aldrich, St. Louis, MO) was used to remove the non-encapsulated pDNA and the pDNA electrostatically adsorbed on the outer surface of the liposomes. For the analysis of the cellular uptake efficiency of the liposomes, the mixed lipids containing *N*-(7-nitrobenz-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmytoyl-*sn*-phosphatidylethanolamine (NBD-PE, Avanti Polar Lipids, Inc., Alabaster, AL), at a concentration of 0.1 mol%, were alternatively prepared. The fluorescence labeled liposomes were then prepared in 20 mM phosphate buffer instead of pDNA solution as described in the preparation of the pDNA-encapsulating liposomes.

2.4. Liposomal contents

The lipid concentration of the liposomes was calculated from a DOPC content determined by a phospholipid *C* Test WAKO kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The liposome dispersions ([lipid] = 5 mg/mL, 10 μL) were mixed with an equal volume of a 10% SDS solution to solubilize the liposomes. After incubation at 60 °C for 5 min, the test reagent (3 mL) was added to the solution. Then, the resulting mixed solution was incubated at 37 °C for 3 min according to the manufacturer's instructions. A DOPC concentration was calculated from the 600 nm absorbance corresponding to production by cholineoxydase. A total lipid concentration was calculated from the DOPC content using the feed lipidic molar ratio. The synthetic cationic

Table 1
Characteristics of pDNA-encapsulating liposomes containing synthetic cationic lipids having a spacer.

Lipid components	Mole ratio	Diameter [nm]	Zeta potential [mV]	DNA–lipid ratio (w/w) [$\times 10^{-3}$]	Encapsulation ratio [%]
DOPC/cholesterol/PEG-Glu2C18	5/5/0.03	198 ± 81	−0.7 ± 1.3	2.9	10
1a/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	174 ± 66	1.3 ± 1.2	6.2	38
2a/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	248 ± 99	3.3 ± 1.0	4.1	30
2b/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	186 ± 69	3.2 ± 0.9	8.0	49
2c/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	229 ± 91	1.8 ± 0.2	4.6	39
2d/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	N.D.	N. D.	N.D.	N.D.
3a/DOPC/cholesterol/PEG-Glu2C18	1/5/5/0.033	203 ± 78	5.5 ± 1.6	5.6	33

lipid content of the resulting cationic liposomes was analyzed using a HPLC system as described earlier. The concentration of pDNA encapsulated within the liposomes was determined from absorbance at 260 nm after extracting pDNA with chloroform/ethanol as described below. The liposome dispersions (50 μL) were ultracentrifuged (100,000×*g*, 30 min) to collect the liposomes. Next, chloroform (900 μL) was added to the liposome pellet to dissolve the lipid in the organic layer. Then, distilled water (100 μL) was added to the chloroform solution to transfer the pDNA into the water layer. Finally, pDNA in the water layer was extracted with isopropanol, followed by 70% ethanol addition. The concentration of pDNA dissolved in 20 mM HEPES buffer was calculated from the 260 nm absorbance using a Nano Drop ND-1000 (SCRUM Inc. Tokyo, Japan). The pDNA encapsulation ratio represented using the following (1):

$$\text{Encapsulation ratio}\,(\%) = (\text{amount of pDNA extracted from the pDNA-encapsulating liposomes}\,(\mu g) / \text{amount of total pDNA used in preparation}\,(100\mu g)) \times 100. \quad (1)$$

2.5. Size distribution of the cationic liposomes

A dispersion of the liposomes (5 μL) containing 10 mg/mL lipid was diluted with 3 mL of 20 mM HEPES buffer to measure the liposome size. Then, the mean particle diameter was measured three times with a dynamic light scattering spectrophotometer (N4 PLUS Submicron Particle Size Analyzer, Beckman-Coulter, Fullerton, FL). The data represents an average diameter and a standard deviation (S.D.) value obtained from measurements performed in triplicate.

2.6. TEM observation of the pDNA-encapsulating cationic liposomes

The pDNA-encapsulating liposomes were observed by transmission electron microscopy (TEM). A drop of the sample dispersion ([lipid] = 100 μg/mL) was placed on a 100 mesh copper grid, and then the excess dispersion was removed with a piece of filter paper. A 2% phosphotungstic acid solution (pH 7.4) was dropped on the grid and then dried for 12 h in a desiccator. The morphology of the cationic liposomes was observed with TEM (JM-1011, JEOL).

2.7. Zeta potential of the cationic liposomes

The zeta potential of the resulting cationic liposomes at pH 7.4 was measured with a Zetasizer (Zetasizer4, Malvern, U.K.). The liposome dispersions ([lipid] = 0.1 mg/mL) in 20 mM HEPES buffer (pH 7.4) were loaded in a capillary cell mounted on the apparatus and measured three times at 37 °C. The data represents average zeta potential and a standard deviation (S.D.) value from measurements carried out in triplicate.

