Anchoring Cationic Amphiphiles for Nucleotide Delivery Significance of DNA Release from Cationic Liposomes for Transfection

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We have designed and synthesized lithocholic acid-based cationic amphiphile molecules as components of cationic liposomes for gene transfection (lipofection). To study the relationship between the molecular structures of those amphiphilic molecules, particularly the extended hydrophobic appendant (anchor) at the 3-hydroxyl group, and transfection efficiency, we synthesized several lithocholic and isolithocholic acid derivatives, and examined their transfection efficiency. We also compared the physico-chemical properties of cationic liposomes prepared from these derivatives. We found that isolithocholic acid derivatives exhibit higher transfection efficiency than the corresponding lithocholic acid derivatives. This result indicates that the orientation and extension of hydrophobic regions influence the gene transfection process. Isolithocholic acid derivatives showed a high ability to encapsulate DNA in a compact liposome–DNA complex and to protect it from enzymatic degradation. Isolithocholic acid derivatives also facilitated the release of DNA from the liposome–DNA complex, which is a crucial step for DNA entry into the nucleus. Our results show that the transfection efficiency is directly influenced by the ability of the liposome complex to release DNA, rather than by the DNA-encapsulating ability. Molecular modeling revealed that isolithocholic acid derivatives take relatively extended conformations, while the lithocholic acid derivatives take folded structures. Thus, the efficiency of release of DNA from cationic liposomes in the cytoplasm, which contributes to high transfection efficiency, appears to be dependent upon the molecular shape of the cationic amphiphiles.

Key words transfection; cationic amphiphile; DNA relese; lipofection; lithocholic acid

Establishment of an efficient and safe method for introducing exogenous nucleotides into mammalian cells is critical for both basic sciences and clinical applications, such as gene therapy.[1)] Among various methods for gene transfection, lipofection using cationic liposomes is considered to be a promising way to deliver a foreign gene to target cells.[2—4)] Although many kinds of cationic liposomes have been developed for lipofection,[5—10)] further studies are still required to achieve a transfection efficiency comparable with that of viral vectors. The results of a recent structural study indicated that a multilamellar or inverted hexagonal structure with alternating lipid bilayer and DNA monolayer might be favorable.[11—13)] Therefore, the structure of the lipid bilayers of cationic liposomes are probably conserved upon complexation with DNA. The width of the bilayer is estimated to be 36 Å.[11—13)] Recently, we have developed several cationic amphiphiles for nucleotide delivery.[14,15)] These are lithocholic acid-based molecules (see Chart 1) which bear *cis*-decalin structures, while the frequently used cholestane-based molecules (such as DC-Chol) have *trans*-decalin structures. The additional hydrophobic region at the 3-hydroxyl group of lithocholic acid derivatives may enforce hydrophobic interaction in the assembly, resulting in anchoring of the amphiphiles to the bilayer. We found that the hydrophobic appendant at the 3-position and the orientation and extension of the hydrophobic regions around the ether linkage both significantly influence the gene transfection. However, the mechanism of the dependence of the transfection efficiency on molecular structure was not elucidated. In liposome-mediated transfection, a liposome–DNA complex is taken into target cells by endocytosis.[16—20)] The internalized exogenous DNA is released by disruptive interaction between liposomal membrane and endosomal membrane.[21—25)] The released DNA in the cytoplasm is translocated into the nucleus, while DNA remaining in the endosomes is degraded in lysosomes. Therefore, the efficiency of cellular uptake of the liposome–DNA complex and the efficiency of release of DNA from both the endosomes and the liposomes are postulated to be major factors that determine the transfection efficiency, which can be estimated by means of luciferase assay. In the present study, we prepared cationic liposomes from lithocholic acid–polyamine conjugates (Chart 1), and investigated two chemico-physical characteristics that may affect the efficiency of the processes mentioned above. Firstly, we examined the encapsulation of DNA and compaction of the liposome–DNA complex, since the liposome–DNA interaction is thought to facilitate the uptake of liposome–DNA complexes by target cells and also to protect the plasmid DNA from enzymatic degradation. Secondly, we examined the ability of cationic liposomes to release DNA upon membrane fusion with anionic liposomes, which mimic the endosomal membrane.[21—25)] This release allows DNA to translocate to the nucleus, where it becomes accessible to the transcription apparatus. We also examined the relationship between these properties. Transfection efficiency was positively correlated with the ability of liposomes to release DNA, which is apparently dependent upon the molecular shape of the cationic amphiphiles.

