Synthesis of a Barbell-like Triblock Copolymer, Poly(L-lysine) Dendrimer-*block*-Poly(ethylene glycol)-*block*-Poly(L-lysine) Dendrimer, and Its Self-Assembly with Plasmid DNA

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Abstract: A barbell-like ABA-type triblock copolymer, poly(L-lysine) dendrimer-*block*-poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer (PLLD-PEG-PLLD), was synthesized by the liquid-phase peptide synthesis method. The self-assembling complex formation of the third and fourth generation of the copolymer with plasmid DNA was studied. ^{1}H NMR and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) were used for the characterization of the synthesized copolymer. The self-assembling behavior of the 3rd and 4th generations of the copolymer with plasmid DNA was investigated by electrophoretic mobility shift assay, DNase I protection assay, and ethidium bromide exclusion assay. We observed great differences in the self-assembling ability of the 3rd and 4th generations of the polymer. This suggests that the number of positively charged amines per polymer molecule should be an important factor for the potential for self-assembling complex formation with DNA. Atomic force microscopy (AFM) and ζ potentials were used for evaluating the shape, size distribution, and surface charge of the complexes at various charge ratios. From AFM images, it was observed that the shape of the complex was nearly spherical and its size was about 50−150 nm in diameter. The *in vitro* cytotoxicity of the copolymer was compared with that of poly(L-lysine), poly(D-lysine), and polyethylenimine.

Introduction

In recent years, cationic polymers that can self-assemble with DNA have been extensively studied for transfecting exogenous genes into the mammalian cells.[1] Several cationic polymers were synthesized but their intrinsic drawbacks (e.g., solubility, cytotoxicity, and low transfection efficiency) limited their clinical use as *in vivo* gene carriers.[2−4] Among them, however, dendrimers were very attractive to many scientists designing gene carriers because of their well-defined structure and ease of control of surface functionality. Already, polyamidoamine[5] and polyethylenimine dendrimers[6] have been tested for their potential utility in the field of gene therapy and exhibited high transfection efficiency. However, these dendrimers have not overcome the problems of solubility of the complex with DNA and their cytotoxicity yet.

Block copolymers containing poly(ethylene glycol) (PEG) have been used for many drug carriers because of their high solubility in water, nonimmunogenicity, and improved biocompatiblity.[7] PEG has been coupled to polycationic polymers (e.g., poly(L-lysine), polyspermine, and polyethylenimine)[8−10] or liposomes[11] to improve the solubility of complexes with DNA and transfection efficiency. These PEG-coupled copolymers were reported to be capable of forming self-assembling complexes with plasmid DNA or antisense oligonucleotides[12] in submicrometer diameter and exhibit fairly high transfection efficiency at certain cell types *in vitro*.[4]

Poly(L-lysine) dendrimer[13] is another polycationic dendrimer containing a large number of surface amines and considered to be capable of electrostatic interaction with polyanions, such as nucleic acids. We previously coupled PEG and poly(L-lysine) dendrimer, an AB-type block copolymer.[14] We have also grafted PEG of small molecular weight to linear poly(L-lysine) (PLL) and reported that this comb-shaped copolymer showed improved transfection efficiency and cell viability in comparison with PLL.[15]