2.8. DNase digestion assay

The pDNA encapsulation within the cationic liposomes was investigated using DNase. The following samples were tested: naked pDNA, liposomes with the pDNA (lipoplexes), and the pDNA-encapsulating liposomes. pDNA (500 ng) was incubated with 500 U of DNase I (Invitrogen, Carlsbad, CA) in a total volume of 100 μL HEPES buffer (pH 7.4) for 60 min at 37 °C. The resulting solution was then extracted by sequential addition of 900 μL of chloroform and 500 μL of ethanol. After centrifugation (20,400×*g*) for 30 min, the supernatant was decanted, and the pDNA was purified with 70% ethanol and dried. The resulting pDNA pellet was dissolved in 10 μL of 20 mM HEPES buffer (pH 7.4) and analyzed by gel electrophoresis using a 1% agarose gel in Tris–acetate-EDTA. After electrophoresis for 60 min, the agarose gel was rinsed in an ethidium bromide solution for 10 min, washed in distilled water for 15 min, and visualized on a UV transilluminator (GelDoc XR system; Bio-Rad, Hercules, CA).

2.9. Cellular uptake of the cationic liposomes

COS-1 cells (Transformed African green monkey kidney fibroblast cells, 1×10^4 cells/well) were seeded on a 96-well plate cell culture dish and incubated in a humidified 5% CO_2 incubator at 37 °C. After overnight incubation, the medium in the cell culture dish was exchanged with fresh Dulbecco's Modified Eagle Medium (DMEM) containing the fluorescent labeled liposomes ([lipid] = 3 μg/mL as a final concentration of the liposomes). After incubation at 37 °C for 2 h, the cells were washed twice with a cold PBS solution, and dissolved in 0.1% SDS-containing 5 mM Tris buffer (100 μL, pH 7.4). The amount of the NBD-PE in the lysate was fluorometrically determined at an excitation wavelength of 488 nm and an emission wavelength at 530 nm with an ARVO MX-3 (PerkinElmer Japan Co., Ltd, Japan). The standard curve of NBD-PE fluorescent intensity to lipid concentration was constructed for each investigated cationic liposome in advance. The amount of lipid introduced into the cells was then calculated from the fluorescent intensity of the NBD molecules, followed by extrapolation using a standard curve. The protein concentration of the lysate was determined using a standard protein assay (Bio-Rad Protein Assay, Bio-Rad). Thus, the cellular uptake efficiency was quantitatively estimated and represented as the amount of liposomes per cellular protein. The data represents average cellular uptake efficiency and a standard deviation (S.D.) value from measurements performed in triplicate.

2.10. Fusogenic potential of the cationic liposomes

Membrane fusion between the cationic liposomes and liposomes composed of a biomembrane mimicking lipid composition was investigated using a fluorescence resonance energy transfer (FRET) assay [20,30]. The biomembrane mimicking lipid composition, DOPC/DOPE/DOPS/cholesterol (45/20/20/15, wt.%), was both incorporated with 1 mol% NBD-PE and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmytoyl-*sn*-phophatidylethanolamine (Rh-PE, Avanti Polar Lipids Inc., Alabaster, AL) to the mixed lipids. For the fusion assay, the labeled liposomes (500 μM, 500 μL) were mixed with the cationic liposomes (lipids; 500 μM, 500 μL). Then, the mixed solution was incubated at 37 °C for the appropriate time. The transition in fluorescence intensity was continuously recorded with excitation at 460 nm and emission at 525 nm. The lipid mixing ratio between the cationic liposomes and the biomembrane mimicking liposomes was determined using the following equation (2):

$$\text{Lipid mixing ratio}\,\% = ((F_t - F_0) / (F_{TX} - F_0)) \times 100 \quad (2)$$

where F_t is the fluorescence intensity at an appropriate time and F_0 is the fluorescence intensity just after dilution with distilled water and F_{TX} is the fluorescence intensity after the addition of Triton X-100 (100 μL) to solubilize the liposomes for estimating the non FRET conditions to provide 100% fusion value.

2.11. Gene expression efficiency of the pDNA-encapsulating liposomes

COS-1 cells were used to evaluate gene expression efficiency of the pDNA-encapsulating cationic liposomes. The COS-1 cells (1×10^4 cells/well) were seeded on 96-well plates and incubated at 37 °C under 5% CO_2 overnight. The medium in the cell culture dishes was exchanged with a fresh medium (100 μL) containing pDNA or the pDNA-encapsulating cationic liposomes diluted with DMEM ([pDNA] = 2 μg/mL as a final concentration). After incubation at 37 °C for 72 h, the cells were washed twice with an ice-cold PBS solution and then lysed with the lysis buffer of a luciferase assay kit (Promega, Madison, WI). The luciferase activity of a 10 μL aliquot of the cell lysate was measured with a Microlumat Plus (EG&G Berthold, BadWilbad, Germany). The protein concentration of each well was determined by a standard protein

assay. The luciferase activity in each sample was normalized to the relative light unit (RLU) per microgram of protein. The data represents average gene expression efficiency and a standard deviation (S.D.) value from independent measurements performed in triplicate. Indeed, for comparison between pDNA-encapsulating liposomes and lipoplexes in terms of gene expression efficiency, the pDNA-encapsulating liposomes and liposome/pDNA lipoplexes related **2c** or **3a** were added to COS-1 cells to investigate the influence of pDNA encapsulation. The lipid-to-pDNA ratio in lipoplexes was set to that of pDNA-encapsulating liposomes (**2a**: 217, **3a**: 179 (w/w)). Then, the gene expression studies were performed as described above. Furthermore, gene expression was compared with that of Lipofectamine™2000 (LA™2000). The LA™2000-mediated transfection was performed according to the manufacturer's guidelines.

