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Synthesis, Characterization and Application of Dendritic Lipids for Gene Delivery

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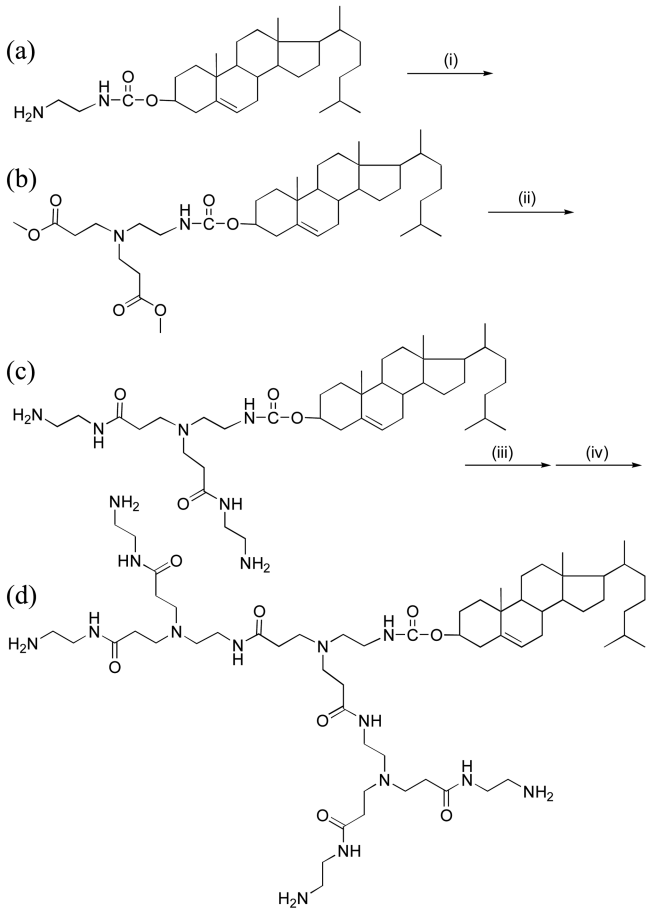
Tomalia and coworkers first reported spherical cationic Starburst poly(amidoamine) (PAMAM) dendrimers that consist of primary amines on the surface and tertiary amines in the interior.[1] Being well-defined and highly-branched macromolecules, they have been used in many fields including gene delivery.[2] Although poly(amidoamine) dendrimer forms a complex with DNA through electrostatic interaction, it shows low transfection efficiency on most mammalian cell lines. Therefore, many researchers developed PAMAM modifying methods such as random degradation of PAMAM (*i.e.*, fractured PAMAM)[3] or PEGylation of PAMAM[4] to enhance its transfection efficiency. Previously we had modified low-molecular-weight polyethylenimine by hydrophobic residues for achieving higher transfection efficiency.[5] Based on these results, we introduce a systematic PAMAM modifying methods for enhancing transfection efficiency as well as maintaining low cytotoxicity.

Generally PAMAM dendrimer is synthesized by repeated Michael addition and amidation from ammonia or ethylenediamime core.[1] Hydrophobic PAMAM derivatives with multivalent cationic residues can be obtained by the same synthetic method from a cholesterol core (Scheme 1). Briefly, the cholesteryl chloroformate was treated with excess ethylenediamine to prepare EDA-Chol (ethylenediamine-cholesterol). PAM-Chol (polyamidoamine-cholesterol, generation 0.5) was obtained by Michael addition of the primary amine of ethylenediamine to double bonds of two methyl acrylate molecules. The resulting two ester groups of PAM-Chol (generation 0.5) were attacked by ethylenediamine to produce PAM-Chol (generation 1). Similarly, PAM-Chol (generation 2) was obtained by the same repeated treatment of PAM-Chol (generation 1) with methyl acrylate and ethylenediamine. To avoid side reaction and to obtain a perfect dendritic lipid, we have used excess methyl acrylate and ethylenediamine in each step. Here, we synthesized novel dendritic cationic lipids based on a hydrophobic cholesterol core and performed characterization for gene delivery systems. Finally, their structure-transfection efficiency relationship was investigated.

[a]The first two authors contributed equally to this work.

