Poly(ethylene glycol)-*block*-poly(L-lysine) Dendrimer: Novel Linear Polymer/Dendrimer Block Copolymer Forming a Spherical Water-Soluble Polyionic Complex with DNA

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Methoxypoly(ethylene glycol)-*block*-poly(L-lysine) dendrimer was designed to form a water-soluble complex with plasmid DNA. The copolymer was synthesized by the liquid-phase peptide synthesis method. It was characterized by ^{1}H NMR and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrum. Agarose gel electrophoresis and DNase I protection assay proved that this linear polymer/dendrimer block copolymer assembled spontaneously with plasmid DNA, forming a water-soluble complex which increased the stability of the complexed DNA. Atomic force microscopy of the complex was evaluated at various charge ratios showing that the copolymer/DNA complex was like a globular shape.

Block copolymers containing poly(ethylene glycol) (PEG) have been used in various drug delivery systems (*1*). PEG is a very attractive polymer in some aspects. The polymer is nontoxic, nonimmunogenic, and effective in solubilizing other hydrophobic polymers coupled to it (*2*). PEG has been conjugated linearly to polycationic polymers such as poly(L-lysine) (PLL) (*3*) and polyspermine (*4*) in order to improve solubility and gene transfer efficiency. Also, we have demonstrated that PEG-*graft*-PLL, a comb-shaped copolymer, showed improved cell viability and transfection efficiency in comparison with PLL (*5*).

The physicochemical characteristics of the methoxypoly(ethylene glycol) (mPEG)-*block*-PLL copolymer/nucleic acid complex have been recently studied. The shape and size distributions of the complex were reported to be dependent on the nature of PLL and/or the nucleic acid. In the case of the mPEG-*b*-PLL (10 kDa) complexed with plasmid DNA, a large size exceeding 100 nm was observed, forming a extended toroidal structure (*3*). In comparison, the mPEG-*b*-PLL (20 mer)/salmon testes DNA complex demonstrated that a smaller sized complex was dominant with a small fraction of secondary aggregates (*6*). Also, the mPEG-*b*-PLL (18 mer) was analyzed to form a small spherical nanoassociate with antisense oligonucleotide (ca. 60 nm) by the dynamic light-scattering method (*7*).

Many synthetic polycationic polymers were synthesized and tested for their potential utility in the field of gene therapy. Among them, dendrimers—hyperbranched macromolecules—were distinctive due to their defined structure and large number of surface amino groups. Polycationic dendrimers are capable of electrostatic interaction with polyanions, such as nucleic acids. Therefore, some dendrimers were introduced for the transfer of antisense (*8*) or plasmid DNA (*9*) in vitro and showed remarkable efficiency.

In keeping with the polymers mentioned above, we report herein a conceptually new approach that is designed to conjugate linear PEG with a dense globular macromolecule, PLL dendrimer. We present the characteristics of the complex formed by plasmid DNA and this linear polymer/dendrimer block copolymer that is composed of biocompatible mPEG and L-lysine.

We synthesized the mPEG-*b*-PLL dendrimer in which 16 surface amines were present (generation 4, Figure 1A). mPEG-amine (Sigma, St. Louis, MO) was used as the polymeric supporter, and the PLL dendrimer was prepared by repeated liquid-phase peptide synthesis (*10*) using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry. mPEG-amine (M_w = 5757, M_n = 5697, M_w/M_n = 1.01, determined by MALDI-TOF mass spectrum) was stirred with 4 equiv of *N*-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-α-*N*-ϵ-di-Fmoc-L-lysine (AnaSpec, Inc., San Jose, CA), and *N*,*N*-diisopropylethylamine (DIPEA), respectively, in anhydrous *N*,*N*-dimethylformamide (DMF). After the coupling reaction reached completion, the mixture was precipitated with 10-fold excess of cold ether and further washed two times with ether. Piperidine (30%) was used for deprotection of the Fmoc group of lysine. The reaction mixture was precipitated and washed with cold ether as mentioned above. From the reaction mixture, only mPEG-coupled products were precipitated in cold ether, and other byproducts were removed by washing with excess ether. To remove small traces of excess reagents, the precipitate was recrystalized in pure ethanol. The precipitates were dried in vacuo and prepared for further coupling reaction. The coupling and deprotection reactions were repeated four times. The amounts of *N*-α-*N*-ϵ-di-Fmoc-L-lysine, HOBt, HBTU, and DIPEA were doubled in each coupling reaction to meet the 4 equiv. Each reaction progress was monitored by ninhydrin test and ^{1}H NMR until completed.[1] The copolymer was dialyzed for 1 day against water using Spectra/Por dialysis membrane (molecular weight cutoff = 6000−8000, Spectrum, Los Angeles, CA) and collected by freeze drying. This linear polymer/dendrimer block copolymer was further characterized by MALDI-TOF

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[1] ^{1}H NMR (D_2O): δ 1.64 [br m, $(CH_2)_3$], 3.08 (br m, CH_2−N), 3.39 (s, CH_3−O), 3.68 (s, CH_2CH_2−O), 4.25 (br m, COCH−N).