2.12. Cytotoxicity of the cationic liposomes

The cytotoxicity of the liposome-containing cationic lipids having different spacer units was investigated using COS-1 cells. The COS-1 cells (1×10^4 cells) were seeded on a 96-well plate and incubated at 37 °C under 5% CO_2 overnight. The COS-1 cells were cultured with DMEM containing 10% FBS. The medium in the cell culture dish was exchanged with a fresh medium (100 μL) containing an appropriate concentration of liposomes. After incubation at 37 °C for 72 h, the medium was exchanged with a medium (110 μL) containing a tetrazolium salt (WST-1) and incubated for 30 min. Formazan (absorbance at 540 nm) is then produced by succinate-tetrazolium reductase in living cells. The absorbance at 540 nm was monitored using a microplate reader (Perkin Elmer Japan Co. Ltd, Tokyo, Japan).

3. Results and discussion

3.1. Liposomal contents

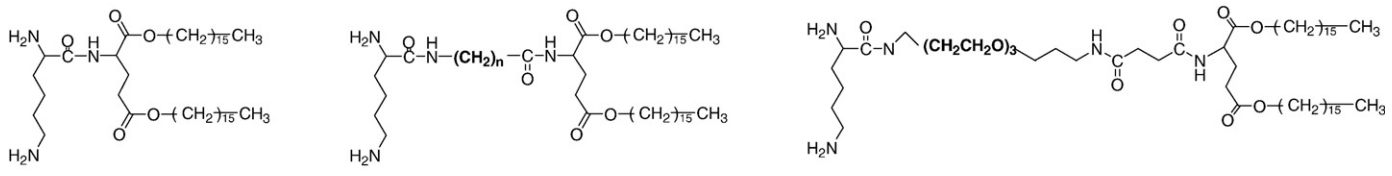
We synthesized a series of lipids with a hydrocarbon chain spacer containing 3, 5, 7, or 11 carbon atoms or an oxyethylene chain spacer with ten carbon and three oxygen atoms. The hydrophobic moiety; 1,5-dihexadecyl-L-glutamate, was utilized for all cationic lipids. To introduce the hydrocarbon chain spacer, ammonioalkanoic acid derivatives with different carbon numbers were reacted with the 1,5-dihexadecyl-L-glutamate *via* an amide linkage as shown in Scheme 1. Then, after introducing a lysine moiety as a cationic head group, we obtained cationic lipids having the different hydrocarbon chain spacers (**2a**–**2d**). Alternatively, **3a** having an oxyethylene chain spacer was introduced from 1,5-dihexadecyl-*N*-succinyl-L-glutamate. Thus, we succeeded in devising an efficient synthetic route to generate a series of cationic lipids containing various spacers using a minimum number of steps. When the cationic liposomes containing the synthetic lipids were prepared, the ratio of the cationic lipids to liposomal membrane was confirmed using HPLC analysis. Then, the concentration of the cationic liposomal components was confirmed to be the same as that prepared in mixed lipids. Indeed, the **2d** assembly tended to aggregate just after preparation. Therefore, no further analysis on **2d** was performed in this study.

The most studied liposomes for gene delivery are composed of phospholipids and mixed membrane cationic lipids. Phospholipids, such as dioleoylphosphatidylcholine (DOPC) [31], dimyristoylphosphatidylcholine (DMPC) [32,33], and dioleoylphosphatidylethanolamine (DOPE) [24,34,35], are frequently utilized as liposomal components, and cholesterol is also utilized to control membrane permeability and fluidity. In this study, DOPC and cholesterol were selected as basic membrane components of the liposome, of which the mixed mole ratio was 1:1. Indeed, incorporation of the cationic lipid or PEG-Glu2C_{18} to the basic membrane was defined as 10 mol% or 0.3 mol%, respectively because PEG-Glu2C_{18} is essential in preventing aggregation during preparation with ethanol. Furthermore, because excess incorporation of the cationic lipids into liposomal membrane would alter the vesicular structure, the incorporation ratio was determined as 10 mol% of the total membrane. We confirmed the tube-like morphology of liposomes incorporating 50 mol% of cationic lipids, particularly in the case of **3a** (data not shown). By contrast, liposomes containing **2d** having an eleven hydrocarbon spacer produced aggregates just after extrusion. Presumably this was due to the particularly hydrophobic nature of the spacer unit of **2d**. Using this approach, we were able to define the limit to the length of the hydrocarbon spacer between the head group and hydrophobic moieties in order to generate stable liposomes.