MATERIALS AND METHODS

Materials 3β-[*N*-(Dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phos-

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phatidylcholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) were purchased from Sigma. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol-amine (NBD-PE) was purchased from Molecular Probes. Plasmid DNA coding luciferase (pGL3) was purchased from Promega.

Synthesis of the Isolithocholic Acid–Polyamine Conjugates (1—3) and the Lithocholic Acid–Polyamine Conjugates (4—6). Synthesis of Isolithocholic Acid (Chart 2) (i) Lithocholic Acid Methyl Ester (**8**): A mixture of lithocholic acid (**7**) and concentrated (98%) sulfuric acid in MeOH was stirred at room temperature (rt) for 2.5 h. The whole was diluted with CH_2Cl_2, and washed with water and brine. The organic phase was dried over Na_2SO_4, and the solvent was evaporated. The residue was column-chromatographed (silica gel; *n*-hexane : AcOEt=4 : 1) to give a colorless solid (**8**).

(ii) Isolithocholic Acid *p*-Nitrobenzoate (**9**): To a solution of **8**, triphenylphosphine and *p*-nitrobenzoic acid in dry THF, a solution of diethyl azodicarboxylate in dry THF was added at rt under an argon atmosphere. The whole was stirred for 2 h. After evaporation of the solvent, the residue was extracted with CH_2Cl_2 and water, and the organic phase was washed with brine, then dried over Na_2SO_4. The residue obtained after evaporation of the solvent was column-chromatographed (silica gel; *n*-hexane : CH_2Cl_2=4 : 1 to 3 : 2 to 1 : 1) to give the *p*-nitrobenzoate (**9**).

(iii) Isolithocholic Acid (**10**): A suspension of **9** in a mixture of 40% aqueous KOH and methanol was heated at reflux at 100 °C for 14 h. The whole was diluted with water and acidified with aqueous HCl, and the precipitate was filtered off. The filtrate was extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated. The residue was flash-chromatographed (silica gel; *n*-hexane : AcOEt=9 : 1 to 3 : 1) to give a colorless solid, **10**.

Synthesis of Polyamine Derivatives (Chart 3) (i) Mono-*N*-Boc-amine (**12**): To a solution of 1,4-diaminobutane (**11**) in methylene chloride, a solution of di-*t*-butyl dicarboxylate in methylene chloride was added dropwise over 20 min at rt. The whole was stirred at rt for 6.5 h, then diluted with methylene chloride, and washed with water and brine. The organic phase was dried over Na_2SO_4. Evaporation of the solvent gave **12** as a colorless oil.

(ii) Mono-*N*-Boc-amine-nitrile (**13**): To a solution of **12** in methanol, acrylonitrile was added at 5 °C. The whole was allowed to warm to rt, and stirred for 12 h. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; $CHCl_3$: MeOH=19 : 1) to give a pale yellow oil (**13**).

(iii) Bis-*N*-Boc-nitrile (**14**): To a solution of **13** in methylene chloride, a solution of di-*t*-butyl dicarboxylate in methylene chloride was added at rt. The whole was stirred for 4.5 h. Water was added, and the whole was extracted with methylene chloride. The organic layer was washed with brine. The organic phase was dried over Na_2SO_4, and the solvent was evaporated. The residue was flash-chromatographed (silica gel; $CHCl_3$: AcOEt=19 : 1) to give a pale yellow oil **14**.

(iv) Bis-*N*-Boc-spermidine (**15**): To a suspension of $LiAlH_4$ in dry diethyl ether (argon-bubbled), a solution of **14** in dry diethyl ether was added at 5 °C over 25 min. The whole was allowed to warm to rt, stirred for 3 h, and poured into water. The mixture was extracted with diethyl ether, and the organic phase was dried over Na_2SO_4. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; $CHCl_3$: $iPrNH_2$=24 : 1) to give a pale yellow oil (**15**).