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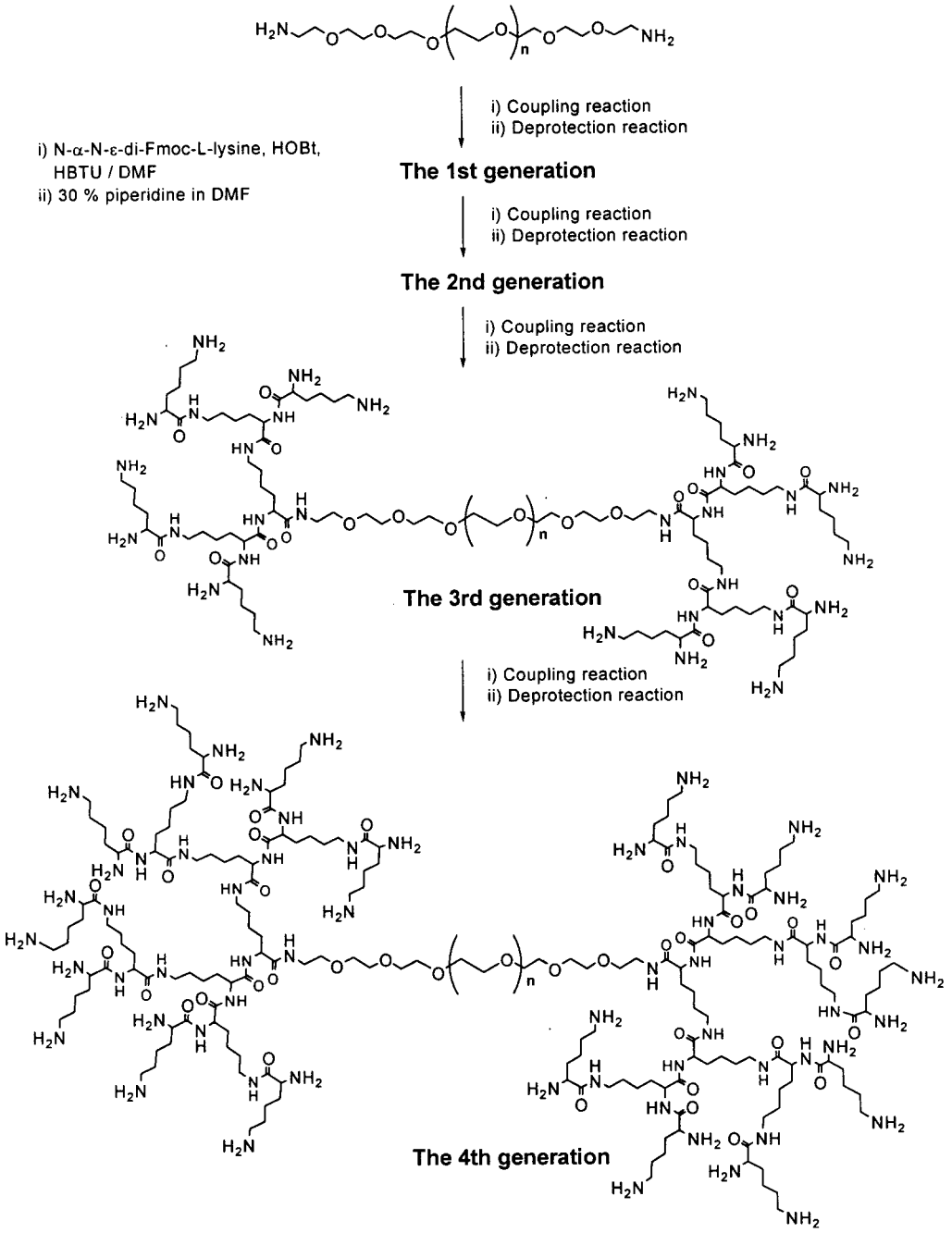
Scheme 1. Synthesis of $PLLD_n$-PEG-$PLLD_n$



Here we report another new concept of copolymer that takes advantage of the above-mentioned copolymers and dendrimers, poly(L-lysine) dendrimer-*block*-poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer (PLLD-PEG-PLLD), a barbell-like ABA-type triblock copolymer. We have designed and synthesized this series of hybrid dendrimer−linear polymer−dendrimer triblock copolymers especially for the formation of polyionic complex with plasmid DNA. To better understand the phenomena of supramolecular self-assembly between the hybrid copolymer with plasmid DNA, we have also reported the effect of the number of surface amines on the formation of complex with plasmid DNA.

These copolymers were synthesized by the liquid-phase peptide synthesis method with a stepwise approach up to the 4th generation (Scheme 1). By the divergent synthesis method, the number of surface amines was increased exponentially as the reaction proceeded (Table 1). The self-assembling behavior of the 3rd and 4th generations of the copolymer/pSV-β-gal plasmid DNA was investigated by electrophoretic mobility shift assay (EMSA), DNase I protection assay, and ethidium bromide exclusion assay. From the results, we observed some differences in the self-assembling capability of the 3rd and 4th generations of the polymer. Atomic force microscopy further revealed that only the 4th generation or possibly higher generations could condense DNA into spherical particles 50−150 nm in diameter. In addition, the cytotoxicity of the copolymer was evaluated in comparison with that of PLL, poly(D-lysine) (PDL), and polyethylenimine (PEI).

Experimental Section

Materials. Poly(ethylene glycol) bis(amine) (H_2N-PEG-NH_2), prepared from PEG with an average molecular weight of 3350, poly(L-lysine) (19.2 kDa), poly(D-lysine) (19.2 kDa), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from

Table 1. Characterization of Poly(L-lysine) Dendrimer-*block*-Poly(ethylene glycol)-*block*-Poly(L-lysine) Dendrimers ($PLLD_n$-PEG-$PLLD_n$)

polymer	peak intensity ratio[a] calcd	peak intensity ratio[a] exptl	M_n	M_w calcd	M_w exptl	no. of surface amines	IC_{50}[b]
H_2N-PEG-NH_2			3411		3473	2	
$PLLD_1$-PEG-$PLLD_1$	26.3	26.7	3682	3788	3729	4	
$PLLD_2$-PEG-$PLLD_2$	8.77	8.75	4178	4250	4241	8	
$PLLD_3$-PEG-$PLLD_3$	3.76	3.84	4908	5269	5265	16	2
$PLLD_4$-PEG-$PLLD_4$	1.75	1.91	7261	7317	7313	32	0.6

[a] Peak intensity ratio of the ethylene protons of PEG segment (δ 3.7 ppm) and the β-, γ-, and δ-methylene protons of PLL dendrimer (δ 1.4−1.8 ppm). [b] The charge ratios of the complex of the 3rd and 4th generations of copolymer with DNA needed to achieve 50% fluorescence inhibition.

