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Transfection Property of a New Cholesterol-Based Cationic Lipid Containing Tri-2-Hydroxyethylamine as Gene Delivery Vehicle

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A novel cholesterol-based cationic lipid containing a tri-2-hydroxyethylamine head group and ether linker (Chol-THEA) was synthesized and examined as a potent gene delivery vehicle. In the preparation of cationic liposome, the addition of DOPE as helper lipid significantly increased the transfection efficiency. To find the optimum transfection efficiency, we screened various weight ratios of DOPE and liposome/DNA (N/P). The best transfection efficiency was found at the Chol-THEA:DOPE weight ratio of 1:1 and N/P weight ratio of 10~15. Most of the plasmid DNA was retarded by this liposome at the optimum N/P weight ratio of 10. The transfection efficiency of Chol-THEA liposome was compared with DOTAP, Lipofectamine, and DMRIE-C using the luciferase assay and GFP expression. Chol-THEA liposome with low toxicity had better or similar potency of gene delivery compared with commercial liposomes in COS-7, Huh-7, and MCF-7 cells. Therefore, Chol-THEA could be a useful non-viral vector for gene delivery.

Keywords: Cholesterol, cationic liposome, gene therapy, hydroxyethylamine, transfection

Gene therapy is the method to treat diseases by delivering nucleic acid therapeutics such as plasmid DNA, antisense oligonucleotides, and siRNA, unlike conventional medication [6]. In the gene therapy for various diseases such as cancer [2, 11] and heart failure [13, 25], the effective delivery of such drugs becomes more important because the introduction of foreign DNA, RNA, or antisense sequence into the target regions is essentially required. The cationic lipids are largely feasible to develop as a gene delivery vector owing to the advantages of handy synthesis, low immune response, and safety [12]. However, a relatively low efficiency of gene delivery is considered as the major hurdle of these vectors [24].

Cationic lipids are commonly composed of three parts – a positively charged polar head group, linker, and lipophilic domain [7]. The head group often consists of amines or those extended formations such as ethylamine, propylamine, lysine, spermidine, and spermine. The linker is mostly composed of an ether, ester, and carbamoyl (urethane) structure, and lipophilic tails composed of long-chain fatty acids or cholesterol derivatives. Among these components, the cholesterol-based cationic lipid has been used as the major lipid of liposomes for the delivery of genes [27] and chemical drugs [1, 22] owing to it being less toxic than other cationic lipids [20]. Generally, the presence of serum decreases the transfection efficiency and intracellular gene expression [33]. However, these cholesterol-based cationic lipids successfully delivered plasmid DNA in the presence of serum. Our preliminary studies [16] suggested that use of an ether linkage rather than the more frequently used carbamoyl linkage in primary ammonium cationic lipids with cholesterol backbone gives advantages such as high transfection efficiency and serum compatibility in gene delivery. We also reported the efficient gene delivery of quaternary ammonium cationic lipids with a cholesterol backbone and ether linker [14, 15].

On the other hand, hydroxyalkyl headgroups were previously reported to have good gene transfer properties [5, 17, 23, 26, 30]. 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 owing to its ability to destabilize endosomal or plasma membranes [4, 9, 10].

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In this study, we synthesized cholesterol-based cationic lipids with an ether linker and tri-2-hydroxyethylamine head group (Chol-THEA), and optimized the formulation of liposome with helper lipid DOPE. We found that Chol-THEA has better or similar potency of gene delivery compared with commercial lipids when measured by luciferase assay and GFP protein assay.

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 this synthetic process were purchased from Aldrich (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 from Merck (1.05554). Column chromatography was performed with silica gel (70−230 mesh, Merck, USA). Lipofectamine and DMRIE-C were purchased from Invitrogen (USA). DOTAP was purchased from Roche Molecular (Germany). DOPE was purchased from Fluka (Switzerland). Fetal bovine serum (FBS) was purchased from GIBCO (Invitrogen, USA). COS-7 (African green monkey kidney cell), Huh-7 (human hepatocarcinoma cell), and MCF-7 (human breast adenocarcinoma cell) cell lines were obtained from the Korean Cell Line Bank. Cells were grown at 37°C in DMEM (Welgene Inc., Korea) for COS-7 and Huh-7, or RPMI 1640 (Welgene Inc., Korea) for MCF-7 with 10% FBS in a humidified atmosphere containing 5% CO_2 /95% air.