3.2. pDNA-encapsulating cationic liposomes

The pDNA-encapsulating cationic liposomes containing synthetic lipids with different spacer units (Chart 1) were prepared by varying the ethanol concentration before and after addition of the pDNA solution. The size of the pDNA-encapsulating liposomes was determined to be within 170–250 nm as shown in Table 1. Furthermore, we observed the pDNA-encapsulating cationic liposomes by TEM (Fig. 1). The structures were confirmed to be vesicular having an approximate diameter of 200 nm, corresponding to the results of the DLS measurements. Morphology of the cationic liposomes was not influenced by the spacer length when 10 mol% cationic lipids (**1a**, **2a**–**2c**, **3a**) were inserted into the liposomal membrane. The pDNA encapsulating efficiency was about 30–49% in the case of liposomes containing cationic lipids. By contrast, the encapsulating efficiency was only about 10% for basic DOPC/cholesterol liposomes.

We succeeded in preparing pDNA-encapsulating liposomes containing synthetic cationic lipids bearing different spacer units. Previous studies indicated that ethanol made the lipid membrane susceptible to structural rearrangements, and ultimately led to liposome formation [14]. Moreover, incorporation of PEG-lipid to the



Non-spacer lipid **1a**

Hydrocarbon-spacer lipids
Lipid **2a**: n=3
Lipid **2b**: n=5
Lipid **2c**: n=7
Lipid **2d**: n=11

PEG-spacer lipid **3a**

Chart 1. Chemical structure of the comparable synthetic cationic lipids.

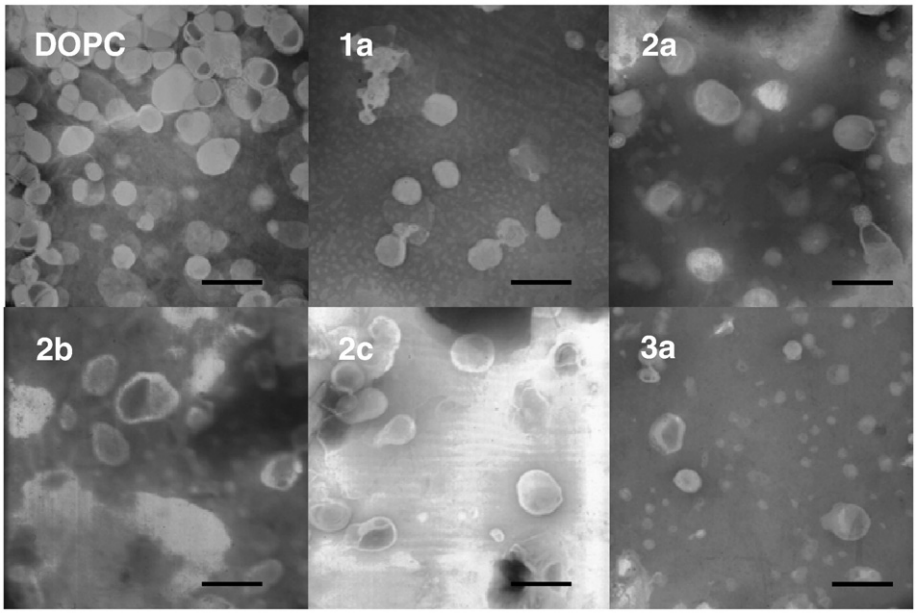


Fig. 1. Transmission electron microscopic images of the pDNA-encapsulating cationic liposomes composed of DOPC, **1a**, **2a**, **2b**, **2c**, or **3a**. Scale bar indicates 200 nm.

membrane is required for liposomal formation in 40% ethanol. In this study, the ratio of 0.3 mol% PEG-Glu2C$_{18}$ was enough to prepare stable liposomes having diameters of 150–200 nm. The cationic liposomes showed extremely higher pDNA encapsulating efficiency than DOPC/cholesterol liposomes. This is presumably because a large number of the encapsulated pDNA molecules are electrostatically adsorbed to the inner leaflet of the cationic liposome membrane.

3.3. Zeta potential of the liposomes

The zeta potential depends on the charge density of the cationic moiety at the surface of the liposomes. We investigated the influence of the spacer unit of the cationic lipids on the zeta potential of the liposomes. DOPC/cholesterol liposomes gave a zeta potential of around zero (Table 1). The zeta potentials of the **1a**, **2a**–**2c**, and **3a** liposomes were 1.3, 3.3, 3.2, 1.8 and 5.5 mV, respectively. A positive zeta potential was attained when the cationic lipids were inserted into the basic DOPC/cholesterol liposomes. Moreover, the degree of the zeta potential of **2a**–**2c** liposomes was similar to that of **1a** liposomes, though **3a** liposomes displayed a slightly greater zeta potential.