Sigma Chemical Co. (St. Louis, MO). Polyethylenimine (PEI, 25 kDa) was from Aldrich (Milwaukee, WI). *N*-Hydroxybenzotriazol (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU), and *N*-α-*N*-ϵ-di-Fmoc-L-lysine were purchased from Anaspec (Inc., San Jose, CA) and pSV-β-gal plasmid DNA (6821bp, 4.33×10^6 Da) from Promega (Madison, WI). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO (Gaithersburg, MD). All other chemicals were purchased and used without any further purification

Techniques. ^{1}H NMR spectra were recorded in D_2O solution on a Bruker DPX-300 (300 MHz) apparatus. From the peak intensity ratio of the ethylene protons of the PEG segment (δ 3.7 ppm) and the β-, γ-, and δ-methylene protons of the PLL dendrimer (δ 1.4−1.8 ppm), the contents of lysine monomers in the copolymers were determined. The MALDI-TOF mass spectra were done on a Voyager Biospectrometry Workstation (Perseptive Biosystems, Inc) in the linear mode. A N_2 laser radiating at 337 nm wavelength with 3 ns pulses was used. The ions generated by the laser pulses were accelerated to 25 kV energy. For MALDI-TOF analysis, polymer solutions in H_2O at a concentration of 5 mg/mL were mixed in a 1:9 (v/v, polymer solution:matrix solution) ratio with a matrix solution of 2,5-dihydroxybenzoic acid (Aldrich) at a concentration of 10 mg/mL in H_2O. One microliter aliquots of sample were then loaded on the sample plate and dried *in vacuo*.

Synthesis of PLLD-PEG-PLLD. PLLD-PEG-PLLD was synthesized by the liquid-phase peptide synthesis method (Scheme 1). Poly(ethylene glycol) bis(amine) was used as the polymeric supporter, and the PLL dendrimer was prepared by repeated liquid-phase peptide synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry.[14,16] Poly(ethylene glycol) bis(amine) was dissolved in anhydrous *N*,*N*-dimethylformamide (DMF) with 4 equiv of HOBt, HBTU, and *N*-α-*N*-ϵ-di-Fmoc-L-lysine. Then, 10 equiv of *N*,*N*-diisopropylethylamine (DIPEA) were added to the reaction mixture. The reaction was allowed to proceed at room temperature for 12 h up to 3 days for the 4th generation of dendritic copolymer. After the coupling reaction was completed, the reaction product was precipitated with a 10-fold excess of cold ether to remove the excess coupling reagents and DMF and further washed twice with ether. All nonpolymeric reagents and byproducts are soluble in ether, including the activated lysine ester. Piperidine (30% in DMF) was used to deprotect the Fmoc group of lysine moiety and the deprotected product was precipitated and further washed with ether as described above. The mixture was then centrifuged to remove the ether, followed by drying in vacuo before the next coupling reaction proceeded. The coupling and deprotection reactions were performed three or four times for the synthesis of the 3rd or 4th generation of the dendritic copolymer. Each reaction's progress was monitored by ninhydrin test and confirmed by ^{1}H NMR and MALDI-TOF MS. The copolymer was dialyzed for 1 day against water using Spectra/Por dialysis membrane (molecular weight cutoff = 3500, Spectrum, Los Angeles, CA) and lyophilized before use for analysis and assay. ^{1}H NMR (D_2O): δ 1.50 (br m, $-CH_2CH_2CH_2-$), 3.00 (br m, CH_2-N), 3.70 (s, CH_2CH_2-O), 4.25 (br m, $COCH-N$).

Electrophoretic Mobility Shift Assay (EMSA) and DNase I Protection Assay of $PLLD_n$-PEG-$PLLD_n$/Plasmid DNA Complexes. PLLD-PEG-PLLD/plasmid DNA complexes at various charge ratios (+/−), ranging from 0.5 to 20, were formed in Hepes buffered saline (HBS, 20 mM Hepes, 150 mM NaCl, pH 7.4). After 30 min incubation for complex formation, each sample was electrophoresed on a 0.7% (w/v) agarose gel containing ethidium bromide (0.5 μg/mL in the gel) for EMSA, then illuminated on a UV illuminator to show the location of the DNA. DNase I protection assay was performed by the method originally developed by Niidome *et al*.,[17] with slight modifications. After the complexes, in which 2.5 μg of the plasmid DNA was mixed with various amounts of PLLD-PEG-PLLD corresponding to the above charge ratios in 50 μL of HBS, were formed as described above, 10 units (1 μL) of DNase I (Sigma) were added to the complex solution. After 20 min at room temperature 75 μL of stop solution consisting of 4 M ammonium acetate, 20 mM EDTA, and 2 mg/mL of glycogen was added, and the reaction mixture was placed on ice. To dissociate the plasmid DNA from the cationic copolymer, 37 μL of 1% SDS was added, followed by extraction with Tris/EDTA buffer-saturated phenol/chloroform, and DNA was then precipitated by absolute ethanol. The precipitated DNA was dissolved in TE buffer and subjected to 0.7% agarose gel electrophoresis (Figure 2). Charge ratios were calculated from the number of phosphate groups in the nucleic acid compared with the number of terminal NH_2 groups on a copolymer. For example, given that the number of bases in 1.0 μg of DNA is about 1.89×10^{15} negative charges are present per 1.0 μg of DNA, whereas the 4th generation of copolymer has about 2.66×10^{15} charges per microgram. Therefore, to obtain a 1:1 charge ratio, 1.0 μg of DNA was mixed with 0.71 μg of the 4th generation copolymer.