Synthesis

The synthesis of compounds **2~4** has been previously reported [15]. To a 50 ml round flask, cholest-5-en-3β-oxypropane bromide **4** (0.5 g, 1.0 mmol) and triethanolamine (75.0 mg, 0.5 mmol) were added in DMSO (2.0 ml) and methylene chloride (2.0 ml). The reaction mixture was stirred at room temperature for 16 h and then the chloroform was removed on a rotary evaporator. The last compound **5**, Chol-THEA, was purified by chromatography from a silica gel column with 10% methanol in chloroform. Yield: 120.0 mg, 0.2 mmol, 36.6% (TLC developer; $CHCl_3$/MeOH/NH_4OH = 4:2:0.5, Rf = 0.6~0.7). ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 1:1) δ (ppm): 0.70 (s, 3H), 0.83−2.07 (m, 40H; -$OCH_2C\underline{H}_2CH_2N$- and cholesterol moiety), 2.14−2.38 (m, 2H), 3.18 (m, 1H), 3.34 (t, 2H; -$OCH_2CH_2C\underline{H}_2N$-), 3.59 (t, 2H; -$OC\underline{H}_2CH_2CH_2N$-), 3.68 (t, 6H; -$N(C\underline{H}_2CH_2OH)_3$), 4.02 (br s, 6H; -$N(CH_2C\underline{H}_2OH)_3$), 5.36 (d, 1 H, J = 5.3 Hz). FAB-MS: 576.50 (M^+-Br^-).

Preparation of Liposomes

For the preparation of liposomes, the Chol-THEA and DOPE were mixed in chloroform/methanol [1/1 (v/v)] at an appropriate ratio, and the solvent was evaporated under vacuum. Solvent trace was removed by keeping these films under vacuum overnight. The film of cationic lipid and DOPE was vortexed hardly for 1 min in Milli Q water (Biocel equipment, Millipore, U.S.A) to give final total lipid concentrations of 1 mg/ml for *in vitro* transfection. The suspension 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 liposomes obtained were stored at 4°C prior to use.

Formation of Complex and Transfection

The pcDNA-Luc containing the firefly luciferase reporter gene and the plasmid coding for green fluorescent protein (pCMVTnT-GFP) were 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 $OD_{260}/OD_{280} \geq 1.8$ was used in this study. The pcDNA-Luc and pCMVTnT-GFP were used at a concentration of 0.3 μg/well unless otherwise specified. A liposome solution was prepared separately by diluting an appropriate amount of the initial liposomal stock solution with transfection optimizing medium (TOM, WelGene Co., Korea) to reach a final volume of 25 μl. To this liposome solution, 25 μl of the DNA stock solution was added. This lipoplex solution was incubated for 15 min at room temperature. The lipoplexes were tested for their ability to transfer DNA in COS-7, Huh-7, and MCF-7 cells. Twenty-four hours prior to transfection, the cells were transferred to 48-well culture plates at a density of 30,000 cells/well for COS-7 and Huh-7, or 80,000 cells/well for MCF-7. 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 received 50 μl of lipoplex solution, and the plates were incubated for 4 h. An additional 200 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 comparison with Chol-THEA, 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 microliters 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).

Gel Retardation Assay

For agarose gel retardation assays, 0.25 μg of pCMVTnT-GFP was mixed with Chol-THEA at various concentrations. Ten microliters of DNA:lipid complex solution was mixed with 2 μl of 6× loading buffer and loaded onto a 1% agarose gel containing ethidium bromide. Electrophoresis was carried out for 45 min in 1× TBE running buffer solution. Images were taken using a UV light illuminator.

Cell Viability

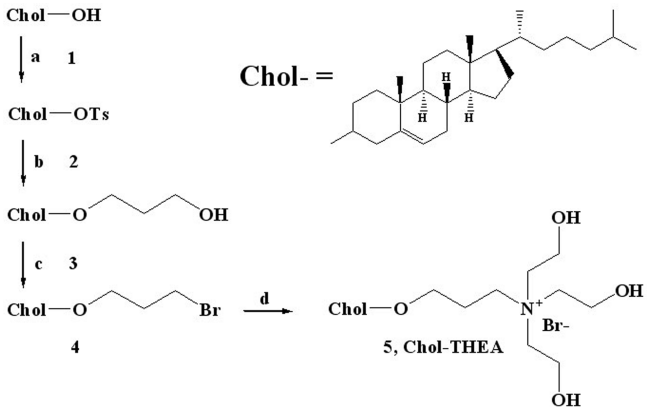
According to the transfection protocol described above, 24 h after transfection, 25 μg of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] in 20 μl of PBS was added to each well and the plates were then incubated for 3 h. The medium was carefully removed and 200 μl of DMSO was added to each well. The formazan crystals were 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:

