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Synthesis and Optimization of Cholesterol-Based Diquaternary Ammonium Gemini Surfactant (Chol-GS) as a New Gene Delivery Vector

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Amongst a number of potential nonviral vectors, cationic liposomes have been actively researched, with both gemini surfactants and bola amphiphiles reported as being in possession of good structures in terms of cell viability and *in vitro* transfection. In this study, a cholesterol-based diquaternary ammonium gemini surfactant (Chol-GS) was synthesized and assessed as a novel nonviral gene vector. Chol-GS was synthesized from cholesterol by way of four reaction steps. The optimal efficiency was found to be at a weight ratio of 1:4 of lipid:DOPE (1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine), and at a ratio of between 10:1~15:1 of liposome:DNA. The transfection efficiency was compared with commercial liposomes and with Lipofectamine, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide (DMRIE-C), and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP). The results indicate that the efficiency of Chol-GS is greater than that of all the tested commercial liposomes in COS7 and Huh7 cells, and higher than DOTAP and Lipofectamine in A549 cells. Confirmation of these findings was observed through the use of green fluorescent protein expression. Chol-GS exhibited a moderate level of cytotoxicity, at optimum concentrations for efficient transfection, indicating cell viability. Hence, the newly synthesized Chol-GS liposome has the potential of being an excellent nonviral vector for gene delivery.

Keywords: Cholesterol, cationic liposome, gene therapy, gemini surfactant, transfection

Gene therapy is one of the newer methods of treating genetic diseases and operates through the delivery of the desired genetic drugs in ways that are unlike those of conventional medication [7]. To further develop treatment, there is a need for ongoing exploration of genetic drugs, such as plasmid DNA, antisense oligonucleotides, and siRNA, alongside vectors for the transfer of these genetic materials into cells. Vectors for a successful delivery of genetic materials can be classified into two main types: viral or nonviral. Viral vectors are known to be the best means for the delivery of genetic materials [14], but bring with them a number of issues, such as antigenicity, toxicity, the limited size of cargo, and the difficulty of large-scale virus production [9, 15, 16, 21]. Although a nonviral cationic liposome has a relatively low efficiency in gene delivery, it is more feasible to develop this gene delivery vector owing to the advantages of ease of synthesis, a low immune response, and safety factors [12, 20].

Since Felgner's group first introduced a gene delivery vector based on a cationic liposome, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [6], the structure of cationic liposome has been further modified for the creation of more effective vectors. Generally, the physicochemical property of cationic lipids is amphipathic, owing to their chemical structure consisting of three regions; the cationic head group, the linker, and the hydrophobic domain. Cationic head groups and polar linkers vary in form quite substantially, whereas hydrophobic regions are mostly composed of long chain fatty acids, as seen in DOTMA, DOTAP, DMRIE, and DOSPA, or of cholesterol derivatives, as observed in DC-Chol, CDAN, and BGTC [17]. DNA–lipid complexes (lipoplex) are formed by the electrostatic interactions between the cationic head of a lipid and the negatively charged plasmid DNA, and typically enter cells by way of adsorptive endocytosis [23].

Recently, reports have been authored regarding bola amphiphiles and gemini surfactants, where two lipid molecules containing hydrophobic domain–linker–cationic heads are joined to each other *via* a linker (Fig. 1). A number of gemini-formed cationic lipids have been noted as being efficient as gene delivery vectors [1–3, 11, 13].

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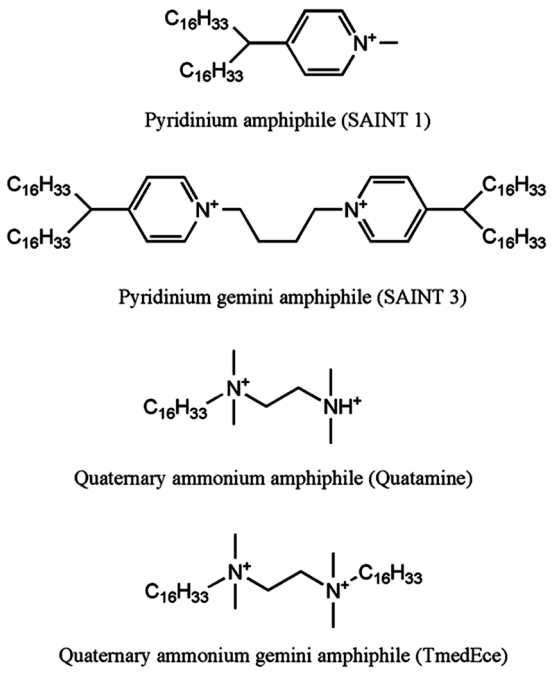


Fig. 1. Examples of gemini surfactants and monomeric counterparts.

