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Synthesis of novel cholesterol-based cationic lipids for gene delivery

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ABSTRACT

The new cholesterol-based cationic lipids B, C, and D with an ether linked spacer were synthesized by using aminopropyl chain extension with acrylonitrile. The cholesterol-based cationic lipid A with carbamoyl linkage were also synthesized in order to compare the difference in transfection efficiency of the two linkage types. To this end, GFP expression of these cationic lipids was confirmed respectively.

Gene therapy represents a medicine of the future owing to its potential to treat various diseases including cancer. In gene therapy, it is exceedingly important not only to manufacture the therapeutic materials such as plasmid DNA and antisense oligonucleotides but also to delivery these therapeutics to target cells.[1] Generally, the vectors for an successful delivery of genetic materials can be classified into two main types: viral or nonviral. Although viral vectors are highly efficient for delivery of DNA into cells, they have many disadvantages such as antigenicity, toxicity, limited size of cargo, and the difficulty of large-scale virus production.[2,3] As an alternative to the limitations of viral vectors, non-viral vectors offer the new hope for DNA delivery systems. Among the non-viral vectors, cationic lipids have been intensively investigated because of the advantages of handy synthesis, low immune response, and safety.[4–8] The cationic lipids are also a class of vectors easily applying rational design and studying structure–activity relationships.[9]

Cationic lipids are commonly composed of three parts (positive-charged polar head group, linker, and lipophilic domain).[10] The head group often consists of amines or those extended formation such as ethylamine, propylamine, lysine, spermidine, and spermine. The linker mostly composes of ether, ester and carbamoyl (urethane) structure, and the lipophilic tails compose 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[11] and chemical drugs[12,13] due to it being less toxic than other cationic lipids.[14] The capacity of the gene delivery using these cationic lipids depends on the combination of cationic head, degree of hydrophobicity of the tails, and the bond that links the lipophilic tails. Neutral lipid, for example, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine (DOPE), is also play a key role when forming cationic liposome for high activity.[15,16] Especially, the presence of serum in gene delivery by almost commercial liposomes decreases the degree of gene expression.[17]

This study focused on the development of optimum cationic lipids for cationic liposome delivery systems that can improve serum stability and transfection efficiency. The synthesis of novel cholesterol-based cationic lipids **B**, **C**, and **D** (Scheme 1) having aminopropyl head groups, ether linker, and cholesterol tail is reported.[18] The current synthetic strategy is based on the cyanoethylation of cholesterol and direct Boc protection via reduction of nitrile group.

This study first introduced the cationic lipids that aminopropyl cationic head groups attached via carbamoyl and ether linkers to cholesterol as shown in Scheme 1. According to the current report[19], ether linker is more efficient than other linkers on the same structure used for gene delivery system. To our knowledge, lipid **B** having an aminopropyl cationic head group has not been compared with other carbamoyl linked cationic lipids having an aminopropyl cationic head group. Therefore, to demonstrate the predominance of the ether linkage, lipid **A** having the carbamoyl linkage was synthesized according to the method of Tsutomu et al.[20] as shown in

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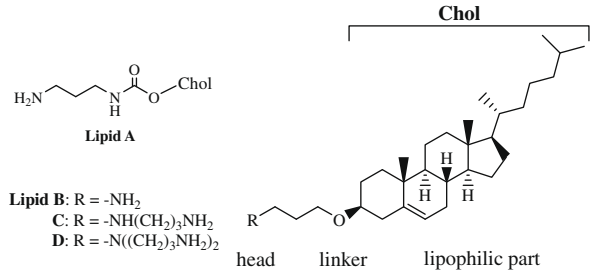
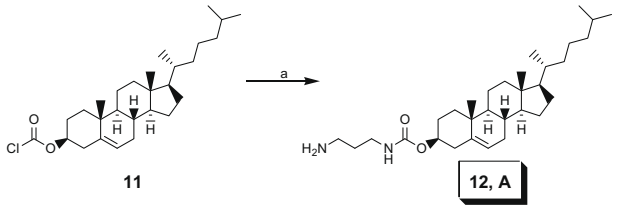


Scheme 1. Structure of cholesterol-based cationic lipids.