(v) Isolithocholic Acid Methyl Ester (**16**) (Chart 4): A mixture of isolithocholic acid (**10**) (3.38 g (8.65 mmol)) and 1.5 ml of concentrated H_2SO_4 in 40 ml of methanol was stirred at rt for 3 h. The whole was diluted with 150 ml of methylene chloride. The mixture was washed with water (100 ml×2) and brine, and the organic phase was dried over Na_2SO_4. The residue, obtained after evaporation of the solvent, was column-chromatographed (silica gel; *n*-hexane : AcOEt=4 : 1) to give a colorless solid (**16**).

(vi) *O*-Allyl Isolithocholic Acid Methyl Ester (**17**): To a solution of **16** in DMF, *N*,*N*-diisopropylethylamine was added, and the whole was heated at reflux at 160 °C for 2 h. Then, allyl bromide was added, and the whole was heated at reflux for 16 h. After removal of the DMF by distillation, the resultant residue was diluted with water, and the whole was extracted with methylene chloride. The organic phase was washed with brine, and dried over Na_2SO_4. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; *n*-hexane : AcOEt=19 : 1 to 4 : 1) to give a pale yellow solid **17**.

(vii) *O*-Isoheptenyl Isolithocholic Acid Methyl Ester (**18a**): To a solution of benzylidene-bis(tricyclohexylphosphine)dichlororuthenium (the Grubbs catalyst) (55.9 mg (8.5 mol%)) in 3 ml of dry chloroform, a solution of **16** (344.7 mg (0.80 mmol)) and 4-methyl-1-pentene (485.7 mg (7.08 eq)) in 3 ml of dry chloroform was added at rt under an argon atmosphere. The whole was stirred at rt for 37 h. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; *n*-hexane : CH_2Cl_2=1 : 1) to give a colorless solid (**17**).

(viii) *O*-Isoheptenyl Isolithocholic Acid (**19a**): To a solution of the ester **18a** in THF, a solution of lithium hydroxide monohydrate in water was added at rt. The whole was stirred for 28 h, diluted with water and acidified with 2 N aqueous HCl. This mixture was extracted with ethyl acetate. The organic phase was washed with brine, and dried over Na_2SO_4. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; $CHCl_3$: AcOEt=9 : 1) to give a colorless wax **19a**.

(ix) *O*-Isoheptenyl Isolithocholic Acid-*N*-Boc-spermidine Conjugate (**20a**): To a solution of the carboxylic acid **19a**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysuccinimide in methylene chloride, a solution of the protected polyamine **15** in methylene chloride was added at rt. The whole was stirred for 40 h, and diluted with methylene chloride. The organic phase was washed with aqueous saturated $NaHCO_3$, water and brine, and dried over Na_2SO_4. The residue, obtained after evaporation of the solvent, was flash-chromatographed (silica gel; *n*-hexane : AcOEt=3 : 2) to give a colorless oil (**20a**).

(x) *O*-Isoheptenyl Isolithocholic Acid–Spermidine Conjugate (**1**): To a solution of **20a** in methylene chloride, trifluoroacetic acid was added at 5 °C. The mixture was stirred at 5 °C for 1.5 h, then the TFA was distilled off. The residue was flash-chromatographed (silica gel; $CHCl_3$: MeOH :

iPrNH$_2$=18 : 1 : 1) to give a yellow oil (**1**).

Other polyamine derivatives (**2**—**6**) were synthesized similarly as shown in Chart 3.

Liposome Preparation DOPE (dioleoyl L-α-phosphatidylethanolamine) (Sigma) in chloroform was mixed with a solution of a steroid polyamine conjugate in chloroform to prepare a mixture of DOPE/synthetic conjugate in a molar ratio of 3 : 2.[10,14] The mixture was evaporated under reduced pressure to give a thin film of lipids. Phosphate-buffered saline (PBS) was added to the lipid film, and the sample was allowed to stand for 1 h. Hydrated samples were sonicated for 2—3 min in a bath-type sonicator (model 5210J, Branson), followed by sonication with a probe-type sonicator (Sonifier 250, Branson) for 10 min to give small unilamellar vesicles (SUVs). Plasmid DNA (pGL3) was mixed with cationic liposomes in a charge ratio (+/−) of 1.4.