However, there remain very few systematic studies of the effects of the molecular architecture on the transfection efficiency of gemini surfactants. In a reported series on pyridinium surfactants, pyridinium gemini amphiphile (SAINT 3) was found not to be superior to monomeric pyridinium amphiphile (SAINT 1), the latter which has just one headgroup (Fig. 1, upper two structures) [19]. However, TmedEce gemini lipid, with a diquaternary ammonium head, was found to be less toxic than the equivalent amphiphile, which was missing one hydrophobic tail (Quatamine) (Fig. 1, lower two structures) [18], and bola amphiphile, with a hydrophobic domain consisting of a homodimeric litocholic acid, exhibited a better transfection efficiency than its monomeric counterparts [22].

The type and mole ratios of helper lipids such as DOPE and cholesterol are one of the factors influencing transfection efficiency. The appropriate addition of neutral DOPE to the liposome formulation significantly enhances the transfection of many cationic lipids in various cell types [5, 10]. In addition, it may contribute to the facilitation of the intracellular delivery of macromolecules, such as plasmid DNA, owing to its ability to destabilize endosomal, or plasma, membranes [4, 8, 10].

In this study, we have newly designed and synthesized a cholesterol-based diquaternary ammonium gemini surfactant (Chol-GS), having an ether-linked propyl spacer. The transfection efficiency of the Chol-GS liposome was optimized by the addition of the neutral lipid DOPE in liposome formulation, and was found to be more efficient than commercial liposomes for *in vitro* gene delivery.

Materials and Methods

General Procedures and Materials

Fast atom bombardment mass spectrometry (FAB−MS) data were acquired with a high-resolution mass spectrometer (JEOL JMS-700, Japan). ^{1}H-NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer. All reagents and chemicals used in these synthetic processes were purchased from the Aldrich Co. (USA). Unless otherwise stated, all of the solvents were purchased from local commercial suppliers and were used without further purification. The progress of reactions was monitored by thin-layer chromatography (TLC) on silica gel plates obtained from Merck & Co. (1.05554). Column chromatography was performed with silica gel (70−230 mesh; Merck & Co., USA). Lipofectamine and DMRIE-C were purchased from the Invitrogen Corp. (USA). DOTAP was obtained from Roche Molecular (Germany). DOPE was acquired from Fluka (Switzerland). Fetal bovine serum (FBS) was purchased from GIBCO (Invitrogen Corp., USA). COS7 (African green monkey kidney cell), Huh7 (human hepatocarcinoma cell), and A549 (human lung adenocarcinoma epithelial cell) cell lines were obtained from the Korean Cell Line Bank. Cells were grown at 37°C in RPMI 1640 (Welgene Inc., Korea) for A549, or DMEM (Welgene Inc., Korea) for COS7 and Huh7, with 10% FBS in a humidified atmosphere containing 5% CO_2 / 95% air.

Synthesis of Cholest-5-en-3β-tosylate 2

Compound **2** was synthesized by modifying the method of Bajaj *et al.* [1]. To an ice-cooled solution of cholesterol **1** (5.3 g, 13.0 mmol) in pyridine (6.5 ml) and chloroform (6.5 ml), *p*-toluenesulfonyl chloride (3.7 g, 20 mmol) was added and stirred for 12 h under argon gas at room temperature. After the cholesterol spot had completely disappeared in the reaction mixture using TLC, chloroform was slowly added until the solid portion had disappeared. Cholest-5-en-3β-tosylate **2** was extracted by dropping enough methanol in the reaction mixture and then filtered twice with filter paper. Yield: white solid, 7.0 g, 13.0 mmol, 100% (TLC developer; Hex/EtOAc=15:1+a droplet of pyridine, Rf=0.2~0.3); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 0.65 (s, 3H; H-18'), 0.85−2.01 (m, 38H, cholesterol moiety), 2.24−2.47 (m, 2H; H-24'), 2.45 (s, 3H; tosyl-C$\underline{H}_3$), 4.32 (m, 1H; H-3'), 5.30 (d, 1H, *J*=5.3 Hz; H-6'), 7.32−7.34 (d, 2H, *J*=8.6 Hz; tosyl moiety), 7.79−7.81 (d, 2H, *J*=8.3 Hz; tosyl moiety).

