Synthesis of Diblock Copolymer, Methoxypoly(ethylene glycol)-*block*-Polyamidoamine Dendrimer and Its Generation-dependent Self-Assembly with Plasmid DNA

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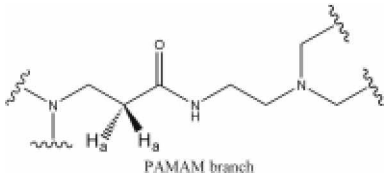
In recent years, various types of cationic polymers that can condense DNA have been extensively studied for gene delivery system.[1,2] Among them, dendrimers are very interesting polymers because of their well-defined structure and ease of surface functionality control.[3] Although these dendrimers such as PEI or PAMAM show high transfection efficiency, they have not overcome the cytotoxicity and the solubility problem of the polyplex with DNA yet.[4-7]

We have reported that methoxypoly(ethylene glycol)-*block*-poly(L-lysine) dendrimer (mPEG-PLLD) and PLLD-PEG-PLLD can form water-soluble nanoparticles with DNA.[8,9] On the one hand, Iyer *et al.* reported the new linear dendritic diblock copolymers with a poly(ethylene glycol) (PEG) block and a dendritic PAMAM block in 1998.[10] But they only showed properties of the block copolymer. So, we thought that it also could be used for DNA condensing because PAMAM dendrimer has been studied for gene delivery[11-13] and PEG brings many advantages such as water solubility, nonimmunogenicity, and improved biocompatibility when it is coupled to cationic polymers that usually form complexes with DNA.[14]

Here, we report the synthesis of the block copolymer, methoxypoly(ethylene glycol)-*block*-polyamidoamine dendrimer (mPEG-PAMAM) and generation-dependent characterization of complex formation of the copolymers with DNA.

The polymers were synthesized through following procedures. Methoxypoly(ethylene glycol)-amine (MW 5000, Shearwater Polymers, Huntsville, AL) was used as the polymeric supporter, and the PAMAM dendrimer was extended from PEG by divergent method. First, 500 mg of mPEG was dissolved in 20 mL methanol and added dropwise to 200 equiv. of methyl acrylate (Sigma-Aldrich, St. Louis, MO) kept at 37 °C for the complete reaction. After 48 h, the methanol and unreacted methyl acrylate were removed under vacuum. The residue was precipitated 2 times with excess of cold ethyl ether to remove residual methyl acrylate and dried under vacuum to remove ethyl ether, leaving a white solid, mPEG-PAMAM 0.5 G. Secondly, mPEG-PAMAM 0.5 G was dissolved in methanol and added dropwise to 200 equiv. of ethylenediamine (Sigma-Aldrich, St. Louis, MO) kept at 37 °C. After 48 h, methanol and ethylenediamine were removed under vacuum. The residue was precipitated with excess of ethyl ether samely to remove residual ethylenediamine and dried under vacuum to remove ethyl ether, leaving a weak yellow solid, mPEG-PAMAM 1.0 G. These 2 steps were performed four times repeatedly for the synthesis of the 5th generation of the dendritic copolymer. Each reaction's progress was monitored by ninhydrin test, and confirmed by 300 MHz ^{1}H NMR (Bruker DPX-300) and MALDI-TOF MS. The MALDI-TOF MS were done on a Voyager Biospectrometry Workstation (Perceptive Biosystems, Inc.) in the linear mode with 2,5-dihydroxybenzoic acid as matrix. To remove impurities, the product was dialyzed for 1 day against ultrapure water using Spectra/Por dialysis membrane (molecular weight cutoff = 3500, Spectrum, Los Angeles, CA) after the 2nd generation, only at full generation to avoid the degradation of ester bonds of each half generation of the copolymer, and lyophilized before use for next synthesis and analysis.



PAMAM branch

We have confirmed the synthesis of mPEG-PAMAM by using ^{1}H NMR and MALDI-TOF MS. For the 0.5th generation copolymer, ^{1}H NMR (MeOD): δ PEG (CH_2CH_2O) = 3.661 (b); δ PAMAM (-$COOCH_3$) = 3.665 (s); δ PAMAM (-CH_2COOCH_3) = 2.519 (t); δ PAMAM (protons next to tertiary amines) = 2.863 (t). For the 1st generation copolymer, ^{1}H NMR (D_2O): δ PEG (CH_2CH_2O) = 3.715 (b); δ PAMAM (-CH_2CONH-) = 2.459 (t); δ PAMAM (-$CONHCH_2$-) = 3.290 (t); δ PAMAM (protons next to tertiary amines) = 2.6-3.0 (m). At each generation, each proton peaks of NMR spectra were observed on almost same positions as above data (data not shown). However, as generation increases, peaks became broader. It is thought to be the general property of polymers. We identified the completion of synthesis by comparing the theoretical number of protons (a) with the experimental data determined by calculating the ratio of integral between proton (a) and methylene protons of PEG used for NMR standard (Table 1). The number (Mn).