Incorporation of cationic lipids **1a**, **2a**–**2c**, and **3a** generated positively charged liposomes as compared to the basic DOPC/cholesterol liposomes. The hydrophobic and hydrophilic spacer unit of the cationic lipids **2a**–**2c**, **3a** did not significantly influence the zeta potential of the liposomes. However, the slightly high zeta potential of **3a** liposomes suggested that the cationic moiety of **3a** might be located on the outer most side of the membrane as a result of the extension of the hydrophilic oligoxyethylene spacer.

3.4. DNase assay

We confirmed pDNA encapsulation within the liposomes by adding DNase to the pDNA-encapsulating liposomes. Naked pDNA alone and pDNA/empty liposomes (complex) were readily degraded by DNase (Fig. 2). By contrast, the DNA in pDNA-encapsulating liposomes was completely protected from degradation. This result suggests that the pDNA was efficiently encapsulated within the cationic liposomes and therefore resistant to exogenous DNase.

DNA degradation is known to be a limiting factor in the application of gene therapy [36,37]. When the lipoplexes are applied to target tissues under intravenous injection or direct injection into organs, DNA is subject to degradation and then cleared from the biological system. Therefore, the protection of DNA from DNase is an absolute requirement for practical gene therapy. In this study, we achieved DNA protection from exogenous DNase by encapsulation of DNA within liposomes, indicating the liposomal membrane is able to limit DNase access to encapsulated DNA within the inner aqueous phase.

3.5. Cellular uptake of the cationic liposomes

We investigated the cellular uptake efficiency of the cationic liposomes containing the synthetic cationic lipids using COS-1 cells

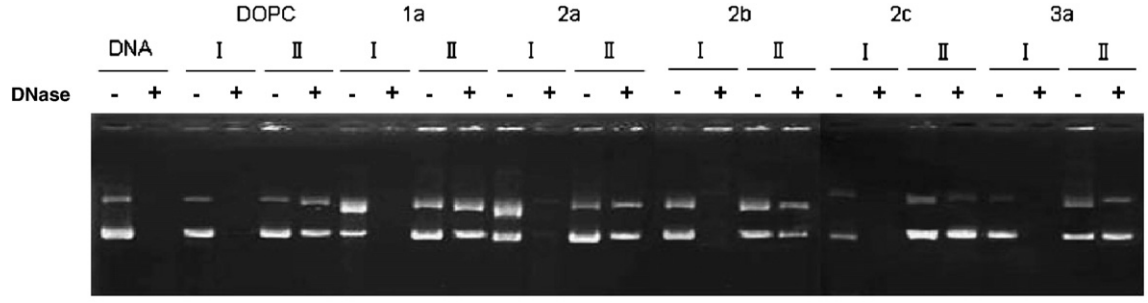


Fig. 2. DNase I digestion assay for pDNA encapsulation within the liposomes containing the space embedded lipids. (I) pDNA/liposomes (Complex). (II) pDNA-encapsulating liposomes. Each sample containing 500 ng pDNA was incubated with (+) or without (−) 500 U of DNase I at 37 °C for 1 h.

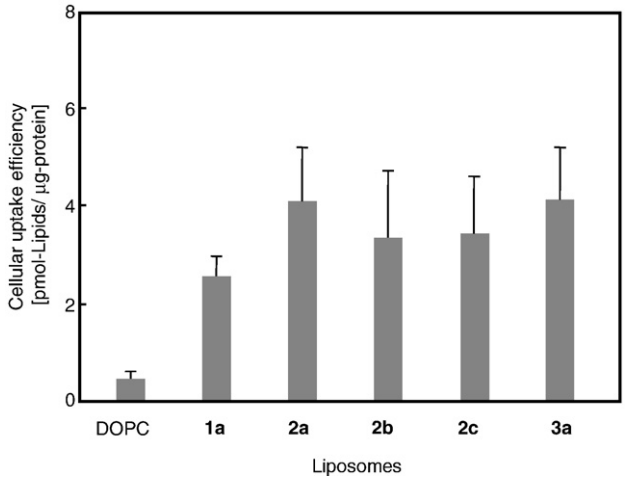


Fig. 3. Cellular uptake efficiency of the cationic liposomes for 2 h at 37 °C.

(Fig. 3). The cellular uptake efficiency of the liposomes containing the lipids having a lysine head group was higher than that of the DOPC/cholesterol liposomes. Specifically, the cellular uptake efficiency of **1a** liposomes was five times higher than that of the DOPC/cholesterol liposomes. Indeed, cationic liposomes containing the synthetic lipids **2a**–**2c** having a hydrophobic spacer unit showed comparable cellular uptake efficiencies, which were similar to **1a** liposomes. Moreover, cationic liposomes containing lipid with an oxyethylene spacer unit showed similar cellular uptake efficiencies to those containing a hydrophobic spacer unit.