Synthesis of Cholest-5-en-3β-oxypropane-3-ol 3

Compound **3** was synthesized by modifying the method of Bajaj *et al*. [1]. Cholest-5-en-3β-tosylate **2** (1.6 g, 3.0 mmol) was dissolved in anhydrous dioxane (5 ml), and then 1,3-propanediol (1.6 g, 21.0 mmol) was added to the mixture. This mixture was refluxed under argon gas for 7 h. The solution was cooled, and the solvent was removed on a rotary evaporator. The brown residue was dissolved in chloroform and the organic solution was sequentially washed with saturated water containing $NaHCO_3$, water, and brine. The organic phase was dried over anhydrous $MgSO_4$ and was followed by filtration. Chloroform was removed on a rotary evaporator. Cholest-5-en-3β-oxypropane-3-ol **3** was purified by silica gel column chromatography using a hexane/ethylacetate (5:1) eluent. Yield: 1.1 g, 2.4 mmol, 81.2% (TLC developer; Hex/EtOAc=3:1, Rf=0.4); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 0.68 (s, 3H; H-18'), 0.85−0.88 (d, 6H, *J*=6.6 Hz; H-26', H-27'), 0.91 (d, 3H, *J*=6.5 Hz; H-21'), 0.92−2.05 (m, 29H, cholesterol moiety), 1.83 (m, 2H; $HOCH_2C\underline{H}_2CH_2O$-),

2.14–2.40 (m, 2H; H-24'), 3.17 (m, 1H; H-3'), 3.68 (td, 2H, *J*=5.5 Hz, *J*=5.9 Hz; $HOCH_2CH_2C\underline{H}_2O$-), 3.78 (t, 2H, *J*=5.4 Hz; $HOC\underline{H}_2CH_2CH_2O$-), 5.35 (d, 1H, *J*=5.2 Hz; H-6').

Synthesis of Cholest-5-en-3β-oxypropane Bromide 4

Carbon tetrabromide (1.9 g, 5.7 mmol) and triphenylphosphine (2.2 g, 8.5 mmol) were added to a solution of cholest-5-en-3β-oxypropane-3-ol **3** (2.5 g, 5.7 mmol) in methylene chloride (20 ml). The reaction mixture was stirred at room temperature for 1 h. The organic solvent was evaporated under vacuum. Cholest-5-en-3β-oxypropane bromide **4** was twice purified by silica gel column chromatography using a hexane/ethylacetate (1:1) and a hexane/ethylacetate (30:1) eluent. Yield: 2.7 g, 5.3 mmol, 93.0% (TLC developer; Hex/EtOAc=15:1, Rf=0.5); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 0.68 (s, 3H; H-18'), 0.85–0.88 (d, 6H, *J*=6.6 Hz, H-26', H-27'), 0.92 (d, 3H, *J*=6.5 Hz; H-21'), 0.92–2.05 (m, 29H, cholesterol moiety), 2.08 (m, 2H; $BrCH_2C\underline{H}_2CH_2O$-), 2.14–2.39 (m, 2H; H-24'), 3.15 (m, 1H; H-3'), 3.52 (t, 2H, *J*=6.5 Hz; $BrC\underline{H}_2CH_2CH_2O$-), 3.59 (td, 2H, *J*=5.7 Hz, *J*=6.1 Hz; $BrCH_2CH_2C\underline{H}_2O$-), 5.35 (d, 1H, *J*=5.3 Hz; H-6').

Synthesis of Cholesterol-Based Diquaternary Ammonium Gemini Surfactant (Chol-GS) 5

To a 10 ml screw-top pressure tube, cholest-5-en-3β-oxypropane bromide **4** (0.5 g, 1.0 mmol) and tetramethyl-1,3-propanediamine (52.0 mg, 0.4 mmol) were added in DMSO (3 ml), and then methylene chloride was added to this mixture until all reagents had dissolved. The reaction mixture was stirred at 80°C for 30 h. The mixture was then cooled and the chloroform removed on a rotary evaporator. Hexane was added to this mixture to furnish a crude solid and this was filtered. The residue was dispersed in ethylacetate by sonication and filtered repeatedly until the TLC indicated the complete disappearance of impurities. The last compound **5**, Chol-GS, was obtained under high vacuum for 24 h to entirely remove residual DMSO. Yield: 0.1 g, 0.1 mmol, 21.8% (TLC developer; $CHCl_3$/MeOH/NH_4OH=4:2:0.5, Rf=0.2~0.4); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 0.67 (s, 6H; H-18'), 0.85–0.87 (d, 12H, *J*=6.6 Hz; H-26', H-27'), 0.91 (d, 6H, *J*=6.4 Hz; H-21'), 0.92–2.05 (m, 58H, cholesterol moiety), 2.10–2.33 (m, 4H; H-24'), 2.12 (br, 4H; $-OCH_2C\underline{H}_2CH_2(CH_2)N$--21'), 0.92–2.05 (m, 58H, $_3)_2CH_2C\underline{H}_2CH_2(CH_3)_2N$-), 3.12 (m, 2H; H-3'), 3.40 (s, 12H; $-N(C\underline{H}_3)_2CH_2CH_2CH_2(C\underline{H}_3)_2N$-), 3.58 (br s, 8H; $-C\underline{H}_2N(CH_3)_2C\underline{H}_2CH_2C\underline{H}_2(CH_3)_2NC\underline{H}_2$-), 3.86 (br s, 4H; $-OC\underline{H}_2CH_2$ $CH_2(CH_2)N$- 3.865.35 (d, 2 H, *J*=5.0 Hz; H-6'). FAB-MS: 1063.6/1065.6 (M^++Br^-), 969.8 (M^+-methyl).