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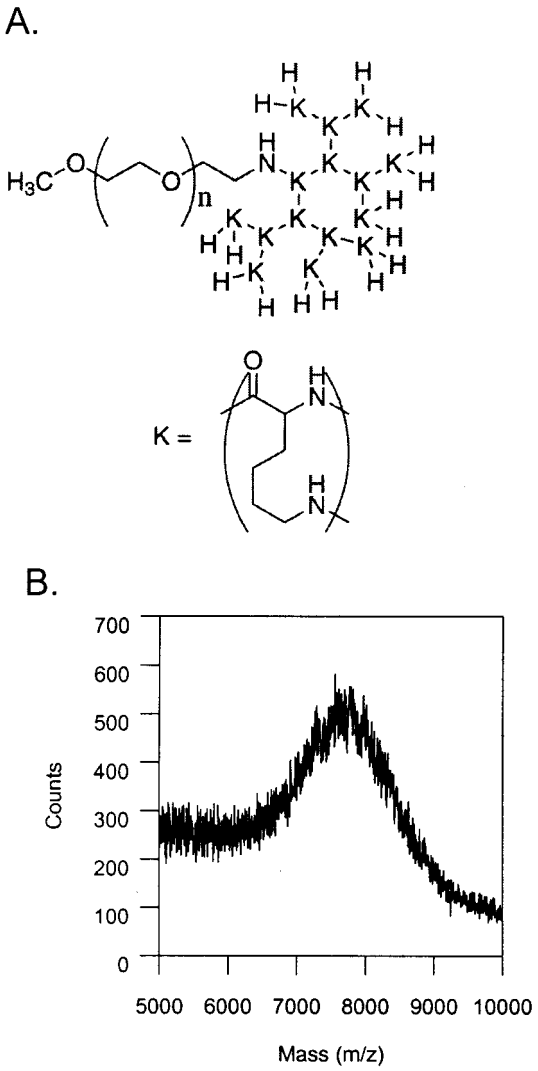

Figure 1. (A) Structural formula of mPEG-*b*-PLL dendrimer. (B) MALDI-TOF mass spectrum of the copolymer. The spectrum was obtained on a PerSeptive Biosystems instrument in the linear mode at 20 kV. The sample was dissolved in water containing 2,5-dihydroxybenzoic acid as a matrix.

mass spectrum (PerSeptive Biosystems, Inc., Framingham, MA) (Figure 1B). The M_w and M_n values of this copolymer were 7594 and 7553, respectively (M_w/M_n = 1.01).[2]

Figure 2 shows the agarose gel electrophoresis data of the copolymer/plasmid DNA associates. Complexes were formed at different charge ratios between the copolymer and pSV-β-gal plasmid DNA (Promega, Madison, WI) by incubation in 15 mM HEPES buffer (0.15 M NaCl, pH 7.4) at room temperature for 30 min. Each sample was then analyzed by electrophoresis on a 0.7% agarose gel containing ethidium bromide (0.5 μg/mL of gel). As the quantity of mPEG-*b*-PLL dendrimer increased, inhibition of migration into the agarose gel increased. At a cationic/anionic charge ratio of 2, complete retardation occurred (lane 3).

The shape and particle size distributions of the copolymer/plasmid complex at various charge ratios were examined by atomic force microscopy (NanoScope IIIa system, Digital Instruments, Inc., Santa Barbara, CA) under the same condition as reported previously with

[2] The M_w and M_n values of the product based on its structural formula were calculated to be 7678 and 7618, respectively.