Cell Culture and Transfection NIH3T3 and COS-7 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Bio-Whittaker). Transfection was carried out following the procedure described in our previous paper.[14,15] In brief, plasmid pGL3 DNA (5.4 μg) in TE buffer was complexed with the above liposomes in SFM101 (Nissui) at room temperature by pipetting well, and then incubated at 37 °C for 15 min to allow formation of the DNA–liposome complex. This DNA–liposome complex was incubated with the cells (1×10^6 cells/60 mm dish) in the SFM101 medium for 4 h at 37 °C. The cells were washed and cultured in growth medium (DMEM) for another 40 h at 37 °C. Luciferase assay was carried out after cell lysis.

Luciferase Assay Transfection efficiency was evaluated by means of luciferase assay using a Picagene luciferase assay kit (Toyo Ink) as previously described.[14,24] The cells were washed three times with PBS and lysed in a cell lysis buffer for 15 min at room temperature. The cell lysate was centrifuged at 12000 rpm at 4 °C for 3 min and the supernatant was subjected to luciferase assay. Light emission was measured with a luminometer (TD-20/20, Turner Designs) and normalized to the amount of protein of each sample, determined by means of BCA assay.

Ethidium Bromide Intercalation Assay The interaction between liposomes and DNA was examined by means of ethidium bromide (EtBr) intercalation assay.[21,26] Fluorescence of EtBr at 595 nm (excited at 520 nm) was monitored with a spectrofluorometer (RF-5300PC; Shimadzu) linked with a personal computer. Values were expressed as the fraction of the maximum intensity obtained when EtBr was added to free plasmid DNA without liposomes.

FRET Assay To examine the efficiency of the release process, the dissociation of DNA from cationic liposomes caused by the addition of anionic membrane, which mimics the endosomal membrane, was monitored by means of fluorescent resonance energy transfer (FRET) assay using NBD-labeled liposomes and rhodamine-labeled DNA.[10,22] Fluorescence-labeled liposomes were prepared by adding NBD-PE (0.5% mol) to the liposomes described above, and c-myc antisense oligonucleotides (phosphorothioate; CACGTTGAGGGGCAT) were labeled with rhodamine (Nihon Gene Research Laboratories Inc., Sendai, Japan). After formation of liposome–DNA complexes with a charge ratio (+/−) of 1.4, dissociation of DNA from liposomes was induced by the addition of anionic liposomes in citrate buffer (pH 5.0). Anionic liposomes were prepared from DOPC, DOPE and DOPG (DOPC : DOPE : DOPG=1 : 2 : 1 (molar ratio)). The fluorescence intensity of NBD (535 nm) excited at 465 nm was monitored with a spectrofluorometer (RF-5300PC; Shimadzu).

Molecular Modeling The energy-minimum structures under three environmental conditions (gas-phase, in $CHCl_3$, and in water) were obtained by conformation searches with pure LowMode methods[27] and subsequent multiple-minimization. All the calculations were done with the OPLS-AA force field.[28] The calculations were carried out with Macromodel, version 8.1, Schrödinger Inc.

RESULTS AND DISCUSSION

Synthesis We synthesized three different polyamine–lithocholic acid conjugates and their isolithocholic acid counterparts (Chart 1). Isolithocholic acid was prepared by Mitsunobu inversion reaction (*p*-nitrobenzoic acid/Ph_3P/DEAD) of the 3-hydroxyl group of lithocholic acid (in the form of the methyl ester) (Chart 2). The synthesis of *O*-allyl isolithocholic (**17**) and lithocholic acid (**22**) derivatives was carried out as reported previously.[15] Synthesis of the polyamine conjugates **1**—**6** was accomplished by olefin metathesis of the *O*-allyl derivatives and a terminal olefin in the presence of the ruthenium-alkylidene catalyst developed by Grubbs.[29—31] Preparation of polyamines (Chart 3) and amide coupling (Chart 4) were also described previously.[15] All new compounds gave satisfactory ^{1}H-NMR and high-resolution mass spectra (HR-MS, EI$^+$) or FAB mass spectra (FAB-MS).