Ethidium Bromide Exclusion Assay. Ethidium bromide (1.0 μg) and 1.0 μg of plasmid DNA were mixed in 20 μL of HBS. After 10 min incubation at room temperature, various amounts of PLLD-PEG-PLLD (the third and fourth generations), ranging from 0.3 to 16 (+/−) were added to the plasmid DNA−ethidium bromide mixture and then incubated for 30 min. Before fluorescence intensity was measured using a spectrofluorometer (JASCO FP-777), the mixtures were diluted to 2 mL with HBS. Excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 260 and 600 nm, respectively. The fluorescence of the DNA solutions in HBS with ethidium bromide was set to 100%, measured against a background of ethidium bromide without DNA (Figure 3).

Atomic Force Microscopy (AFM). Atomic force microscopy (Nanoscope IIIa system, Digital Instruments, Inc., Santa Barbara. CA) was used for imaging the shape and particle size of the copolymer/plasmid DNA complexes at various charge ratios. One microliter aliquots of the aqueous complex solutions with final DNA amounts of 10 ng were loaded onto the center of a freshly split untreated mica disk. After adsorption for 1−2 min 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 (Figure 4).

ζ Potential Measurements. The 4th generations of copolymer/DNA complexes at four different charge ratios were formed in HBS. ζ potentials were measured at 25 °C using a Malvern 3000 system "Zetasizer" (Malvern Instrument Ltd, Malvern UK) equipped with a He−Ne laser at a wavelength of 680 nm.

Cytotoxicity Assay. Evaluation of the cytotoxicity was performed by the MTT assay.[18] A mouse embryonic fibroblast cell line (NIH3T3) was seeded in 96 well microplates at a density of 10000 cells per well in 0.1 mL growth medium DMEM containing 10% FBS. Each reagent was introduced to the cells and incubated for 48 h. The old medium was removed and replaced with new growth medium containing MTT. The plate was incubated for an additional 4 h at 37 °C and each medium was then removed prior to the addition of dimethyl sulfoxide to dissolve the formazan crystal formed by proliferating cells. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA) and demonstrated as a percent relative to untreated control cells.

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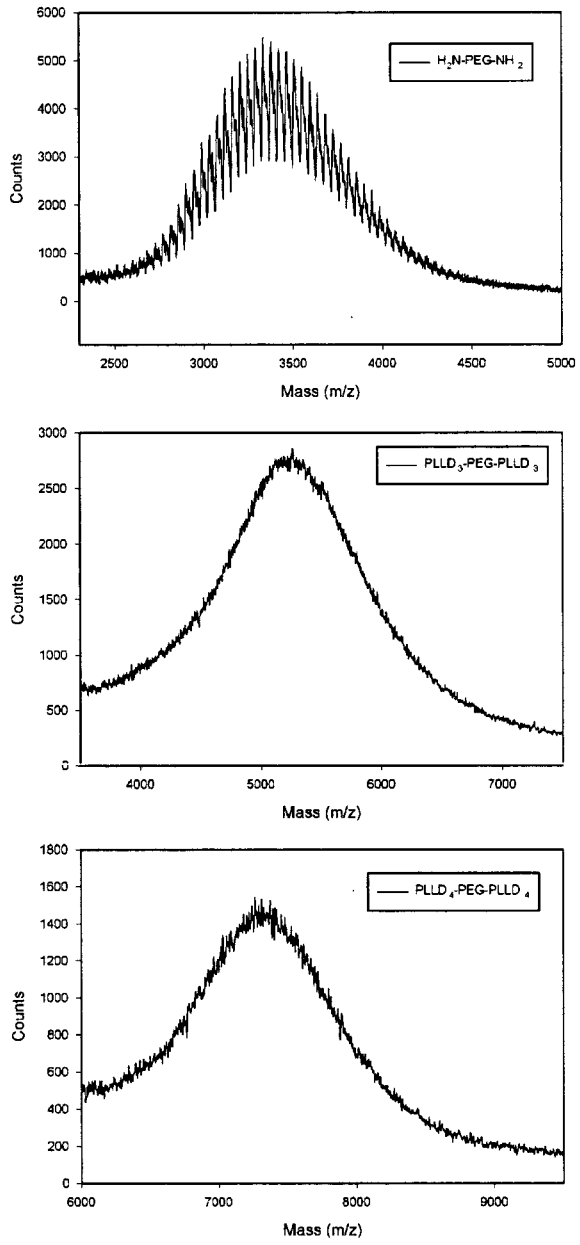

Figure 1. MALDI-TOF mass spectra of polyoxyethylene bis(amine) and PLLD-PEG-PLLD. These spectra were obtained in the continuous extraction mode. The samples were dissolved in water containing 2,5-dihydroxybenzoic acid as a matrix. The spectrum is the sum of 256 laser shots and it was 19-point Savizky−Golay smoothed. Top: poly(ethylene glycol) bis(amine) (H_2N-PEG-NH_2). Middle: 3rd generation of polymer. Bottom: 4th generation.