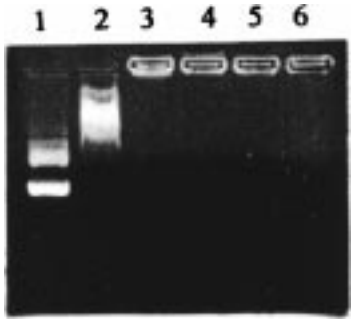

Figure 2. Analysis of complex formation at various charge ratios by agarose gel electrophoresis. pSV-β-gal plasmid DNA (1.0 μg) only (lane 1), charge ratio of copolymer/DNA = 1, 2, 3, 5, and 6 (lanes 2, 3, 4, 5, and 6, respectively). A total of 1.89×10^{15} negative charges are present per 1.0 μg of plasmid DNA, whereas the copolymer has 1.26×10^{15} charges/1.0 μg.

some modification (*3*). From the aqueous complex solution, 1−2 μL containing a final 10 μg/mL of plasmid DNA was deposited onto the surface of freshly split mica. After 1−2 min for adsorption, excess solution was removed by filter paper. The mica was further dried in room temperature and prepared for imaging. When the copolymer was mixed below the charge ratio enough to neutralize DNA, partially associated particles were observed (charge ratio of copolymer/DNA = 1, Figure 3A) as already shown in agarose gel electrophoresis (Figure 2, lane 2). Globular complexes were predominant in the case of charge ratio of copolymer/DNA = 2 (Figure 3B). When the charge ratio was raised to 4, even though a few further associated aggregates between some complexes were observed, most of the complexes were like globular shapes (Figure 3C). In both cases when the charge ratio of copolymer/DNA = 2 or 4, the size range was from ~50 to 250 nm. The average diameter was calculated to be 105 ± 67 nm (copolymer/DNA = 2, n = 30). It could be thought that the complex composed of the mPEG-*b*-PLL dendrimer/pSV-β-gal DNA forms an apparent globular structure, in contrast to the extended toroidal structures of the mPEG-*b*-PLL block copolymer/DNA complex (*3*). It was supposed that PLL dendrimer contained more condensed surface amines in a spatial arrangement than linear PLL and that the dendrimer behaved as a nondraining globular biopolymer (*11*), which led to the ability of this linear polymer/dendrimer block copolymer to form a spherical complex with plasmid DNA.

Nuclease resistance of the complexed DNA was examined at different charge ratios. After adding the copolymer to 4.0 μg of plasmid DNA at various charge ratios from 0 to 4 in 50 μL of 20 mM HEPES buffer (0.15 M NaCl, pH 7.4), the mixtures were incubated for 30 min at room temperature. After incubation, the mixtures were further incubated in the presence of 8.9 units of DNase I (Sigma, St. Louis, MO) for 20 min at room temperature. A total of 75 μL of stop solution (4 M ammonium acetate, 20 mM EDTA, and 2 mg/mL glycogen) was added to each mixture, which was kept in ice. After addition of 37 μL of 1% SDS, DNA was extracted with TE-saturated phenol/chloroform and precipitated by ethanol. The DNA was analyzed by 0.7% agarose gel electrophoresis (Figure 4). In the case of low charge ratios (lanes 2, 3, and 4), plasmid DNA was fragmented and linearized. The position of linearized plasmid DNA was compared with the molecular weight marker. But, nuclease resistance of the complexed DNA was improved much as the charge ratio of the copolymer/DNA increased up to 2 or 4 (lanes 5 and 6). No fragmentation was observed, and both supercoiled and open circular forms of plasmid DNA were obtained.