Experimental Section

Materials. Ethylenediamine, cholesteryl chloroformate, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and methyl acrylate were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dioleoyl-sn-glycero-3-phos-



Scheme 1. Synthesis of PAM-Chol lipids. (a) EDA-Chol is a core which was synthesized from cholesteryl chloroformate and excess ethylenediamine, (b) PAM-Chol g = 0.5[a], (c) PAM-Chol g = 1.0[a], (d) PAM-Chol g = 2.0[a]. [a]Conditions: (i) methyl acrylate/MeOH, (ii) ethylenediamine/MeOH, (iii) methyl acrylate/MeOH, (iv) ethylenediamine/ MeOH.

phatidylethanolamine (DOPE) and 3β [*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). PAMAM dendrimer (generation 6) was purchased from Dendritech (Midland, MI).

Synthesis. 1) Synthesis of EDA-Chol (A) 80 g (1.3 mol) of ethylenediamine and 4 g (8.9 mmol) of cholesteryl chloroformate were dissolved in 10 mL of chloroform. The mixture was warmed to 35 °C and stirred for 48 hours. The reaction mixture was washed by brine three times. The organic layer was separated, dried over anhydrous sodium sulfate, and evaporated. EDA-Chol (A) was obtained as 2.8 g of colorless powder. ^{1}H NMR (300 MHz, $CHCl_3$) δ 2.60 (t, 2H, $NH_2C\underline{H}_2CH_2NHCO$-); 3.05 (t, 2H, $NH_2CH_2C\underline{H}_2NHCO$-); 4.25 (t, 1H, $NH_2CH_2CH_2N\underline{H}CO$-); 5.3 (s, 1H, -C=C$\underline{H}CH_2$-); 0.6-2.3 (broad, 44H, cholesterol). Matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF MS), *m/z* 494[M+Na]$^+$. 2) Synthesis of PAM-Chol g = 1.0 (C) 2.1g (4.5 mmol) of EDA-Chol and 300 mL (3.3 mol) methyl acrylate were dissolved in 300 mL MeOH. The mixture was warmed to 35 °C and stirred for 48 hours. The organic solvent and excess methyl acrylate were evaporated. The product, PAM-Chol g = 0.5 (B), was obtained as 3.0 g of yellow viscous oil. It was dissolved in 300 mL MeOH. 300 mL (4.5 mol) of ethylenediamine was added to this solution dropwise at 0 °C. The mixture was warmed to 35 °C and stirred for 48 hours. The organic solvent and excess ethylenediamine were evaporated. The product, PAM-Chol g = 1.0 (C), was purified on silica gel with an eluent of *n*-propanol: ammonia water: water (70:10:20 to 70:20:10 v/v/v). ^{1}H NMR (300 MHz, MeOD/$CDCl_3$) δ 2.4 (t, 4H, -$CH_2CH_2NHCOC\underline{H}_2$-); 2.62 (t, 2H, -$NC\underline{H}_2CH_2NHCOO$-); 2.8 (m, 8H, (-$C\underline{H}_2$-)$_3$N and $NH_2\underline{C}H_2CH_2NHCO$-); 3.1 (t, 4H, $NCH_2C\underline{H}_2NHCOO$-); 3.5 (t, 4H, $NH_2CH_2C\underline{H}_2NHCO$-); 5.3 (s, 1H, -C=C$\underline{H}CH_2$-); 0.6-2.3 (broad, 44H, cholesterol). MALDI-TOF MS, *m/z* 723 [M+Na]$^+$ 3) Synthesis of PAM-Chol g = 2.0 (D) 1.0 g (1.4 mmol) of PAM-Chol g = 1.0 and 100 mL (1.1 mol) methyl acrylate were dissolved in 100 mL MeOH. The mixture was warmed to 35 °C and stirred for 48 hours. The organic solvent and excess methyl acrylate were evaporated. The product, PAM-Chol g = 1.5, was obtained as 1.0 g of yellowish viscous material. It was dissolved in 100 mL MeOH. 100 mL (1.5 mmol) of ethylenediamine was added to this solution drop wise at 0 °C. The mixture was warmed to 35 °C and stirred for 48 hours. The organic solvent and excess ethylenediamine were evaporated. The product, PAM-Chol g = 2.0 (D), was purified on silica gel with eluent of *n*-propanol: ammonia water: water (70:20:10 to 60:30:10 v/v/v). ^{1}H NMR (300 MHz, MeOD/$CDCl_3$) δ 2.4 (t, 12H, -$CH_2CH_2NHCOC\underline{H}_2$-); 2.6 (t, 8H, $NH_2\underline{C}H_2CH_2NHCO$-); 2.8 (m, 18H, (-$C\underline{H}_2$-)$_3$N-); 3.3 (t, 4H, $NH_2CH_2C\underline{H}_2NHCO$-); 5.3 (s, 1H, -C=C$\underline{H}CH_2$-); 0.6-2.3 (broad, 44H, cholesterol). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra, *m/z* 1179 [M+Na]$^+$. All synthesis steps are shown in Scheme 1.