Results and Discussion

Synthesis of PLLD-PEG-PLLD. We have synthesized the triblock copolymer using the liquid-phase peptide synthesis method with a stepwise approach (Scheme 1). Each synthesis step was monitored by a ninhydrin test, ^{1}H NMR, and MALDI-TOF MS. The peak intensity ratio of the ethylene protons of the PEG segment (δ 3.7 ppm) and the β-, γ-, and δ-methylene protons of PLL dendrimer (δ 1.4−1.8 ppm) are listed in Table 1. A good correspondence was found between the theoretically expected and experimentally obtained values for all the products synthesized. From this, it was confirmed that the synthesis was complete. The number (M_n) and weight (M_w) average molecular weight and polydispersity index were determined by MALDI-TOF (Table 1). Polydispersity indices calculated from the spectra were between 1.01 and 1.02 for all the copolymers synthesized. The MALDI-TOF results agreed well with the theoretically expected molecular weights for the copolymers. Figure 1 shows MALDI-TOF mass spectra of polymeric supporter PEG and the 3rd and 4th generations of dendritic copolymers. In the case of PEG, each peak appeared discretely according to its degree of polymerization but such discrete peaks disappeared in the copolymer, perhaps due to the various ionic states of primary amines in the copolymer.

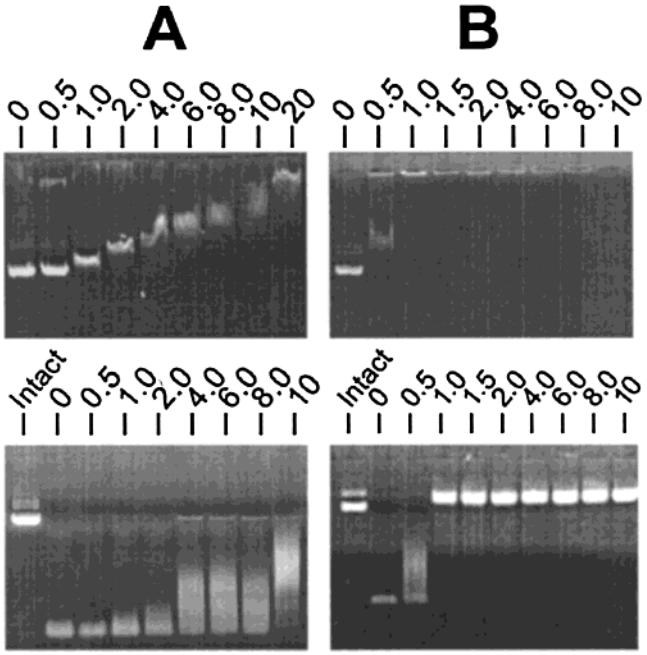

Figure 2. Electrophoretic Mobility shift assay (EMSA) and DNase I protection assay of PLLD$_n$-PEG-PLLD$_n$/plasmid DNA complexes. (A) EMSA (above) and DNase I protection assay (below) of PLLD$_3$-PEG-PLLD$_3$/plasmid DNA complexes. (B) EMSA (above) and DNase I protection assay (below) of PLLD$_4$-PEG-PLLD$_4$/plasmid DNA complexes. Charge ratios (+/−) are indicated above each lane and the first lane of the two lower figures is the intact DNA that is not attacked by DNase I.

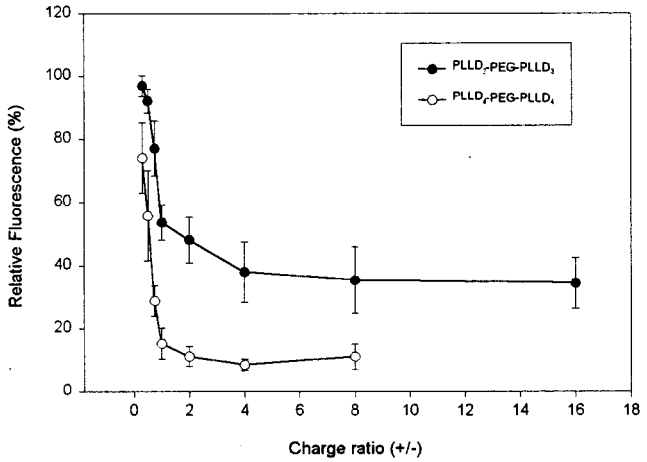

Figure 3. Ethidium bromide exclusion assay. The polymer/DNA charge ratio (+/−) was 0.3, 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, and 16, respectively. Assay at a charge ratio of 16 was tested only in the case of the complex of the 3rd generation of the polymer. Results are represented as relative fluorescence intensity (%) at each charge ratio and mean ± standard deviations are given ($n = 3$).