We confirmed that incorporation of the synthetic cationic lipids to the basic DOPC/cholesterol membrane increased cellular uptake as a result of an electrostatic adsorption of the cationic liposomes to the negatively charged biomembrane, followed by cellular uptake into COS-1 cells. Liposomes containing lipids **2a**–**2c** and **3a**, having a hydrophobic or hydrophilic spacer unit, displayed similar cellular uptake efficiencies. These observations suggest that the hydrophobic or hydrophilic characteristics of the spacer unit do not significantly affect cellular uptake. That can be explained by applying the equivalent zeta potential of the liposomes containing a non-spacer lipid (**1a**) and cationic lipids having a spacer unit.

3.6. Fusogenic potential of the cationic liposomes

We also investigated the fusogenic potential of the cationic liposomes, which is the most important factor for intracellular pDNA trafficking and for gene expression efficiency. As shown in Fig. 4, the DOPC/cholesterol liposomes showed very limited fusogenic potential to the biomembrane-mimicking liposomes (DOPC/DOPE/DOPS/cholesterol). By contrast, liposomes containing the synthetic cationic lipids with a hydrocarbon-type spacer enhanced the fusogenic potential. The order of fusogenic potential over a 5 min period was **2c**>**2b**>**2a**>**3a**>**1a**. In the case of the **2c** liposomes, about 10% were able to fuse after 5 min. Indeed, fusogenic potential depended on the length of the hydrophobic spacer unit. Consequently, liposomes displaying the highest fusogenic potential were those containing **2c**. The fusogenic potential of **3a** liposomes, containing an oxyethylene chain spacer, was low. In the case of **2a** liposomes, fusion decreased after showing a maximum value at 150 s.

The membrane fusion behavior between the cationic liposomes and the biomembrane-mimicking liposomes is known to provide information about gene expression efficiencies [38–40]. Specifically, the experiment mimics fusion of liposomes with the endosome membrane following endocytosis. After endocytosis of the cationic liposomes, they are transferred to early endosomes within 5 min [41]. Liposomes that are retained by the endosomes are subsequently transferred into the lysosome within 30 min. For estimating effective intracellular pDNA delivery, we measured the fusogenic potential of the liposomes for 5 min, implying pDNA release from the early endosome. In our study, **2c** containing a seven hydrocarbon spacer revealed the highest fusogenic potential among the series of cationic lipids. Kono et al. suggested that the hydrophobic nature of the spacer unit exposes the hydrophilic moiety of the lipids thereby enhancing the fusogenic potential to a biomembrane by hydrophobic interaction [42,43]. Therefore, a cationic lipid with a large hydrophobic spacer, such as **2c**, would be expected to give the highest fusogenic potential. By contrast, the hydrophilic nature of the oxyethylene spacer, as in **3a**, is anticipated to give a low fusogenic potential due to hydrophilic repulsion with the endosomal membrane. In the case of **2a** liposomes, the fusion dynamics was different from the other cationic liposomes. When **2a** liposomes adhered to the anionic liposomes by electrostatic interaction just after mixing, **2a** was not diluted into the anionic membrane and gradually became aggregated. The aggregation would therefore reduce fluorescent intensity to provide an apparent reduction of fusogenic potential. Thus, we demonstrated the role of the hydrocarbon chain spacer between the cationic moiety and the hydrophobic moieties for improving fusogenic potential.

3.7. Gene expression efficiency of the pDNA-encapsulating cationic liposomes

We investigated the gene expression efficiency of the pDNA-encapsulating cationic liposomes using COS-1 cells. The pDNA-encapsulating liposomes were added to the cells for various incubation periods. All the cationic liposomes exhibited the highest gene expression at 72 h. Therefore, the gene expression efficiency at 72 h was compared between the pDNA-encapsulating liposomes to evaluate spacer effects (Fig. 5). Our results suggest that the gene expression level is influenced by the length and type of spacer unit. Gene expression efficiency varied in the following order: **2c**>**2a**≥**2b**>**3a**≥**1a**. By contrast, **1a**, as a non-spacer type lipid, showed the lowest gene expression efficiency among the investigated lipids. DDAB-containing liposomes, which acted as a positive control, showed equivalent gene expression efficiency to that of the **3a** liposomes.

We ranked the cationic liposomes in terms of level of gene expression. Intriguingly, this ranking corresponded precisely to that of the cationic liposomes in terms of fusogenic potential. When cationic liposomes are introduced into cells by endocytosis, the release of encapsulated pDNA occurs after fusion of the cationic liposomes to endosomes. Therefore, because the level of cellular uptake efficiency

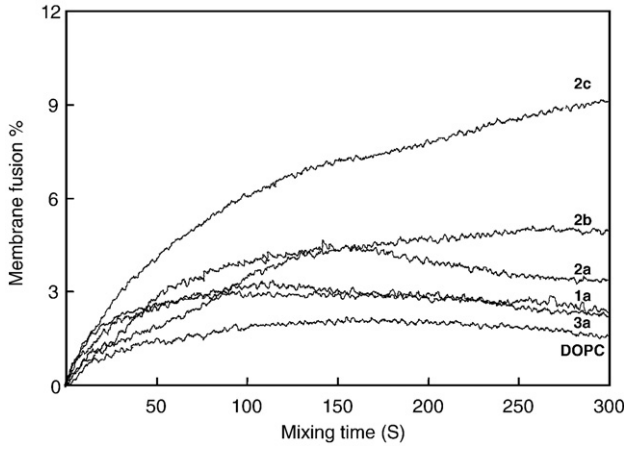


Fig. 4. Time courses of the fusogenic potential of the cationic liposomes to the biomembrane mimicking membrane (DOPC/DOPE/DOPS/cholesterol).