Preparation of Liposomes

For the preparation of liposomes, the Chol-GS and DOPE were mixed in chloroform/methanol [1:1 (v/v)] at an appropriate ratio, and the solvent was evaporated under vacuum. The last traces of organic solvent were removed by keeping these films under a vacuum overnight. The film of cationic lipid and DOPE was briefly vortexed for 1 min in Milli Q water (Biocel equipment; Millipore, USA) to give a final total lipid concentration of 1 mg/ml for *in vitro* transfection. The mixture was subsequently sonicated for 20 min to form the liposomes, and passed 10 times through a Mini-Extruder (Avanti Polar Lipids, USA) equipped with double-layered 200 nm polycarbonate membrane filters. The obtained liposomes were stored at 4°C prior to use.

Size Measurement and Zeta Potential Analysis of Chol-GS Liposome

The particle size distribution and zeta potential of a liposome's dispersion were determined at 25°C by the dynamic light scanning method on a ELS-Z (Otsuka Electronics Co., Japan). The concentration of liposome was 100 μg in 3 ml of Milli Q water. The particle size was measured twice in a set of 50 repetitions, and the zeta potential was measured three times. Data were analyzed using a software package (ELS-Z software) supplied by the manufacturer.

Preparation of Plasmids DNA

The pcDNA-Luc used for *in vitro* transfection was a plasmid of 5.149 kb containing the firefly luciferase reporter gene sequence. The plasmid coding for green fluorescent protein (pCMVTnT-GFP) was obtained from Welgene (Korea). DNA plasmids were amplified in the *Escherichia coli* XL1-Blue strain and purified by a maxi-kit (Qiagen Inc., USA) according to the manufacturer's instructions. DNA purity was determined by agarose gel electrophoresis and by measuring the optical density (OD). DNA having $OD_{260}/OD_{280}\geq1.8$ were used in this study.

***In Vitro* Transfection**

The pcDNA-Luc and pCMVTnT-GFP were used at a concentration of 0.3 μg/well unless specified otherwise. A liposome solution was separately prepared by diluting an appropriate amount of the initial liposomal stock solution with a transfection optimizing medium (TOM, WelGene Co., Korea) to reach a final volume of 50 μl. To this liposome solution, 50 μl of the DNA stock solution was added. This lipoplex solution was then incubated for 10 min at room temperature. The lipoplexes were tested for their ability to transfer DNA in COS7, A549, and Huh7 cells. Twenty-four hours prior to transfection, the cells were transferred to 48-well culture plates at a density of 40,000 cells/well for A549, and 30,000 cells/well for COS7 and Huh7. Thirty minutes before transfection, the medium was removed and the cells from each well were briefly washed with 100 μl of sterile phosphate-buffered saline (PBS); then 150 μl of TOM was added to each well. The wells then received 100 μl of lipoplex stock solution, and the plates were incubated for 4 h. An additional 250 μl of medium (20% FBS) was added to each well so as to achieve a final serum concentration of 10%, and the plates were incubated for a further 24 h. For comparisons with Chol-GS, commercial liposomes were also tested in order to reveal the best conditions within the range of the manufacturer's protocol, and used here in those optimal conditions.