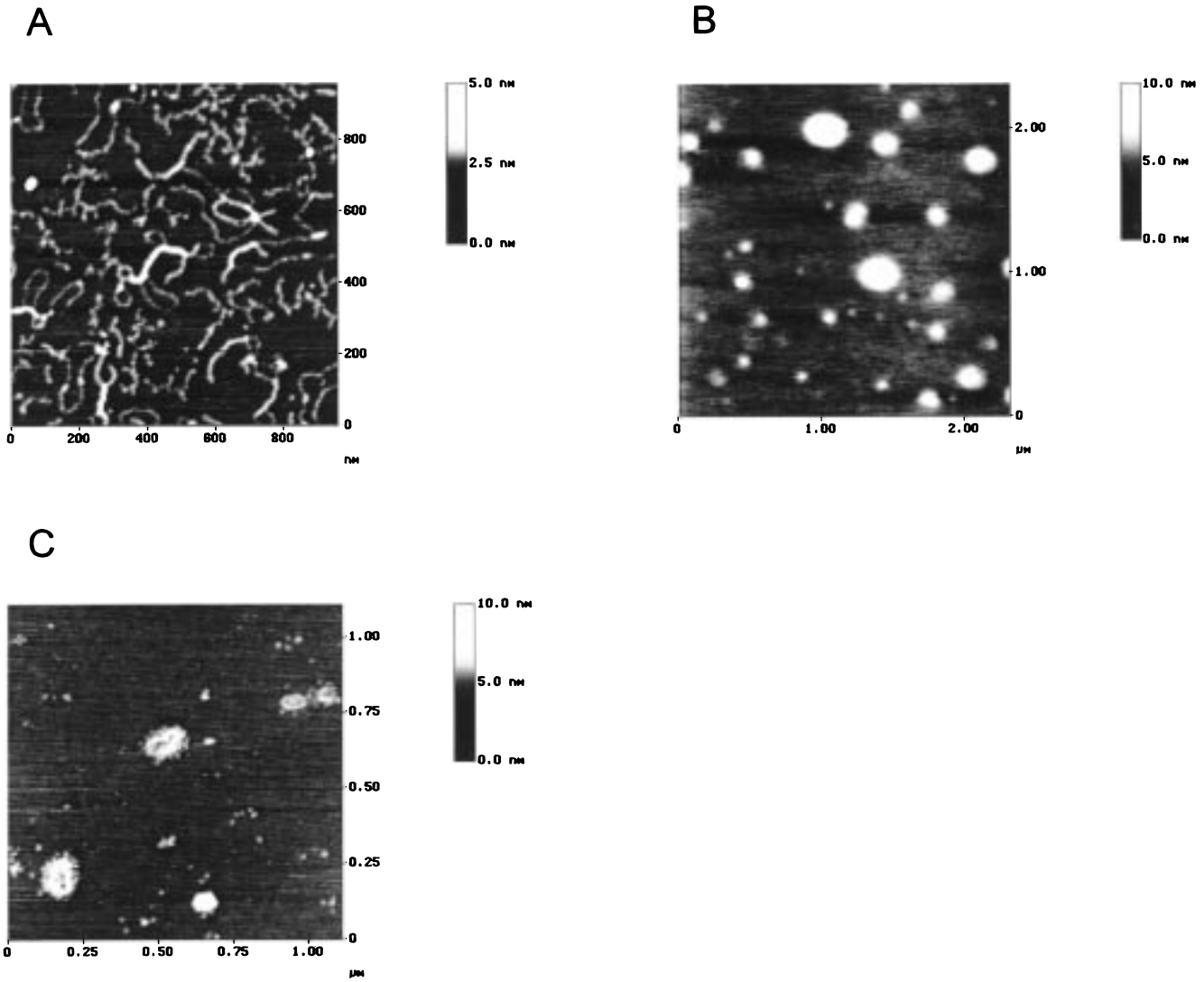

Figure 3. The atomic force microscopy images of the mPEG-*b*-PLL dendrimer/pSV-*β*-gal complex. Charge ratios of copolymer/DNA = 1, 2, and 4 (A, B, and C). The image mode was set to tapping mode. The white color indicates a height more than the designated nm above the mica surface. The *x* and *y* dimensions are scaled as shown.

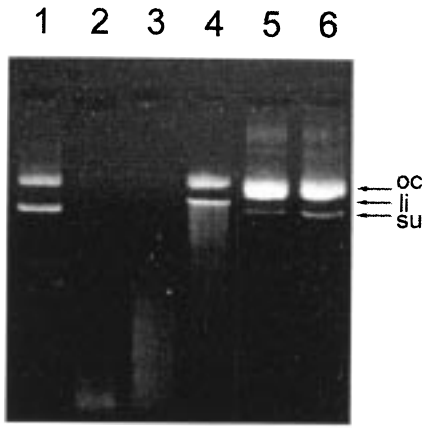

Figure 4. Stability of mPEG-*b*-PLL/plasmid DNA complexes to DNase I at various charge ratios. Undigested plasmid DNA (lane 1), Charge ratio of copolymer/DNA = 0, 0.5, 1, 2, and 4 (lanes 2, 3, 4, 5, and 6, respectively). The positions of the open circular (*oc*), linear (*li*), and supercoiled (*su*) forms are indicated on the right.

Moreover, the amount of supercoiled DNA increased as the charge ratio increased. Even though the complex could not completely protect the plasmid DNA, it is thought to be valuable in considering the recent report that the open circular form of plasmid DNA gave almost the same gene expression efficiency relative to supercoiled DNA (12).

In conclusion, it was proved that this novel linear/dendrimer block copolymer could self-assemble with plasmid DNA at physiological condition, forming a compact and water-soluble polyionic complex. The formed complex was found to take a globular shape with a relatively narrow size distribution. Nuclease resistance of the complex proved that the copolymer increased much the stability of the complexed plasmid DNA. In viewing the results obtained, this copolymer could be valuable in in vitro and/or in vivo delivery of genetic materials, such as antisense or plasmid DNA. Such a study is now in progress in our laboratory.

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