Liposome Preparation and Gel Retardation Assay. 0.5 mg of DOPE (Sigma, St. Louis, MO) and 0.5 mg of synthetic lipids (PAM-Chol g = 1.0 and g = 2.0) in chloroform were dried under a stream of N_2 gas and vacuum. After overnight hydration with 1 mL of distilled water at 4 °C, the mixture was sonicated using a bath-type sonicator. Lipoplexes between cationic lipid and pGL3 plasmid (Promega, Masison, WI) were prepared at different charge ratios by incubation in serum-free DMEM at room temperature for 30 minutes. Each sample was then analyzed by electrophoresis on a 0.7%(w/v) agarose gel containing ethidium bromide (0.5 μg/mL) as shown in Figure 1(a) and 1(b).

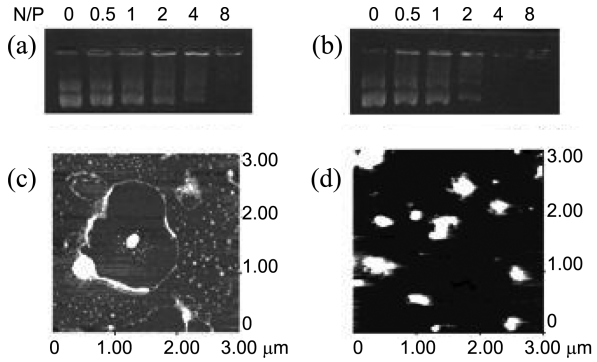


Figure 1. Formation of complexes between DNA and PAM-Chol: (a) gel retardation assay of PAM-Chol g = 1.0, (b) gel retardation assay of PAM-Chol g = 2.0, (c) AFM image of PAM-Chol g = 1.0/DNA lipoplex, (d) AFM image of PAM-Chol g=2.0/DNA lipoplex.

Atomic Force Microscopy. Atomic force microscopy (Nanoscope IIIa system, Digital Instruments, Inc., Santa Barbara, CA) was used for imaging the shape and particle size of the lipoplexes, as previously reported.[2(a)] Briefly, 2 μL aliquots of lipoplex solution were loaded onto the center of a freshly split mica disk. After adsorption for 1-2 minutes at room temperature, excess complex solution was removed by absorption onto filter paper and the mica surface was further dried at room temperature before imaging. The image mode was set to tapping mode and the average scanning speed was 5 Hz.

Cell Culture and Cytotoxicity Assay. Human liver carcinoma HepG2 cells (Korean Cell Line Bank) were propagated in MEM (Hyclone, Logan, UT) supplemented with 10%(v/v) FBS (Gibco, Gaithersburg, MD). HepG2 cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37 °C in a humidified 5% CO_2/95% air containing atmosphere. HepG2 cells were seeded in 96 well plates (10^4 cells per well) and incubated for one day prior to experiment. Each reagent was introduced to the cells and incubated for 48 hours. The cytotoxicity was determined by comparing the amount of MTT reduced by the cells treated with reagents to that reduced by control cells.