Luciferase Assay and GFP Expression

Twenty-four hours after transfection, the medium was aspirated and the wells were washed twice with 200 μl of ice-cold PBS. To each well, 100 μl of 1× reporter lysis buffer (Promega Corp., USA) was added and the cells were lysed for 1 h in an ice tray. The cell lysates were completely collected into Eppendorf tubes and centrifuged (12,000 rpm, 4°C) for 5 min. The supernatant was transferred to Eppendorf tubes in ice and used for luciferase and protein assays. For the luciferase assay, 20 μl of cell lysate was transferred to a white opaque 96-well plate for measurement and assessed directly by means of a LMax II 384 luminometer (Molecular Devices Corp., USA) using a luciferase assay kit (Promega Corp., USA). The

protein content was quantified using a bicinchoninic acid (BCA) assay (PIERCE, USA). The BCA assay was prepared as specified by the manufacturer. Forty μl of cell lysate was mixed with 1 ml of BCA reagent in an acrylic cuvette, and the solution was incubated for 1 h at 37°C. The light absorption of the solution was then read at 562 nm by means of a DU-600 spectrophotometer (Beckman Coulter, USA), and the protein content was estimated by a comparison with bovine serum albumin standards. The luciferase efficiency was normalized by the protein content and expressed as relative luminescence units/μg of protein (RLU/μg protein). To observe GFP expression, 24 h after transfection, the medium was removed and the cells were rinsed twice with PBS. Fluorescence protein was observed on a Nikon ECLIPSE TE300 fluorescence microscope (Japan).

Cell Viability

According to the transfection protocol described above, 24 h after transfection, 20 μg of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] in 20 μl of PBS was added to each well and the plates were then incubated for 3 h. The medium was carefully removed and 200 μl of DMSO was added to each well. The reduced crystal in violet was completely dissolved in DMSO and then the absorbance of the solution was determined at 550 nm. The cells treated with only pcDNA-Luc solution without liposome were used as the OD control. Cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = \text{OD sample}/\text{OD control} \times 100$$

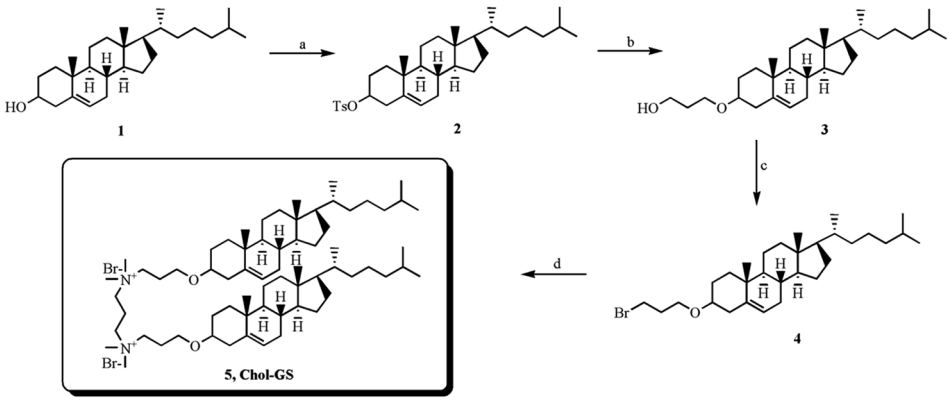
Results and Discussion

Synthesis

A cholesterol-based diquaternary ammonium gemini surfactant (Chol-GS) was synthesized in a four-step reaction from cholesterol. The main structure of Chol-GS is bilateral symmetric, where two of the hydrophobic domain–linker–cationic heads are symmetrically joined to tetramethyl-1,3-propanediamine. These structures are commonly called bola amphiphiles or gemini surfactants [18, 21]. Compounds **2** and **3** were synthesized by modifying the methods of Bajaj *et al*. [1]. To an ice-cooled solution of cholesterol **1** in pyridine:chloroform [1:1 (v/v), *p*-toluenesulfonyl chloride was added and stirred for 12 h at room temperature. Cholesterol tosylate **2** that had tosylated from cholesterol **1** in a practical yield was used in the next reaction without a purification process, because it was sufficiently purified by extraction as a result of TLC and ^{1}H-NMR analyses, and was unstable in a silica gel column. Cholesterol tosylate **2** and 1,3-propanediol 7 equiv. in anhydrous dioxane were refluxed for 7 h to afford cholest-5-en-3β-oxypropane-3-ol **3** in 81.2% yield. To join the cholesterol moiety to both ends of propanediamine, compound **3** was bromized by reacting with carbon tetrabromide 1 equiv. and triphenylphosphine 1.5 equiv. in methylene chloride in 93.0% yield. The last compound, Chol-GS **5**, was synthesized by a reaction with cholest-5-en-3β-oxypropane bromide **4** and tetramethyl-1,3-propanediamine as a mediator for gemini surfactant in DMSO/methylene chloride in a 21.7% yield. The structures of all the synthetic intermediates and Chol-GS shown in Scheme 1 were confirmed by ^{1}H-NMR. The final compound was characterized by FAB–MS to confirm the identity of the molecular ions.