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Table 1. Theoretically calculated and experimentally obtained ratio between proton (a) and PEG methylene proton of the full generation copolymer, and molecular weights. Each molecular weight and PDI were estimated by MALDI-TOF

polymer generation	theoretical # of proton (a)	theor. ratio	exp. ratio	theoretical MW	Mn	Mw	Mz	PDI (=Mw/Mn)
G3	28	0.056	0.054	7090	6697	6752	6806	1.01
G4	60	0.120	0.117	8914	8247	8299	8350	1.01
G5	128	0.256	0.250	12562	11171	11484	11778	1.03

weight (Mw) and z average molecular weight (Mz) and polydispersity index were determined by MALDI-TOF (Table 1).

Polydispersity indices calculated from the spectra were between 1.00 and 1.03 for all the polymers. The MALDI-TOF results agreed well with the theoretically expected molecular weights of the polymers. However, experimental values were always a few smaller than theoretical values and the differences between them increased according to generation of the polymer. It is because that in the case of dendrimer synthesis, as the generation of dendrimer increased, the loss from the defects increased. Also the counts of MALDI-TOF decreased according to generation of the polymer because of the difficulty of ionization. Figure 1 shows the spectra of MALDI-TOF MS of the 5th generation polymer.

We carried out gel retardation assay to identify the self-assembly of mPEG-PAMAM with plasmid DNA. Polyplexes were prepared at various N/P ratios ranging from 0.5 to 8.0. Polymer solutions were prepared from the 3rd to the 5th generation and each of them was added to DNA solutions carefully and samples were incubated for 30 min at room temperature. In general, other polyplexes of polymer such as, poly-L-lysine, PEI tend to aggregate in the solution. So, they have been prepared in the presence of 5% glucose to decrease the formation of aggregates.[15] However, we could not observe any formation of aggregates of mPEG-PAMAM polyplexes in the water even at 4 °C. It is thought that PEG enhance the solubility of polyplexes. The samples were electrophoresed on 0.7% agarose gel containing ethidium bromide in 1X TBE buffer at 100 V. After electophoresis, the gel was analyzed on UV illuminator to show the locations of polyplexes.

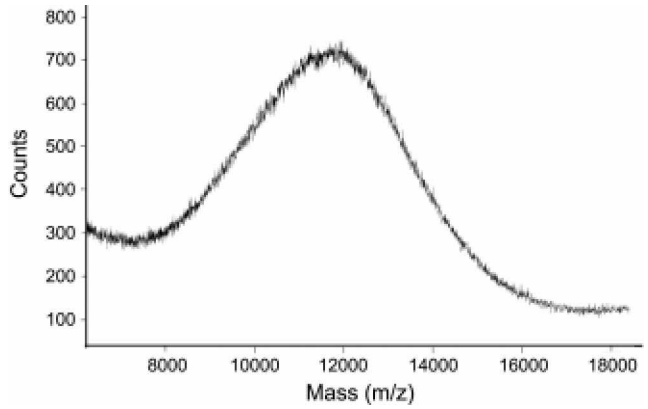

Figure 1. MALDI-TOF MS of the 5th generation polymer.

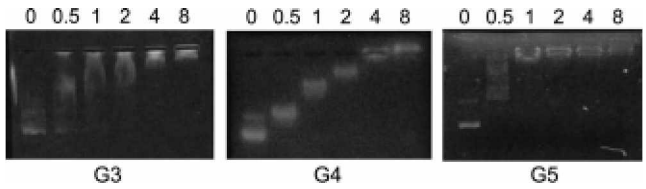

Figure 2. Gel Retardation Assay. The numbers above the gel represent the N/P ratio of the polyplex.

To our interest, when then the N/P ratio reached no less than 8, the 3rd generation polymer could not condense DNA. However, the 4th generation polymer could condense DNA at N/P ratio 4 and at the 5th generation, the polymer could make strong complex with DNA even at N/P ratio 1 (Figure 2). This result shows that as the charge density of the polymer increases, the condensing capability of the polymer increases consequently.

Then the size of polyplex was investigated by dynamic light scattering (DLS).[16] The measured average diameter of polyplex of the 5th generation copolymer was 165.2 ± 6.6 nm at N/P ratio 8. Based on this result, we came to a conclusion that the copolymer condensed plasmid DNA into nanoparticles efficiently.

In summary, we synthesized the diblock copolymer, methoxypoly(ethylene glycol)-*block*-polyamidoamine dendrimer and identified that it could self-assemble with plasmid DNA and form water-soluble nanoparticles. In a recent study, Luo *et al.* reported that the PEG-conjugated PAMAM dendrimer showed biocompatibility and high transfection efficiency for gene delivery.[17] Although they prepared the block copolymer by direct conjugation of PEG to PAMAM dendrimer contrary to our copolymer, it suggests the direction and the potentiality for gene delivery carrier of our further study. We will further study how the complex size and the transfection efficiency change depending upon the polymer generation, and measure the cytotoxicity and transfection efficiency of the block copolymer.

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References and Notes

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16. Light scattering experiments were performed at room temperature with BI-200SM Goniometer (Brookhaven Instruments Corporation) using a Lexel laser model 95 argon laser (100 mW output power at a wavelength of 514.5 nm). Correlator was PD2000 (Precision Detectors), and the scattering angle was 90°. Data were analyzed using the CONTIN algorithm. The sample was prepared by directly mixing each solution of polymer and DNA in water. The final DNA concentration was adjusted to 20 μg/mL.
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