Transfection Assay. For transfection, 1~1.5 × 10^5 cells per well were seeded in 24 well plates one day prior to transfection experiments, and grown in MEM with 10% FBS. The cell lines were 60-70% confluent at the time of transfection. Complexes were prepared by mixing PAM-Chol/DOPE liposome with plasmid DNA (2.0 μg per well) in FBS-free medium. Each complex solution was further

incubated for 30 minutes at room temperature and added to the cells. Transfection was performed in a serum-free medium for 4 hours and each medium was replaced with a fresh complete medium after the transfection. Gene expression was assayed after 48 hours. Control transfections were performed with PAMAM g = 6.0 and DC-Chol. Luciferase gene expression was measured by a luminescence assay. The growth medium was removed, and the cells were rinsed twice with PBS and treated with Reporter Lysis Buffer (Promega, Masison, WI) for 20 min at room temperature. The lysate was cleared by centrifugation, and protein content was determined by using Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). 10 μL of the lysate was dispensed into a luminometer tube, and luciferase activity was integrated over 10s with 2s measurement delay in a Lumat LB 9057 luminometer (Berthold, Germany) with automatic injection of 100 μL of Luciferase Assay Reagent (Promega, Masison, WI). Results were expressed as relative light units per mg of cellular protein.

Results and Discussion

The number of positive charges of PAM-Chol is easily and systematically controlled according to the generation of dendritic structure. PAM-Chol g = 1.0 has two primary amines and one tertiary amine, and PAM-Chol g = 2.0 has four primary amines and three tertiary amines. In the n^{th} generation of PAM-Chol, there are 2^n primary amines and (2^n-1) tertiary amines.

PAMAM surface is coated with primary amines of ethylenediamine. If we merely change the reagent of the final step from ethyleneamine to *N,N*-dimethylethylenediamine or other amines, the surface charges of PAM-Chol can be easily exchanged by other functional groups. Until now, the relationship between structure and function of gene delivery lipids has been poorly understood partially because there was no easy method of controlling the structure and number of charged groups. Therefore, the PAMAM modifying method that we had developed could make a contribution to the understanding of the relationship.

We first examined the effect of charge numbers on the formation of DNA/lipid complex (lipoplex), which was examined by well-known gel retardation assay (Figure 1(a), 1(b)). PAM-Chol g = 1.0 could form a complex with DNA at N/P ratio ≥ 8, whereas PAM-Chol g = 2.0 could do so at ratio ≥ 4. The stronger complex formation ability of PAM-Chol g = 2.0 was primarily due to the existence of more positive charges for binding with DNA. Since the interior tertiary amines of the PAMAM unit are not protonated at neutral pH,[6] the complexes composed of PAM-Chol/DNA may have the proton buffering effect for gene transfection efficiency.[7] PAMAM dendrimer g = 0 or g = 1.0 without any hydrophobic moiety (which correspond to PAM-Chol g = 1.0 or g = 2.0 respectively) cannot form a complex with DNA even at high N/P ratios (data not shown). Only PAMAM dendrimer of the generation ≥ 3.0 was able to form the complex with DNA. The tendency of strong complex formation of our cholesterol derivatives of PAMAM (PAM-Chol) over naked PAMAM is presumably due to the hydrophobic interaction as well as electrostatic interaction.

The shape and size of liposome (PAM-Chol:DOPE = 1:1)/DNA complex was examined by atomic force microscopy. DOPE (dioleoylphosphatidylethanolamine) is a fusogenic helper lipid which is generally used to enhance transfection efficiency by destabilizing endosomal membrane. When PAM-Chol g = 1.0/DOPE liposome was mixed with DNA, the liposome/DNA complex showed spaghetti (DNA) and meatball (liposome) shapes[8] (Figure 1(c)). Whereas, the mixture of PAM-Chol g = 2.0/DOPE liposome and DNA formed 100-500nm sized nanoparticles (Figure 1(d)). The difference might be due to the structure and charge density difference between g = 1.0 and g = 2.0.