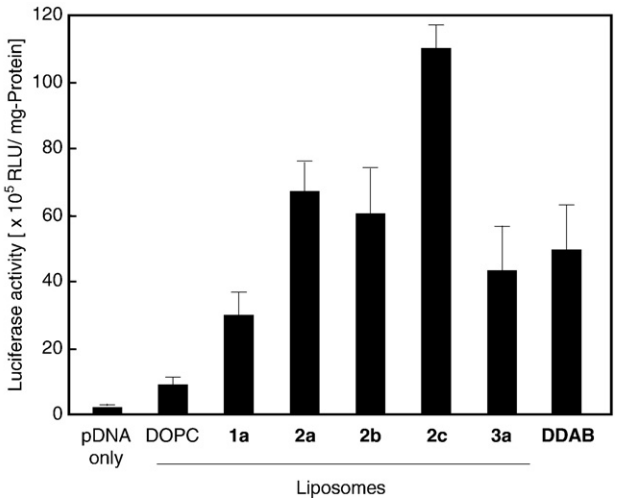


Fig. 5. Luciferase activity of the pDNA-encapsulating liposomes composed of cationic lipids. The activity was measured at 72 h after the addition of the liposomes to COS-1 cells.

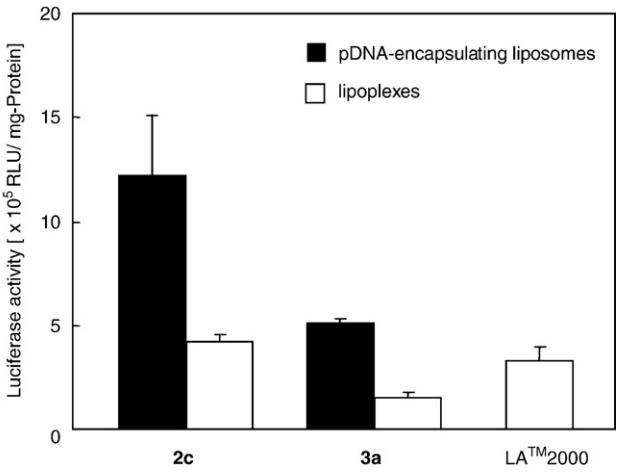


Fig. 6. Gene expression efficiency of the pDNA-encapsulating liposomes, liposome/pDNA lipoplexes and Lipofectamine™2000 in COS-1 cells. Closed bars indicate gene expression efficiency of pDNA-encapsulating liposomes containing **2c** or **3a**, respectively. Open bars indicate gene expression efficiency of liposome/pDNA lipoplexes with a same lipid-to-pDNA ratio to pDNA-encapsulating liposomes.

for the cationic liposomes was almost the same, gene expression efficiency is likely to correspond to fusogenic potential. Considering spacer effects, Bajaj et al. reported that cationic liposomes composed of the cationic gemini lipids having a hydrophilic spacer between the two head groups showed higher gene expression efficiency in comparison with the non-spacer type lipid. It was considered that gemini lipids having the hydrophilic spacer unit between head groups were able to stabilize the lipoplexes in serum protein [44, 45]. Indeed, length of the spacer with a hydrocarbon unit was extensively investigated to clarify influences of the spacer on phase transition, and fusion of liposomes by Bhattacharya et al. [28]. The study suggested that the appropriate length of a spacer between head groups of gemini lipids displayed high fusion potential. Mahidhar et al. analyzed cationic lipids having a hydrocarbon spacer unit between the cationic moiety and the functional sugar [46]. Their results indicated that the long hydrocarbon spacer diminished gene expression efficiency in comparison with the non-spacer type glycolipid [47]. These previous studies used lipoplex system. By contrast, in our study the pDNA is located on the internal phase of the liposomes and clarified the spacer effect. Thus, the different location of the pDNA within the liposome structure may explain why the role of the spacer is different from previous studies using spacer embedded lipids. Using this data we may be able to predict the influence of the spacer unit between the cationic head group and hydrophobic moieties in the pDNA-encapsulating liposomes. Furthermore, the level of gene expression with **2c** was 2-fold higher than that of cationic liposomes containing DDAB, which is extensively utilized in gene delivery systems [48,49]. The pDNA-encapsulating liposomes containing DDAB were also constructed and their properties examined [17,50,51]. Zhang et al. achieved successful transfection on HepG2 and SMMC-7721 human hepatocellular carcinoma cell lines using the pDNA-encapsulating liposomes containing DDAB [52]. Therefore, we were able to demonstrate the high gene expression capacity by using the cationic amino acid-based lipids having a spacer between the cationic and hydrophobic moieties.