Agarose Gel Electrophoresis and Ethidium Bromide Exclusion Assay. The copolymer/DNA complex was formed by simple mixing copolymer and DNA. Unlike other polyelec-

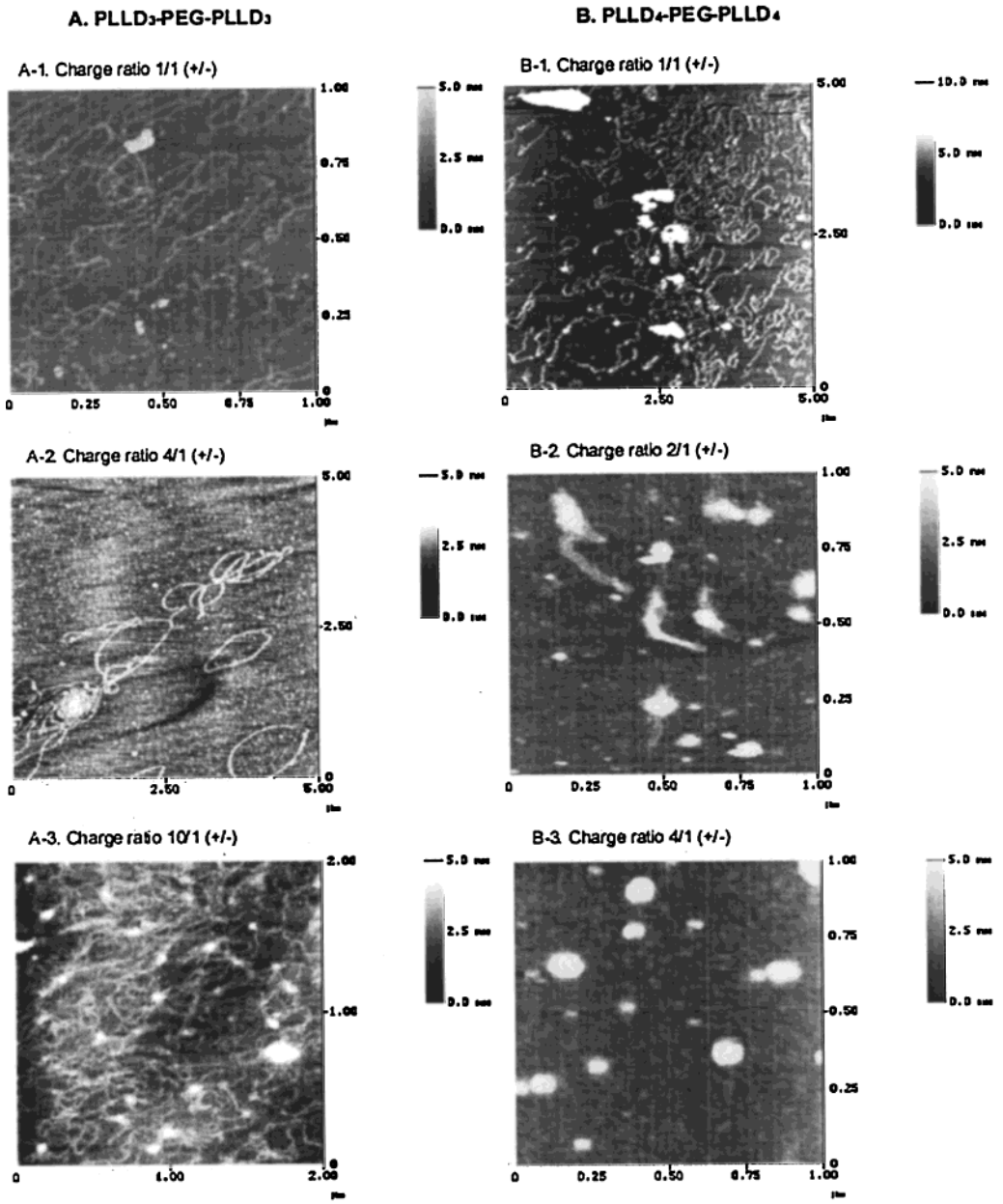

Figure 4. AFM images of polymer/DNA complexes: (A) 3rd generation and (B) 4th generation. Respective charge ratios are given in the figure.

trolytes, this PEG-coupled block copolymer was not precipitated and showed improved solubility in water when it formed a complex with DNA at all charge ratios studied.

The electrophoretic mobility shift assay of the complex of copolymer with plasmid DNA is shown in Figure 2. The complex of the 3rd generation of copolymer and DNA was not retarded completely even at a charge ratio of 20(+/−) (Figure 2A, above) but the complex of the 4th generation with DNA was retarded completely at a charge ratio of 1.0 (Figure 2B, above). While the 3rd generation could not protect DNA against DNase up to a charge ratio of 10 (Figure 2A, below), the 4th generation protected the DNA even at a charge ratio of 1.0 although most of the DNA was nicked once or twice by DNase (Figure 2B, below). This indicates that the interaction between the 3rd generation and DNA is not so strong as that with the 4th generation, and hence it cannot overcome the attack by DNase.