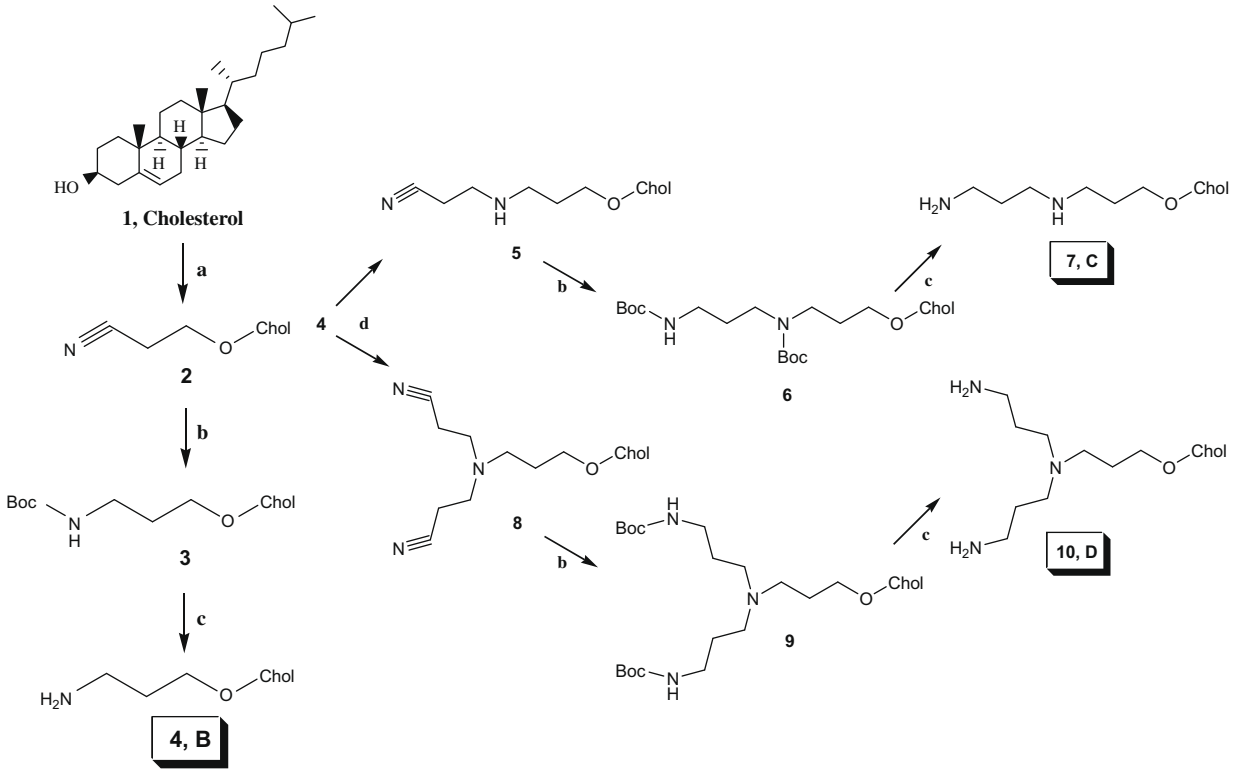


Scheme 2. Synthesis of carbamoyl linked cationic lipid **A**. Reagent and condition: (a) 1,3-diaminopropane, toluene/r.t.

Scheme 2. This study also designed with a view to probe the role of an additional elongated cationic amine center in modulating the gene transfer properties of novel cholesterol-based cationic lipids **7**, **10**.