Transfection Efficiency of Polyamine–Lithocholic Acid Conjugates The transfection efficiency of the three differ-

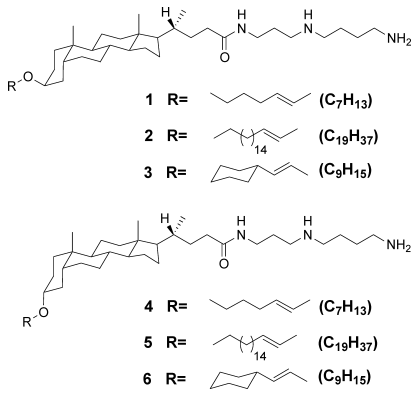
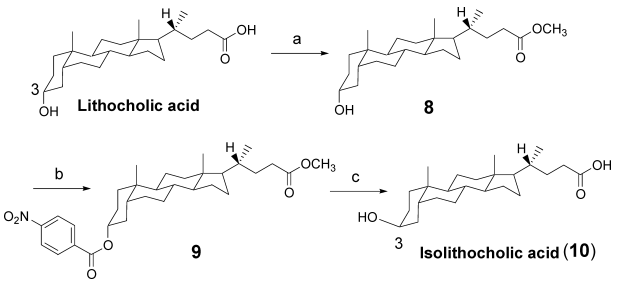


Chart 1. Structures of Cationic Amphiphiles Used in This Study



a) conc-H_2SO_4, MeOH, rt, 2.5 h, yield 98%, b) *p*-nitrobenzoic acid, PPh_3, DEAD, THF, rt, 2 h, yield 97%, c) 40% aq. KOH, MeOH, reflux, 5 h, 90%.

Chart 2

ent polyamine–lithocholic acid conjugates and their isolithocholic acid counterparts (Chart 1) was estimated by means of luciferase assay (Fig. 1). Cationic liposomes were prepared by mixing of the polyamine–lithocholic acid conjugate and DOPE (dioleoyl L-α-phospatidylethanolamine), followed by mixing of a plasmid DNA, pGL3, which encodes luciferase. NIH3T3 cells were transfected with the liposome–DNA complex and the transfection efficiency was evaluated by means of luciferase assay. As shown in Fig. 1, the transfection efficiencies of the isolithocholic acid conjugates (**1**—**3**) were higher than those of the corresponding lithocholic acid conjugates (**4**—**6**). The transfection efficiencies of the isolithocholic acid conjugates were higher than that of the commercially available cationic cholesterol derivative (DC-Chol). The efficiency of the derivative **3** was about 6 times higher than that of DC-Chol. On the other hand, the lithocholic acid conjugates had lower efficiencies than DC-Chol. Similar results were obtained in COS-7 cells (data not shown). Thus, the stereochemistry of the hydrophobic appendant at the 3-position, *i.e.*, the orientation and extension of the hydrophobic regions around the ether linkage at the 3-position, is crucial for the transfection efficiency. The present results are consistent with previous observations.[12)]

Efficiency of Encapsulation of DNA As described above, the isolitocholic acid conjugates had higher transfection efficiency than the lithocholic acid conjugates, so we further investigated the isolithocholic acid conjugates. Electrostatic interaction between the cationic liposomes and the anionic DNA results in encapsulation and compaction of the liposome–DNA complexes. This interaction facilitates the uptake of the liposome–DNA complexes by the target cells and the complexation protects the plasmid DNA from enzymatic degradation. Thus, we evaluated the strength of the interaction of the cationic liposomes of the isolithocholic acid conjugates with DNA by using the ethidium bromide (EtBr) intercalation assay. EtBr is a fluorescent label that is widely used to detect DNA. When it intercalates between the base pairs of the DNA double helix, its fluorescence is greatly enhanced. Aqueous solutions of DNA stained with EtBr consequently have a high initial level of fluorescence. Tight interaction of liposomes with DNA results in encapsulation of the DNA and prevents intercalation of EtBr with the DNA, resulting in a decrease in the fluorescence intensity of EtBr. The efficiency of encapsulation estimated with the EtBr intercalation assay is shown in Fig. 2. All three isolithocholic acid conjugates showed higher efficiency than DC-Chol. However, the efficiencies of these isolithocholic acid conjugates (**1**—**3**) were roughly constant at about 50%, and a higher magnitude of encapsulation was observed with the lithocholic acid derivatives (**4**—**6**), which nevertheless showed much less potent gene transfection activities than the isolithocholic acid conjugates. Thus, the efficiency of encapsulation of DNA by cationic amphiphiles does not directly parallel the transfection efficiency in this system.