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

RESULTS AND DISCUSSION

Synthesis

The main structure of cholest-5-en-3β-oxypropane-*N,N,N*-tri(2-hydroxyethyl) ammonium bromide (Chol-THEA) consists of a cholesterol-based hydrophobic domain, ether linker, and tri-2-hydroxyethylamine modified cationic head. We tried to combine the cholesterol-based hydrophobic domain and ether linker reported to have the good property of gene delivery [14–16] and the hydroxyalkyl headgroups known to have impressive gene transfer properties [5, 17, 23, 26, 30]. The syntheses of compounds **2**~**4** have been reported elsewhere [15]. The last compound **5**, Chol-THEA, was synthesized by reacting cholest-5-en-3β-oxypropane bromide **4** with triethanolamine in DMSO/methylene chloride in 36.6% yield. The structures of all the synthetic intermediates and Chol-THEA 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.



Scheme 1. Synthesis of novel cholest-5-en-3β-oxypropane-*N,N,N*-tri(2-hydroxyethyl) ammonium bromide (Chol-THEA).
Reaction condition: (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) Triethanolamine/DMSO/CH_2Cl_2, 16 h, r.t. (yield: 37%).

Optimization of Liposome Formulation (Chol-THEA:DOPE and N/P Weight Ratios)

The proper addition of DOPE for the liposome formulation has been known to enhance the transfection efficiency of many cationic lipids in various cell types by destabilizing endosomal or plasma membranes [4, 5, 9, 10]. In order to find out the most effective liposome formulation, the transfection efficiency was measured at different DOPE weight ratios. Thus, the luciferase assay was performed from Chol-THEA only to 1:2 of Chol-THEA:DOPE weight ratios at the N/P weight ratio of 10. At this N/P weight ratio, the equal weight ratio of Chol-THEA:DOPE (1:1) showed the highest luciferase activity (Fig. 1). To find the optimum N/P weight ratio, luciferase activity was measured by increasing the amount of liposome in 0.3 μg of constant DNA amount. The most effective N/P weight ratio was 10 to 15 (Fig. 2), and particular cytotoxicity of Chol-THEA liposome observed by microscopy was not seen up to an N/P weight ratio of 15. For further assessment of their transfection properties, the successive experiments were done at the Chol-THEA:DOPE weight ratio of 1:1 and N/P weight ratio of 15.

DNA Binding Affinity

For cationic lipid- and polyamine-mediated transfection, the first step is believed to be the electrostatic interaction between the DNA/carrier complex and the anionic plasma

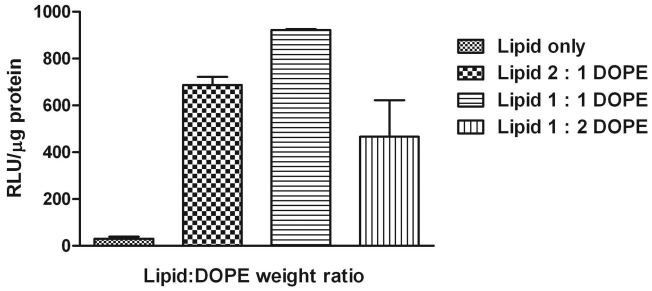


Fig. 1. Transfection efficiencies of Chol-THEA liposome with various weight ratios of DOPE.
The concentration of DNA = 0.3 μg/well was kept constant and the N/P weight ratio was 10. Data were measured with the luciferase assay at different Chol-THEA:DOPE weight ratios in COS-7 cells. Each bar value represents the mean ± SD of duplicate experiments performed on the same day.

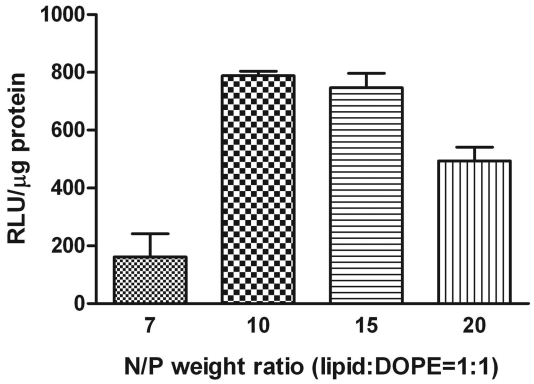


Fig. 2. Transfection efficiencies of Chol-THEA liposome at different N/P weight ratios.
In vitro transfection efficiencies of liposome/DNA complexes were measured with the luciferase assay at various N/P weight ratios in COS-7 cells, while the concentration of DNA = 0.3 μg/well was kept constant. Each bar value represents the mean ± SD of duplicate experiments performed on the same day.