A typical procedure for the synthesis of lipids **B–D** from cholesterol is as follows: Acrylonitrile was added to a mixture of aqueous KOH, 18-crown-6 and cholesterol **1**, 5-cholesten-3β-ol in CH_2Cl_2 at room temperature. Compound **2**, 2-cyanoethyl-*O*-β-cholesterol ether synthesized by cyanoethylation was dissolved in dry MeOH with 2 equiv of $NiCl_2{\cdot}6H_2O$ and 3 equiv of crystalline Boc-ON were added. After the mixture stirred 5 min, 10 equiv of $NaBH_4$ were slowly added under nitrogen atmosphere at room temperature and gave the carboxyamine **3** by the usual workup. Compounds **2**, **5**, **8** were reduced only by $NiCl_2{\cdot}6H_2O$ and $NaBH_4$ to directly obtain final compounds. However, the yield was low (up to 50% in the case of compound **3**) because of by-products and treatment process that is fastidious due to the gluey reaction mixture. Therefore, Boc-protection method was used in this study in order to obtain high yields and easy treatment. Trifluoroacetic acid (TFA) treatment is necessary procedure to retain cationic form of amine head groups and Boc-deprotection is easily reacted by TFA. Ether spacer linked amine **4** attached to cholesterol was obtained by Boc-deprotection using TFA. The intermediate **5** and **8** were obtained by reacting 1 equiv of the lipid **4** with 5 equiv of acrylonitrile in methanol under reflux condition in one step. Finally, the one or two elongated 3-aminopropyl chain cationic lipids **7**, **10** were synthesized by reductative Boc protection and deprotection as described above. Structures of all of the synthetic intermediates and the target lipids as shown in Scheme 3 were confirmed by ^{1}H NMR. Final compounds **4**, **7**, and **10** as shown in Scheme 3 were also characterized by mass spectrometry including GC or FAB (fast atomic bombardment) to confirm the identity of the molecular ions.

This study next examined the GFP expression of liposomes containing lipids **A**–**D**. The liposomes were prepared from cationic lipids with appropriate volume of DOPE in $CHCl_3$ and the solvent was evaporated in a rotary evaporator under reduced pressure. The thin lipid films were resuspended in sterile water and vigorously vortexed. The mixture was subjected to extrusion 10 times through a polycarbonate membrane of 200 nm pore size using an extruder device. The optimized medium for transfection (TOM) and plasmid coding for Green Fluorescent Protein (pCMVTnT-GFP) were obtained from Welgene (Korea) to test the ability of the lipoplexes to transfer DNA in AGS cell (Human Stomach Epithelial—Gastric



Scheme 3. Synthesis of ether linked cationic lipids **B**–**D**. Reagents and conditions: (a) acrylonitrile, 18-crown-6, aqueous KOH/CH_2Cl_2 (yield: 100%); (b) $NiCl_2{\cdot}6H_2O$, Boc_2O, $NaBH_4$/MeOH (yield: 3, 93%; 6, 80%; 9, 68%); (c) TFA/CH_2Cl_2 (yield: 4, 85%; 7, 65%; 10, 67%); (d) acrylonitrile/MeOH, reflux (yield: 5, 42%; 8, 51%; overall, 93%).