Efficiency of DNA Release from Cationic Liposomes

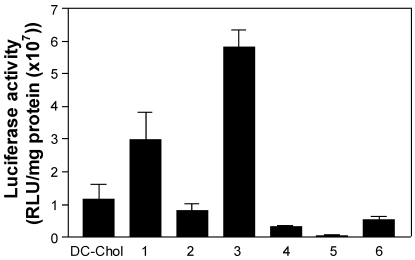


Fig. 1. Transfection Efficiency of Lithocholic Acid-Based Molecules

Transfection activities of cationic liposomes prepared with DC-Chol or lithocholic acid derivatives were determined by means of luciferase assay. Each value represents the mean±S.E. (n=3).

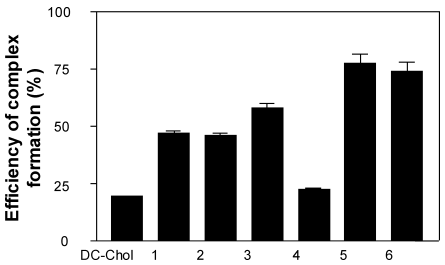
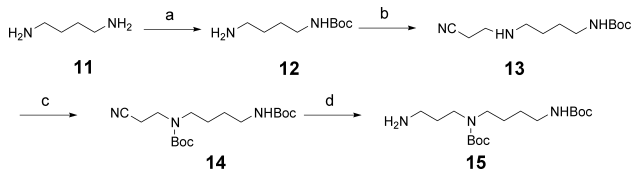


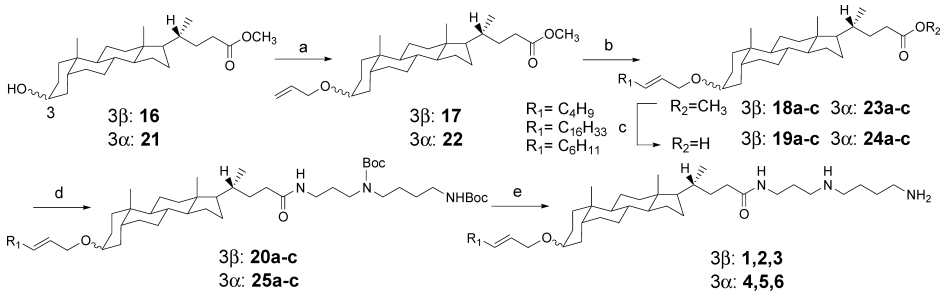
Fig. 2. DNA Encapsulation Ability of Cationic Liposomes

The efficiency of formation of liposome–DNA complex was determined by means of EtBr assay. Each value represents the mean±S.E. (n=3).



a) Boc_2O, CH_2Cl_2, rt, 6.5 h, yield 76%, b) acrylonitrile, MeOH, 0 °C—rt, 12 h, yield 90%, c) Boc_2O, CH_2Cl_2, rt, 4.5 h, yield 92%, d) $LiAlH_4$, Et_2O, 0 °C—rt, 3 h, yield 65%.