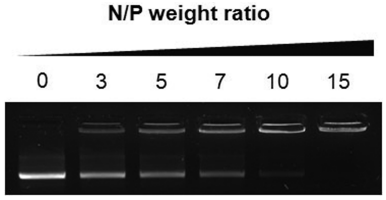


Fig. 3. Gel retardation assay of Chol-THEA liposome/DNA complexe at lipid:DOPE weight ratio of 1:1.
Lane marked "0" contained DNA alone and was used as a control. The presence of a lower band indicated that the DNA has migrated and has not been bound by Chol-THEA liposome.

membrane [31]. The relative DNA binding affinities of Chol-THEA liposome were performed to determine whether transfection activities correlated with DNA binding. Electrophoretic gel retardation assay was used to evaluate binding affinities between DNA and Chol-THEA liposome. To perform this assay, Chol-THEA liposome was mixed with pCMVTnT-GFP at various ratios, and the liposome/DNA complex (lipoplex) were loaded onto an agarose gel (Fig. 3). At N/P weight ratio of 10, most of the plasmid DNA was retarded by Chol-THEA liposome. This result is concordant that the transfection efficiency of Chol-THEA liposome was the highest in luciferase assay at an N/P weight ratio of 10~15 (Fig. 2). These results imply that Chol-THEA liposome is not enough to bind DNA at an N/P weight ratio under 10, and an N/P weight ratio over 15 is excessive for the interaction with cell membrane.

Comparison of Chol-THEA and Commercial Liposomes in Luciferase and GFP Expression

Luciferase gene expression assay of Chol-THEA was performed to compare its activity with commercial liposomes

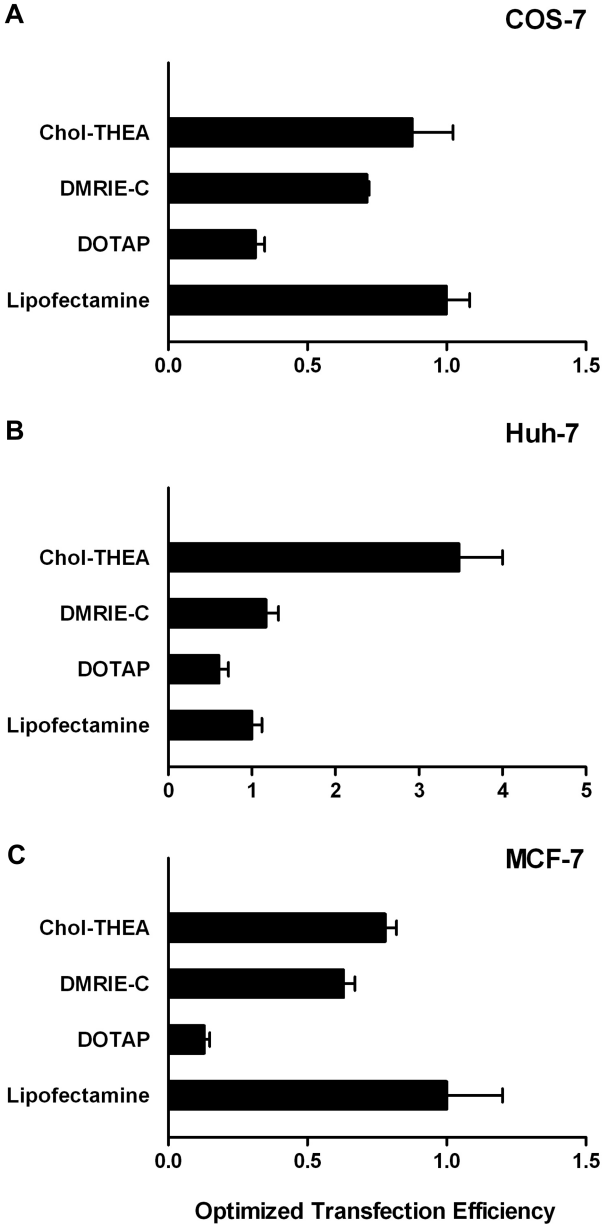


Fig. 4. Comparison of transfection efficiencies (normalized to Lipofectamine) with commercial liposomes.
The transfection efficiency of Chol-THEA liposome was compared with commercial liposomes at optimal N/P weight ratios of 5 for DMRIE-C and Lipofectamine, 7 for DOTAP, and 15 for Chol-THEA in COS-7 (**A**), Huh-7 (**B**), and MCF-7 (**C**) cells, using the luciferase assay. Each bar value represents the mean ± SD of triplicate experiments performed on the same day.