The transfection efficiencies of PAM-Chol g = 1.0, 2.0, DC-Chol, and PAMAM g = 6.0 were compared in HepG2 cells by a luciferase reporter system (Figure 2(a)). DC-Chol is a commercially available cationic lipid containing a tertiary amine head and a cholesterol tail. In some cases, chloroquine, an endosome destabilizer, was added to the transfection media for enhancing transfection efficiency. The transfection efficiencies were compared at the concentration at which each reagent showed the maximal transfection efficiency. The transfection efficiency followed the order of PAM-Chol/DOPE g = 1.0 > DC-Chol/DOPE > PAMAM g = 6.0 (with chloroquine) > PAM-Chol/DOPE g = 2.0 > PAMAM g = 6.0 (without chloroquine). The transfection

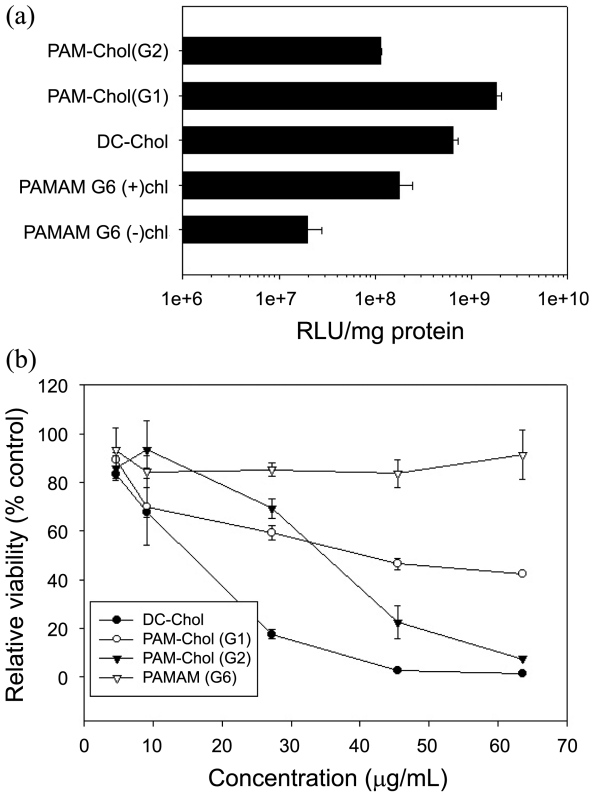


Figure 2. Transfection efficiencies (a) and cytotoxicity profiles (b) of PAMAM G6, PAM-Chol g = 1.0, PAM-Chol g = 2.0 and DC-Chol.

efficiency of PAM-Chol/DOPE g = 1.0 at the optimal condition was about 3- and 100-fold more efficient than those of DC-Chol/DOPE and PAMAM g = 6.0 (without chloroquine), respectively. PAM-Chol g = 1.0, which requires only one Michael addition/amidation step, showed significantly higher efficiency than PAMAM g = 6.0, which requires seven iterative Michael addition/amidation steps. Moreover, PAM-Chol might be a good candidate for *in vivo* gene delivery partially because it did not require a toxic molecule (chloroquine) to exhibit high efficiency. On the other hand, PAM-Chol g = 2.0 showed lower level of transfection efficiency than PAM-Chol g = 1.0. Although it was shown that multivalent lipids were more efficient than monovalent lipids in some research, the balance between the hydrophobic and hydrophilic groups was also important for high transfection efficiency.[9] Synthesis of further generations of PAM-Chol and analysis of their transfection efficiency could help more systematic approach to studying the charged group structure - transfection efficiency relationship.

The cellular toxicity of PAM-Chol derivatives were analyzed quantitatively by MTT assay (Figure 2(b)). The cytotoxicity of PAM-Chol liposomes was lower than DC-Chol, but higher than PAMAM g = 6.0. The cytotoxicity of cationic lipid was due to its incorporation within the plasma membrane.[10] The cationic moiety of PAM-Chol could be incorporated within the plasma membrane more easily by the hydrophobic modification to induce higher toxicity than the unmodified PAMAM dendrimer. However, because the toxicity of PAM-Chol was somewhat lower than that of commercial cationic lipid (DC-Chol), PAM-Chol was expected to be an attractive candidate as a transfection reagent for in vivo research and application.

In summary, PAM-Chol is the first dendritic lipid with a PAMAM head synthesized by a *de novo* approach. Like those of PAMAM dendrimer, the structure and number of charges of this dendritic lipid are well defined according to generation. Systematical investigation of the structure-function relationship of cationic lipid can be achieved by this synthetic approach. High transfection efficiency as well as low toxicity of PAM-Chol can help its utilization as a potential transfection reagent for efficient and safe gene delivery.

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