Chart 3



a) $(i\text{-}C_3H_5)_2EtN$, DMF, reflux, 2 h, then allyl bromide, reflux, 14 h, yield 83%, b) $Cy_2(PPh_3)_2Ru{=}CHPh$, $H_2C{=}CHR_1$, CH_2Cl_2, rt, (**18a**, 37 h, 51%; **18b**, 26 h, 39%; **18c**, 22 h, 54%; **23a**, 17 h, 62%; **23b**, 36 h, 39%; **23c**, 25 h, 48%), c) 40% aq. KOH, MeOH, rt, or LiOH–H_2O, THF, rt (**19a**, 22 h, 61%; **19b**, 22 h, 74%; **19c**, 32 h, 75%; **24a**, 24 h, 50%; **24b**, 19 h, 78%; **24c**, 22 h, 97%), d) **15**, *N*-hydroxysuccinimide, EDC, CH_2Cl_2, rt, (**20a**, 22 h, 61%; **20b**, 22 h, 74%; **20c**, 32 h, 75%; **25a**, 24 h, 50%; **25b**, 19 h, 78%; **25c**, 22 h, 97%), e) TFA, CH_2Cl_2, 0 °C (**1**, 1.5 h, 77%; **2**, 1 h, 85%; **3**, 1 h, 86%; **4**, 1 h, 70%; **5**, 1.5 h, 35%; **6**, 1 h, 78%).

Chart 4

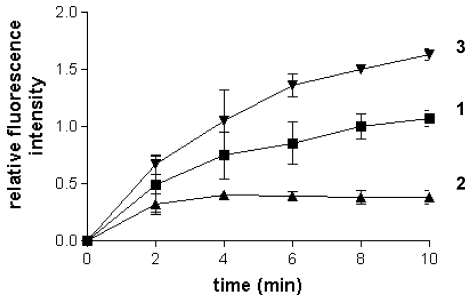


Fig. 3. Efficiency of DNA Release from Cationic Liposomes

Time course of dissociation reaction of cationic liposomes and DNA. Relative fluorescence intensity of NBD was plotted against time (mean±S.E., $n=3$). Anionic liposomes were added at 0 min. Values of relative fluorescence intensity (RFI) were calculated as follows; RFI(t)=(F(t)−F(0))/F(0), where F(t) is the fluorescence intensity of NBD at the indicated time and F(0) is the fluorescence intensity of NBD before addition of anionic liposomes.

Next, we examined the efficiency of release of exogenous DNA from the endosomes and the cationic liposomes. Liposome–DNA complexes are taken up into the cells by endocytosis, and transported to lysosomes, where the complexes are degraded.[19] Therefore, for high transfection activity, DNA must be released from the endosomes and liposomes before the lysosomal degradation, so that it can be transported intact to the nucleus. To evaluate the influence of the molecular structure of the 3-axial appendant (**1**, **2**, **3**) on the efficiency of this release process, the dissociation of DNA from the cationic liposomes caused by the addition of anionic liposomes containing phosphatidylglycerol (PG), which mimics the endosomal membrane, was monitored by means of fluorescent resonance energy transfer (FRET) assay using rhodamine-labeled DNA and NBD-labeled liposomes. The endosomal membrane contains anionic lipids, since the endosome is formed by pinching off of invaginated plasma membrane that contains anionic phospholipids. Anionic lipid is reported to be critical for efficient fusion between cationic and anionic liposomes, whose lipid composition is similar to that of endosomes.[21,23] Anionic lipid mixing neutralizes the positive charge of the cationic liposome–DNA complex and displaces DNA from the complex. Lipid mixing between cationic and anionic liposomes also causes membrane fusion, and this allows the displaced DNA to be released from endosome.[21,23] FRET assays are widely used to monitor dynamic changes in the distance between two fluorescent probes. In the present system, the fluorescence of the NBD fluorophore is quenched by the neighboring rhodamine fluorophore through the FRET mechanism in the DNA–cationic liposome complex. When the rhodamine-labeled DNA is released from the cationic liposomes following interaction with the anionic liposomes containing DOPE, DOPC and DOPG, the FRET from excited NBD to rhodamine was diminished, and the fluorescence intensity of the NBD chromophore was increased. Figure 3 shows the time course of the fluorescence intensity of NBD at 535 nm, where the increase of the fluorescence reflects dissociation of DNA from the liposomes. The efficiency of the DNA release was highest when cationic liposomes containing the derivative **3** were used, while the lowest release was observed when the derivative **2** was used. These results are consistent with the observed transfection efficiency. Several lines of evidence suggest that lipid phase formation in DNA–liposome complex (lipoplex) is crucial for endosomal escape of DNA.[32,33] Using small-angle X-ray spectroscopy (SAXS), important roles of non-lamellar structure of lipoplex, such as micellar cubic and hexagonal, in disruption of endosomal membrane and DNA release from lipoplex.[34,35] High transfection efficiencies of the isolithocholic acid conjugates might relate to their ability to induce non-lamellar structure in lipoplex.