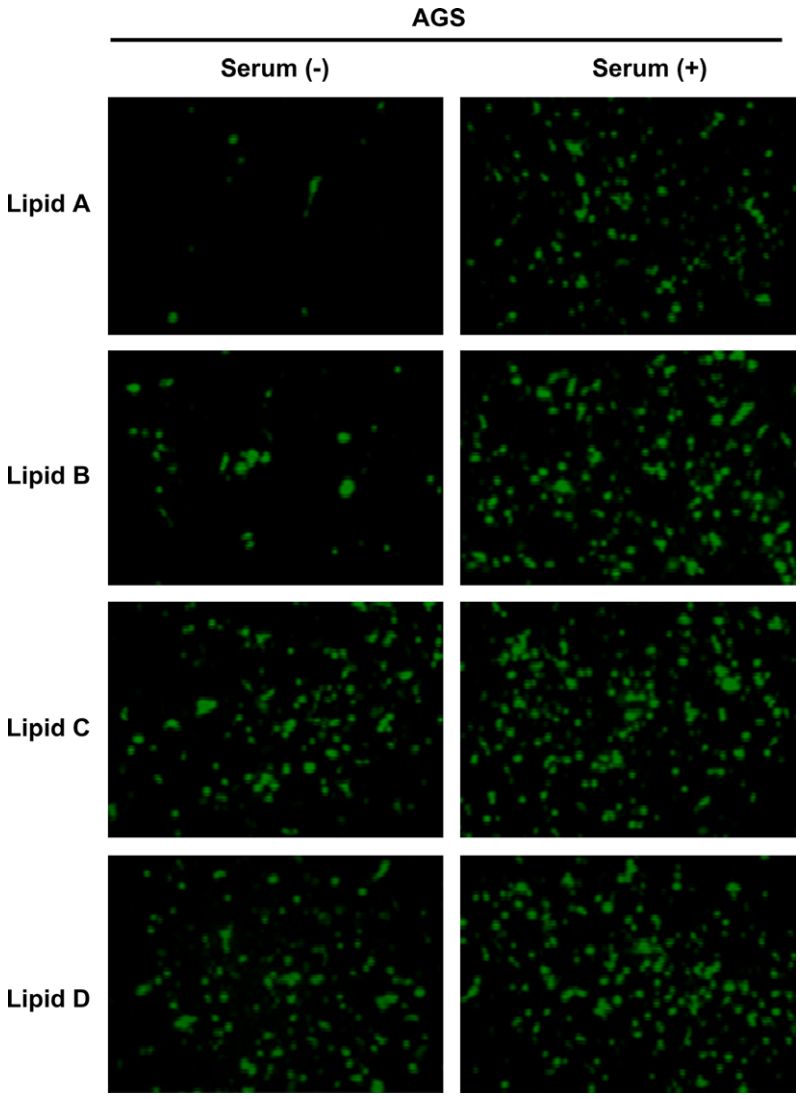


Figure 1. Expression of GFP using lipids **A**–**D**. Plasmids pCMVTnTGFP (0.3 μg) complexed with lipids **A**–**D** were added to AGS cell observed under fluorescence microscope.

Adenocarcinoma). The day before transfection, AGS cells (4×10^4 per well) were seeded into a 48-well plates. Each well was briefly washed with 200 μL of sterile PBS (Phosphate Buffered Saline), and added with 100 μL of TOM before transfection. Each liposome (0.6 μg) were diluted to 100 μL of TOM, and mixed with pCMVTnT-GFP plasmid DNA (0.3 μg) in 50 μL of TOM. The lipoplexes, consists of liposomes and DNA, were incubated for 15 min in room temperature, and then added to each well. After 24 or 48 h of incubation, the media was removed, and cells were washed twice with PBS. Fluorescence expression was observed with a Leica DM-IRE2 fluorescence microscope (Germany). The ether linked cationic lipid **B** containing aminopropyl head group was shown to be more effective in vitro transfection when compared to carbamoyl linked cationic lipid **A**. Also, this study confirmed higher transfection efficiency in the presence of serum than in the absence of serum and elongating one or two 3-aminopropyl chain was benefited to plasmids DNA uptake as shown in Figure 1.

In conclusion, this study describes the synthesis of the novel cholesterol-based cationic lipids **B**–**D** and confirmed that the ether linker is more effective than carbamoyl linker in the cholesterol-based cationic lipids in vitro transfection. Generally, the presence of serum decreases the transfection efficiency and intracellular gene expression.[17] However, these cholesterol-based cationic lipids successfully delivered plasmid DNA in the presence of serum. Transfection biology and characterization of lipids **A**–**D** are now under investigation.

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