Liposome Formulation of Chol-GS with Helper-Lipid DOPE in an Aqueous Solution

The optimum transfection efficiency was showed with the addition of an appropriate amount of helper lipids, such as DOPE and cholesterol, in many cationic liposomes [4, 5, 8, 10]. In this study, DOPE was selected as a helper lipid and the suitable weight ratio of Chol-GS:DOPE was determined in a range of 1:1~1:4 by a preliminary experiment. The transfection efficiency was greater with a higher weight ratio of DOPE in liposome/DNA (N/P), but the cytotoxicity levels were also higher. Thus, the higher weight ratio of DOPE (1:4) was not used. To form liposome



Scheme 1. Synthesis of novel cholesterol-based diquaternary ammonium gemini surfactant.
Reaction conditions: **(a)** *p*-toluenesulfonyl chloride/pyridine/$CHCl_3$, 12 h, r.t. (yield: 100%); **(b)** 1,3-Propanediol, anhydrous dioxane, 7 h, reflux (yield: 81%); **(c)** Carbon tetrabromide, triphenylphosphine/CH_2Cl_2, 1 h, r.t. (yield: 93%); **(d)** Tetramethyl-1,3-propanediamine/DMSO/CH_2Cl_2, 30 h, 80°C (yield: 22%).

formulations, lipid film made from an appropriate weight ratio of Chol-GS and DOPE was briefly dispersed in an aqueous solution, followed by sonication at 30°C for 20 min. Next, cationic liposome was passed 10 times through a 200 nm polycarbonate membrane in order to remove any oversized liposomes. All liposomes in the various weight ratios of Chol-GS:DOPE were seen to be optically transparent and no precipitation was observed within 6 months when stored at 4°C. The optimum weight ratio of Chol-GS:DOPE was 1:4, and the size and zeta potential at this formulation were 79.6±37.9 nm and 46.66 ±1.65 mV. The electrical repulsion of this liposome was large enough (>±30 mV) to retain a suitable size in an aqueous system. Therefore, electrostatic interaction is possible with negatively charged DNA because of the positive zeta potential.

Optimization of Transfection Efficiency (Chol-GS:DOPE and N/P Weight Ratio)

In order to find out the most effective liposome formulation, the optimum transfection efficiency was tested at different DOPE weight ratios. The transfection efficiencies of Chol-GS only, and Chol-GS:DOPE at a weight ratio of 1:1, were very low (data not shown). Thus, luciferase assays were performed at weight ratios of between 1:1~1:4 for Chol-GS:DOPE. Both conditions of N/P weight ratios 10 and 15 were tested owing to there being different transfection efficiencies dependent on the N/P weight ratio conditions. The optimum weight ratio of Chol-GS:DOPE was 1:3 in N/P weight ratio 10, and 1:4 in N/P weight ratio 15 (Fig. 2). To further assess their transfection properties, the weight ratio of Chol-GS:DOPE was set at 1:4.

To discover the optimum N/P weight ratio, the luciferase activity was measured by increasing the amount of liposome in 0.3 μg of a constant DNA quantity. Although the most effective N/P weight ratio was 15 (Fig. 3), both conditions

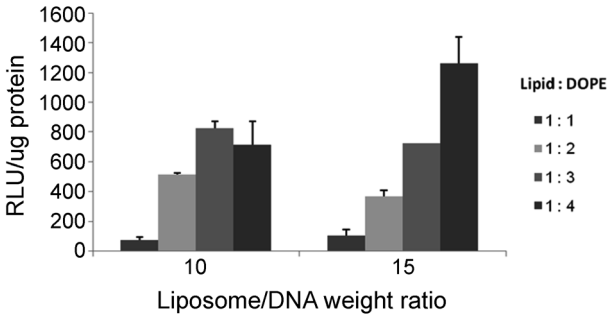


Fig. 2. Transfection efficiencies of the Chol-GS liposome with various weight ratios of DOPE.
The concentration of DNA=0.3 μg/well was kept constant, and the N/P weight ratio was 10 or 15. Data were measured with the luciferase assay at different Chol-GS:DOPE weight ratios in COS7 cells. Each bar value represents the mean ±SD of triplicate experiments performed on the same day.