in three cell lines. In COS-7 and MCF-7 cells, the transfection efficiency of Chol-THEA liposome was higher than DMRIE-C and DOTAP, and similar to Lipofectamine, which showed the highest transfection efficiency among the three commercial liposomes (Fig. 4A and 4C). In Huh-7, the transfection efficiency of the Chol-THEA liposome was 3.5 times higher than Lipofectamine (Fig. 4B). GFP

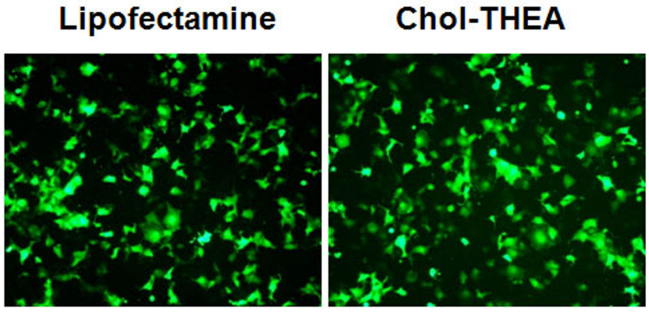


Fig. 5. Expression of GFP using Chol-THEA liposome and Lipofectamine.
The pCMVTnT-GFP (0.3 μg) complexed with Chol-THEA liposome (N/P weight ratio = 15) and Lipofectamine was added to COS-7 cells, and green fluorescence was observed under a fluorescence microscope.

expression was also observed to confirm the results shown by luciferase assay in COS-7 cells. The transfection of GFP plasmid using Chol-THEA liposome showed similar expression efficiency to Lipofectamine (Fig. 5). The cytotoxicities of Chol-THEA 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 of 15 of Chol-THEA liposome and the control, in which plasmid DNA solution was treated without liposome. The cell viability of Chol-THEA liposome was higher than most of the commercial liposomes in Huh-7 and MCF-7 cells (Table 1).

For the development of an efficient gene delivery vehicle beyond the *in vitro* application, serum compatibility is considered as the major hurdle. Despite the desirable efficiency of cationic lipids, the excessive positive charge generally cause nonspecific electrostatic binding with charged molecules such as serum albumin and lipoproteins [18, 28, 33, 34]. Therefore, several researches have been performed to derive efficient non-viral vector system having the stability in serum [3, 8, 19, 21, 29, 32]. Because recent study done by us showed the serum stability of a similar structure of cholesterol-based cationic lipids and hydroxyalkyl headgroups was previously reported to have serum-resistant properties [5, 17, 23, 26, 30], we hope the novel cholesterol-based cationic lipid, Chol-THEA, also will have similar potency in future study.

Table 1. Cell viability of Chol-THEA and commercial liposomes in Huh-7 and MCF-7 cells.

	Cell lines	
Liposomes	Huh-7	MCF-7
Chol-THEA	99 ± 7.0%	102 ± 7.3%
Lipofectamine	81 ± 4.6%	102 ± 3.4%
DMRIE-C	89 ± 5.2%	105 ± 2.8%
DOTAP	90 ± 5.1%	98 ± 2.7%

Cells were treated with the concentration of transfection protocol at N/P weight ratio of 15. Cell viability was measured by MTT assay. The results are represented as the mean ± SD of triplicate experiments performed on the same day.

In summary, cholest-5-en-3β-oxypropane-*N,N,N*-tri(2-hydroxyethyl) ammonium bromide was newly designed and synthesized for stable lipoplex formulation. In the preparation of the cationic liposome, the addition of DOPE as helper lipid significantly increased the transfection efficiency. To find the optimum transfection efficiency, we screened various weight ratios of DOPE and N/P. The best transfection efficiency was found at Chol-THEA:DOPE weight ratio of 1:1 and N/P weight ratio of 10~15. Most of the plasmid DNA was retarded by this liposome at the optimum N/P weight ratio of 10. The transfection efficiency of Chol-THEA liposome was compared with DOTAP, Lipofectamine, and DMRIE-C using the luciferase assay and GFP expression. Chol-THEA liposome was superior to commercial liposomes in Huh-7 cells and similar to Lipofectamine, which showed the highest transfection efficiency among the three commercial liposomes in COS-7 and MCF-7 cells. The cell viabilities of Chol-THEA showed a reasonable cytotoxicity at optimal concentrations for efficient transfection. Therefore, Chol-THEA could be one of the useful non-viral vectors for gene delivery.

Acknowledgment

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Abbreviations

DOPE, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride; DMRIE, 1,2-dimyristyl oxypropyl-3-dimethylhydroxyethylammonium bromide

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