Figure 3 shows ethidium bromide exclusion assay by which the ability of complex formation with DNA can be measured. DNA fluorescence with ethidium bromide is inhibited when the DNA is condensed into particles by interaction with cationic copolymers. In both complexes of the 3rd and 4th generation, fluorescence intensity sharply decreased by increasing charge ratio to 1.0. The charge ratios of the complexes of the 3rd and 4th generations of copolymer with DNA needed to achieve 50% inhibition (IC_{50}) are shown in Table 1. Relative fluorescence intensity did not decrease below 35% in the complexes of the 3rd generation even at a charge ratio of 16, but it decreased below 10% in the case of the 4th generation. These differences between the 3rd and 4th generation copolymer may be due, at least in part, to the different number of surface amines per copolymer molecule, as reported by Wolfert *et al.*, who described that particle formation was dependent on the molecular weight of cationic polymer,[19] that is, on the number of positive charges per polymer molecule. Consequently, the 3rd generation copolymer requires twice as many moles as the 4th to electroneutralize plasmid DNA. Hence, the presence of the hydrophilic PEG block, which already exists in the partially complexed DNA, may provide steric hindrance to the further electrostatic

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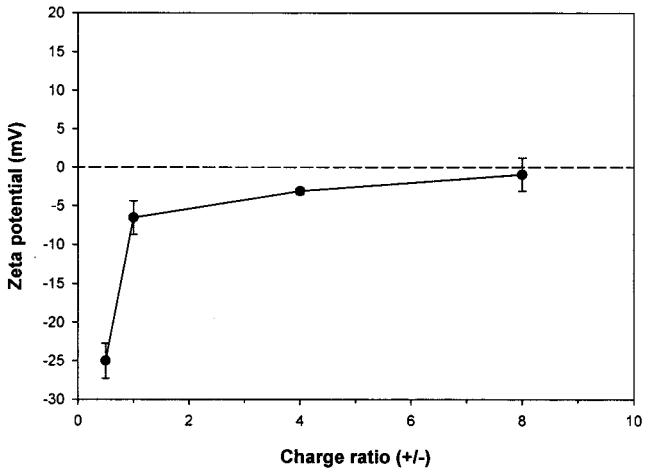

Figure 5. ζ potentials of polymer/DNA complexes at various charge ratios (+/−). Results are expressed as mean ± standard deviation ($n = 5$).

polyionic interaction[20] of free copolymers that is needed to fully neutralize plasmid DNA in the case of the 3rd generation copolymer.

Atomic Force Microscopy (AFM). AFM images of complex with polymer/DNA are shown in Figure 4. In the case of the complex composed of the 3rd generation/DNA, we could not observe compact particles at charge ratios ranging from 1.0 to 10. Only partially complexed DNA was imaged on AFM (Figure 4A), although the extent of complexation increased according to the increase of charge ratio. Figure 4B shows AFM images of complexes composed of 4th generation/DNA. At a charge ratio of 1.0, although the polymer could neutralize the negative charges of DNA (Figure 2B), the polymer could only partially condense DNA as in the case of the 3rd generation. As the charge ratio increased, the complex gradually assumed a spherical shape. At a charge ratio of 2.0, most DNA was condensed into a particle but a small portion of the DNA, which could not be fully compacted into a spherical particle, was still observed. And at a charge ratio of 4.0, nearly all complexes observed formed roughly spherical particles about 50−150 nm in diameter, in contrast to PEG-PLL which showed an extended toroidal structure when complexed with DNA.[4]

ζ Potential Measurements. The results of ζ potential analysis are depicted in Figure 5. Complexes were made of the 4th generation copolymer/DNA at four different charge ratios. Although the charge ratio was increased up to 8.0, ζ potential did not rise to positive values. From this, we suggest that PLL dendrimer blocks conjugated to both ends of PEG electrostatically interact with DNA, and accordingly the surface of the polyionic complex is coated with nonionic hydrophilic PEG chains (Figure 6). Therefore, condensates are electrostatically neutralized. This result corresponds to the report of Vinogradov *et al.*, who studied the self-assembly of polyamine−PEG copolymers with phosphorothioate oligonucleotides,[21] although their copolymers were made by coupling only one side of the PEG with polyamine, an AB-type block copolymer.