3.8. Influence of pDNA encapsulation on gene expression efficiency

We compared the gene expression efficiency of the pDNA-encapsulating liposomes with the liposome/pDNA lipoplexes and clarified that the pDNA-encapsulating liposomes showed complete DNase resistance (Fig. 6). In the case of **2c**, which has a hydrocarbon spacer, the gene expression efficiency of the pDNA-encapsulating liposomes with a lipid-to-pDNA ratio of 217 was higher than that of the liposome/pDNA lipoplexes with the same lipid-to-pDNA ratio (Fig. 6). Indeed, the gene expression efficiency of the pDNA-encapsulating liposomes was also higher than the liposome/pDNA lipoplexes with a lipid-to-pDNA ratio of 179, as shown in the case of **3a** with an oxyethylene spacer. The gene expression efficiency of the pDNA-encapsulating **2c** liposomes was 4-fold higher than that of LA™2000 as a lipoplex system. Therefore, we were able to demonstrate that pDNA encapsulation within liposomes could effectively improve gene expression efficiency.

The degradation of pDNA by endogenous nucleases can result in low gene expression efficiency. Therefore, inhibition of pDNA degradation is required in order to obtain efficient transfection. In this regard, we have already confirmed that pDNA-encapsulating liposomes are resistant to DNase (Fig. 4). Mok et al. previously reported that pDNA encapsulated in the stabilized plasmid-lipid particles inhibited pDNA degradation during intracellular delivery [53]. Our results for enhancement of gene expression efficiency also indicate that pDNA encapsulation in liposomes can facilitate intracellular delivery by preventing pDNA degradation.

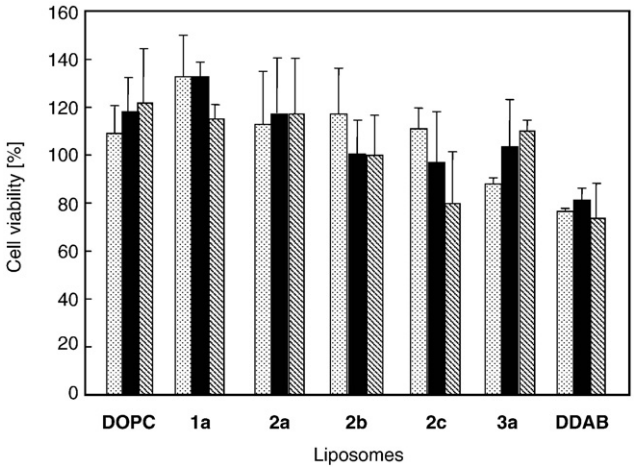


Fig. 7. Cytotoxicity of the liposomes containing cationic lipids having a spacer. The activity was measured at 72 h after the addition of the liposomes to COS-1 cells. The lipid concentration in medium represented as ash bar: 1 μg/mL, closed bar: 10 μg/mL, and line bar: 100 μg/mL.

3.9. Cytotoxicity of the cationic liposomes

The cationic liposomes containing synthetic lipids with a spacer unit displayed relatively low levels of cytotoxicity (Fig. 7). Amongst these, **2c** liposomes showed low cytotoxicity i.e., all cells were survived for 1 μg/mL of **2c**. By contrast, liposomes containing DDAB as a conventional cationic lipid showed higher levels of cytotoxicity than any of the cationic lipids with a spacer unit. For example, 30% of the cells seeded on a culture dish were damaged by the presence of 1 μg/mL DDAB liposome concentration. With the increasing length of hydrocarbon spacer, the cell cytotoxicity tended to increase slightly among **1a**, **2a**–**2c**.

Thus, the low level of cytotoxicity was confirmed for the amino-acid based lipids with a spacer unit. Generally, hydrophobicity of the lipids, which oriented onto the liposomal surface, would be expected to lead to cytotoxicity because hydrophobic materials facilitate adherence to cellular membranes by hydrophobic interaction. The tendency was also confirmed in our study. When we introduced the hydrophobic spacer into cationic lipid to enhance fusogenic potential, the cytotoxicity was slightly increased with increasing length of spacer.

3.10. Conclusions

We synthesized a series of cationic lipids with various spacer units between the cationic head group and hydrophobic moieties and investigated their utility in a pDNA-encapsulating liposome mediated gene delivery system. The pDNA encapsulation, cellular uptake and fusogenic potential of the cationic liposomes were also investigated. Fusogenic potential between liposomes and biomembranes depended on the nature of the spacer (i.e., length and hydrophobicity), which played an important factor in intracellular pDNA delivery. In our system, **2c** was the most appropriate structure for obtaining the highest gene expression efficiency. In this report, we enhanced the gene expression of a pDNA-encapsulating liposome mediated gene delivery by varying the spacer between the cationic head group and hydrophobic moieties of the cationic lipids. Our results clearly demonstrate the importance of spacer embedded cationic lipids for enhancing gene delivery.

Acknowledgements

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