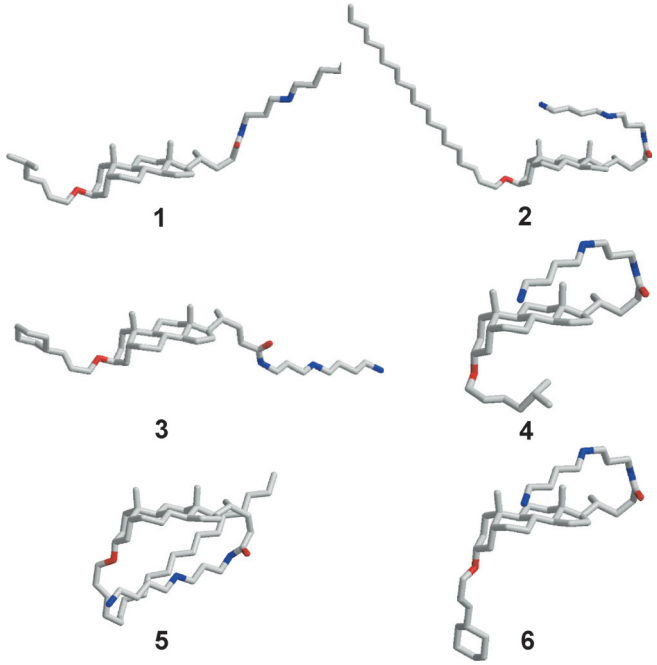


Fig. 4. Energy-Minimum Molecular Structures of Isolithocholic Acid (**1**—**3**) and Lithocholic Acid (**4**—**6**) Derivatives in Water, Obtained by Computational Modeling

Molecular Structure of Lithocholic Acid Derivative–Polyamine Conjugates To understand the relationship between the molecular structure and transfection efficiency, we carried out molecular modeling of the compounds used in this study. Energy-minimized structures of these compounds (**1**—**6**) were obtained for three different environmental conditions (gas-phase, in $CHCl_3$ and in water), and the energy-minimum structures in water are shown in Fig. 4. There was little difference among the preferred structures in the different environments. The polyamine–lithocholic acid conjugates took folded conformations, while the polyamine–isolithocholic conjugates took relatively extended conformations. The former structural trend was also found in the superposition of 100 frames of *N*-protonated structures of other related polyamine–lithocholic acid conjugates previously reported.[14] Among the polyamine–isolithocholic conjugates, derivative **3** took the most stretched-out conformation. Interestingly, the derivative **3** exhibited the highest efficiency of both transfection and DNA release.

CONCLUSIONS

We investigated the mechanism causing the different transfection efficiencies of cationic liposomes prepared from lithocholic acid– and isolithocholic acid–polyamine conjugates. As we reported previously,[14] the orientation and extension of the hydrophobic regions around the ether linkage at the 3-position of the lithocholic acid-based molecules signifi-

cantly affect the gene transfection efficiency. To elucidate the factors involved, we examined two crucial chemico-physical characteristics of the cationic liposomes. One is the robustness of the liposome–DNA complex. The other is the ease of release of DNA from the liposome–DNA complex and endosomes. Compaction of the liposome–DNA complex is required for efficient uptake into the target cells *via* endocytosis, and also for protection of the exogenous DNA from enzymatic degradation. Once the DNA is internalized into the target cells by endocytosis, the exogenous DNA must be released from the complex and the resultant endosomes for transport into the nucleus. This release process is thought to be induced by disruptive membrane interaction between the endosome membrane and the liposome membrane of the complex.[21—25] In the present study, we found that isolithocholic acid derivatives enhance transfection efficiency by facilitating the release of DNA from the liposome–DNA complex and endosomes.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research (Category S, No. 17109001) to TO from the Ministry of Education, Science, Sports, Culture and Technology. A part of the calculations was carried out at the Computer Center of the Institute for Molecular Science. We thank this computational facility for generous allotments of computer time.

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