Cytotoxicity Assay. The cytotoxicity of PLLD-PEG-PLLD ($G = 3, 4$) was compared with that of PLL, PDL, and PEI which are generally used as gene delivery agents. As shown in Figure 7, the viability of NIH3T3 cells decreased abruptly according

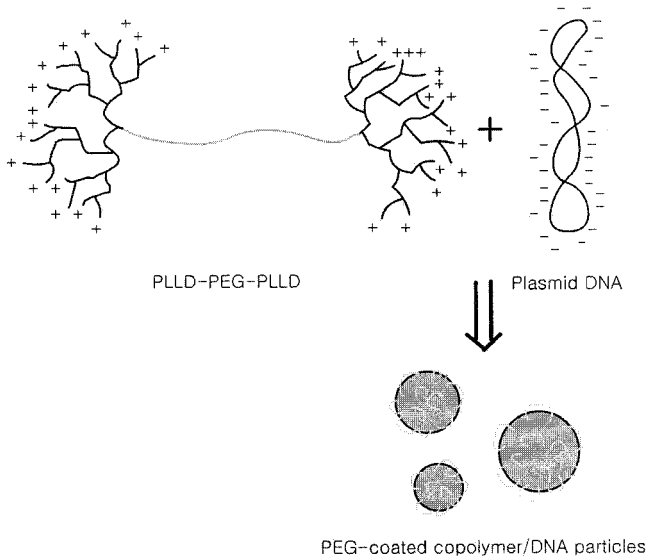

Figure 6. Schematic view of the formation of self-assembling complexes. The copolymer/DNA complex is coated with many hydrophilic PEG chains.

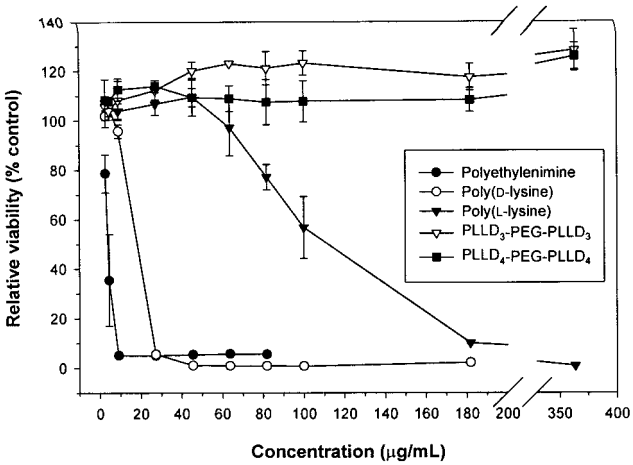

Figure 7. Effect of polyethylenimine, poly(D-lysine), poly(L-lysine), and PLLD-PEG-PLLD ($G = 3, 4$) on the viability of the NIH3T3 cell. Relative viability is expressed considering the absorbance at 570 nm of intact cells as 100%. Each data point is the average ± standard deviation of three different experiments.

to the increase of the concentration of nonbiodegradable polymers, such as PDL and PEI. Significant cytotoxicity was also observed in the case of biodegradable PLL. However, the PLLD-PEG-PLLD copolymers did not show any toxicity toward the cells even at higher concentration ranges. This is an important feature of this copolymer, for cytotoxicity is one of the major barriers in the *in vitro* and/or *in vivo* application. It should be noted that these *in vitro* assay data support the biocompatibility of the copolymer.

Conclusion

In this paper, we synthesized a biocompatible cationic block copolymer and examined its potential utility as a gene carrier. This copolymer showed remarkable biocompatibility relative to PLL, PDL, and PEI. These copolymers were easily synthesized up to the 4th generation by the liquid-phase peptide synthesis method without any problem. It is another type of hybrid triblock copolymers composed of PEG and cationic PLL dendrimers. By introducing PLLD blocks at both ends of linear PEG, the number of surface amines per microgram increased

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about 2-fold in comparison with PEG-PLLD, an AB-type block copolymer recently reported by our group[14] (2.66×10^{15} charges per μg for PLLD-PEG-PLLD; 1.26×10^{15} charges for PEG-PLLD). Aqueous solubility of the complexes formed by PLLD-PEG-PLLD copolymers is an advantage compared to homopolymer polycations and cationic lipids.[22,23] The positively charged PLL dendrimers interact with the negatively charged phosphates of plasmid DNA to form the inner hydrophobic core of particles. This inner core is surrounded by the hydrophilic PEG segment, which determines the aqueous solubility of these condensates.

The 3rd and 4th generations of these copolymers spontaneously formed a complex with plasmid DNA. Only the 4th generation, however, could form a spherical particle with DNA and protect the DNA from DNase attack. Hence the 4th or possibly the higher generations of copolymer can be used as gene carriers. We observed that the number of charges per copolymer is an important factor in condensing DNA into small particles and in determining other physicochemical characteristics of a polymer/DNA complex. Therefore, because the number of primary amines in our block copolymer can be easily controlled by increasing the order of generations, the physicochemical characteristics of these copolymer/DNA complexes can be optimized for clinical use. Furthermore, we are studying the effect of the length of the PEG chain on the copolymer/DNA complexes. It is expected that physicochemical characteristics and transfection efficiency might be optimized by changing the length of the PEG chain. This type of self-assembly is quite interesting from both a theoretical and practical point of view in designing polymers for gene delivery vectors, since it can serve as a suitable model for polyionic complex formation of other hybrid copolymers with DNA.

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