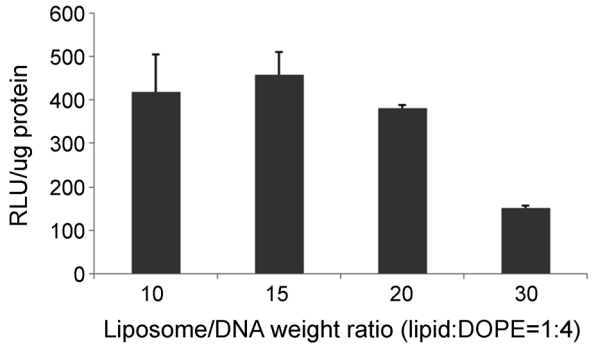


Fig. 3. Transfection efficiencies of the Chol-GS liposome at different N/P weight ratios.
In vitro transfection efficiencies of lipid-DNA complexes were measured with the luciferase assay at various N/P weight ratios in COS7 cells, while the concentration of DNA=0.3 μg/well was kept constant. Each bar value represents the mean ±SD of triplicate experiments performed on the same day.

of N/P weight ratios 10 and 15 were used in this study to compare the Chol-GS liposome to commercial liposomes, again because the transfection efficiencies of N/P weight ratios 10 and 15 were a little different, and the cell viability of N/P weight ratio 10 was higher than that of N/P weight ratio 15.

Comparison of Chol-GS and Commercial Liposomes in Luciferase Assay and GFP Expression

Luciferase gene expression assay of Chol-GS was performed in order to compare its activity with commercial liposomes in COS7, A549, and Huh7 cells. An N/P weight ratio of 7 for DOTAP, and 5 for Lipofectamine and DMRIE-C, were used in the best conditions (data not shown). In COS7 cells, the transfection efficiency of the Chol-GS liposome in both conditions of N/P weight ratios 10 and 15 was higher than Lipofectamine, which showed the highest transfection efficiency amongst the three commercial liposomes (Fig. 4a). Cell viability was also higher than with other liposomes (Table 1). In A549 cells, the efficiency of Chol-GS liposomes was superior to two liposomes, but not to DMRIE-C (Fig. 4b). In Huh7 cells,

Table 1. Cell viability of Chol-GS and commercial liposomes in various cells.

	Cancer cells		
Liposomes	COS7	A549	Huh7
Chol-GS N/P 10	96±3.7%	99±12.3%	112±2.9%
Chol-GS N/P 15	85±1.4%	88±1.3%	102±3.0%
DOTAP	81±2.0%	110±5.7%	122±1.4%
DMRIE-C	82±1.6%	100±1.2%	109±1.7%
LFA	77±1.4%	101±3.2%	105±2.1%

Cells were treated with the concentration of transfection protocol at optimized N/P weight ratios. Cell viability was measured by a MTT assay. The results represent the mean ±SD of triplicate experiments performed on the same day.

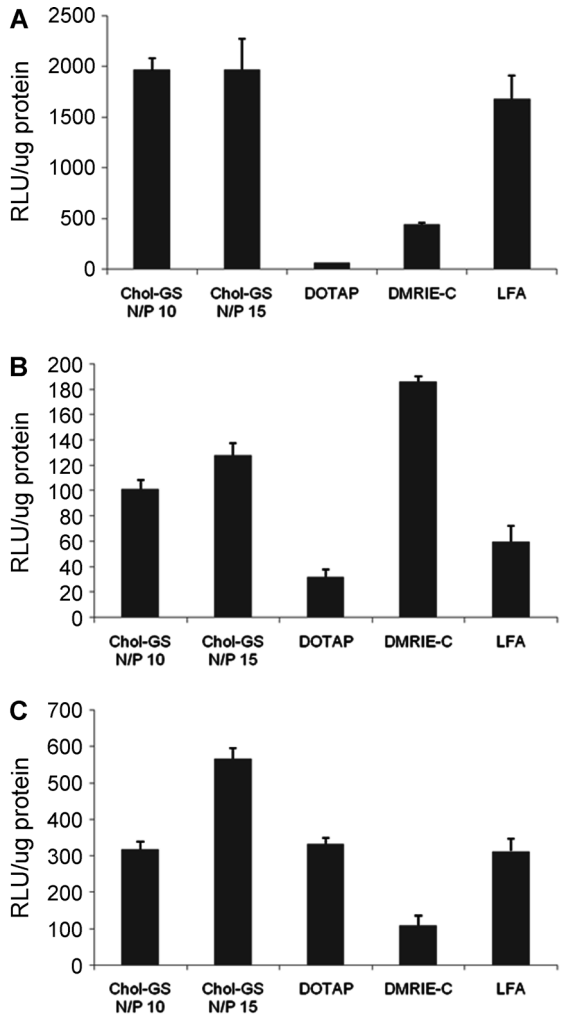


Fig. 4. Comparison of transfection efficiencies with commercial liposomes.
The transfection efficiency of the Chol-GS liposome was compared with the commercial liposomes DOTAP, DMRIE-C, and Lipofectamine (LFA) at optimal N/P weight ratios, in COS7 (**A**), A549 (**B**), and Huh7 (**C**) cells, using the luciferase assay. Each bar value represents the mean ±SD of triplicate experiments performed on the same day.

the N/P weight ratio 15 of Chol-GS liposome performed the best in this experiment, taking both cytotoxicity and activity into consideration (Fig. 4c, Table 1). The transfection efficiency of N/P weight ratio 15 was higher than that of N/P weight ratio 10 in Huh7 cells; however, there were no significant differences in the transfection efficiencies of both N/P weight ratios 10 and 15 in A549 and COS7 cells. This result supposed that the transfection efficiency of the Chol-GS liposome might be affected by cytotoxicity (Table 1). All things considered, the Chol-GS liposome could prove to be highly versatile and suitable for further various research. GFP expression was also performed to confirm the results in the two cells. A greater amount of GFP expression using Chol-GS liposome was observed than when utilizing Lipofectamine (Fig. 5).

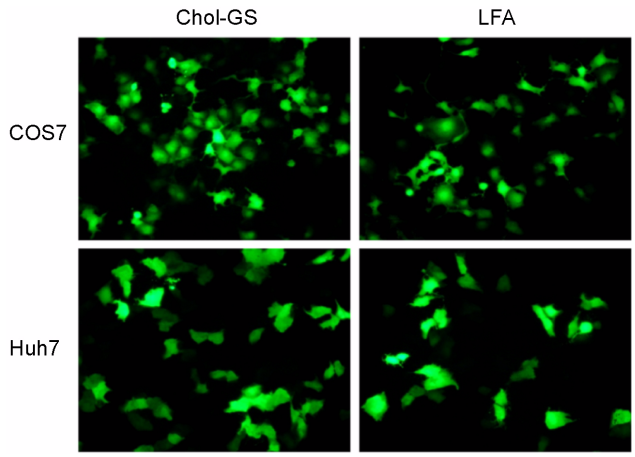


Fig. 5. Expression of GFP using Chol-GS liposome and LFA.
The pCMVTnT-GFP (0.3 μg) complex with Chol-GS liposome (N/P weight ratio=15) and LFA were added to COS7 and Huh7 cells, and green fluorescent protein was observed under a fluorescence microscope.

Cell Viability

The cytotoxicities of Chol-GS and commercial liposomes were tested with the transfection protocols described above and measured by MTT-based cell viability assay. There were no significant differences in cell viability between N/P weight ratio 10 of Chol-GS liposome and the control, in which DNA was treated without liposome solution. Although the cell viability of Chol-GS liposome, at an N/P weight ratio of 15, was slightly lower than commercial liposomes in A549 cells, its cell viability was superior to the others in COS7 cells. The higher viability of all liposomes used in this study was also observed in Huh7 cells (Table 1). Therefore, the cell viabilities of Chol-GS were seen to be reasonable at optimal concentrations for efficient transfection.

In summary, a cholesterol-based diquaternary ammonium gemini surfactant was newly synthesized. In preparation for cationic liposomes, the addition of DOPE as helper lipid is well known to be one of the factors that can increase transfection efficiency. To find out the optimum transfection efficiency, we screened various weight ratios of DOPE and N/P, the result being that the best transfection efficiency was found at a Chol-GS:DOPE weight ratio of 1:4 and an N/P weight ratio of 10~15. The particle size and zeta potential of this liposome at the optimum weight ratio were 79.6±37.9 nm and 46.66± 1.65 mV. The electrical repulsion of this liposome was large enough (>±30 mV) to disperse a stable particle size in colloidal systems. The transfection efficiency of the Chol-GS liposome was compared with DOTAP, Lipofectamine, and DMRIE-C using the luciferase assay and GFP expression. The Chol-GS liposome was noted as being superior to commercial liposomes in this study, except with DMRIE-C in the case of A549 cells. The cell

viabilities of Chol-GS showed a reasonable cytotoxicity at optimal concentrations for efficient transfection. Therefore, the newly synthesized cholesterol-based diquaternary ammonium gemini surfactant could prove to be a useful nonviral vector for gene delivery.

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Abbreviations

DOPE, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine; DMRIE, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl-ammonium bromide; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; DOSPA, 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate; DC-Chol, 3β-[*N*,(*N*,*N*-dimethylaminoethane)-carbamoyl]cholesterol; CDAN, N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; BGTC, *bis*-guanidinium-tren-cholesterol; DMEM, Dulbecco's modified Eagle's medium.

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