Characterization of Linear Polymer-Dendrimer Block Copolymer/Plasmid DNA Complexes: Formation of Core-shell Type Nanoparticles with DNA and Application to Gene Delivery *in Vitro*

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Received April 2, 2004

A hybrid linear polymer-dendrimer block copolymer, poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer, was synthesized and introduced to form polyionic complexes with DNA. The copolymer formed core-shell type nanoparticles with plasmid DNA. From dynamic light scattering experiments, the mean diameter of the polyplexes was observed to be 154.4 nm. The complex showed much increased water solubility compared to poly(L-lysine). The plasmid DNA in polyplexes was efficiently protected from the enzymatic digestion of DNase I. The cytotoxicity and transfection efficiency for 293 cells was measured in comparison with poly(L-lysine).

Key Words : Dendrimer, Block copolymer, Polyplex, Cytotoxicity, Gene delivery

Introduction

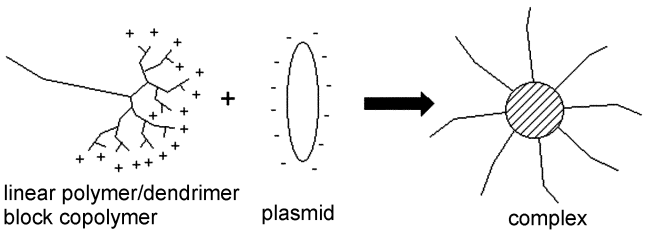
Research and development related to nonviral gene carriers comprising chemically synthesized molecules has increased enormously during the past decade. Polycationic polymers and cationic lipids have constituted the main subjects of the studies. Various polymers from synthetic to naturally occurring ones have been introduced and tested for their suitability in the field of gene therapy. Several cationic polymers were found to be promising but their intrinsic drawbacks, such as solubility, cytotoxicity and low transfection efficiency, limited their use as *in vivo* gene carriers.[1] Among them, however, dendrimers are still very attractive to many scientists for the design of gene carriers because of their well-defined structure and ease of control of surface functionality. Already, both polyamidoamine dendrimer and polyethylenimine dendrimer have been tested for their potential utility and have exhibited high transfection efficiency *in vitro* and *in vivo*.[2,3] However, these dendrimers have not yet overcome the problems of solubility of the complex with DNA and cytotoxicity.

Block copolymers containing poly(ethylene glycol) (PEG) have been used for many drug carriers owing to their high solubility in water, non-immunogenicity and improved biocompatiblity.[4] PEG has also been coupled to numerous polycationic polymers, such as poly(L-lysine), polyspermine, and polyethylenimine or liposomes.[5-9] Therefore, the conjugated PEG helps the reagents to improve the half-life in the blood stream, to increase the solubility, and to reduce the immune reaction of complexes with DNA.

The focus of this paper is to present the characteristics of polyionic complex formation of the copolymer with plasmid DNA *via* supramolecular self-assembly and the application to practical *in vitro* tests. The globular macromolecule, poly(L-lysine) dendrimer (PLLD) was coupled to the linear PEG by the repetitive liquid-phase peptide synthesis method.[10] Poly(L-lysine) dendrimer[11-13] is another polycationic dendrimer containing a large number of surface amines and considered to be capable of electrostatic interaction with nucleic acids.[14] As presented in Scheme 1, the copolymer is considered to self-assemble with plasmid DNA forming core-shell type nanoparticles. The enhanced aqueous solubility of the complex is an advantage compared to that of polycationic polymers and cationic lipids. The complexed plasmid DNA showed greatly increased stability against the enzymatic digestion of DNase I. The results of the cytotoxicity tests and transfection experiments for the copolymer were compared to those of poly(L-lysine) for 293 cells.

Materials and Methods

Materials. Methoxy-poly(ethylene glycol)-amine (mPEG-



Scheme 1. Schematic view of the formation of self-assembling complexes. The polyionic complexes are coated with hydrophilic PEG chains.

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NH_2, M_w = 5757, M_n = 5697, M_w/M_n = 1.01, determined by MALDI-TOF mass spectrum), poly(L-lysine) (PLL, 19.2 kDa), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and Herring testes DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Piperidine, *N,N*-dimethylformamide (DMF), and *N,N*-diisopropylethylamine (DIPEA) were from Aldrich (Milwaukee, WI). *N*-hydroxybenzotriazol (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) and *N*-a-*N*-e-*di*-Fmoc-L-lysine were purchased from Anaspec (Inc., San Jose, CA) and pSV-β-gal plasmid DNA (6821 bp, 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).

Synthesis of the hybrid block copolymer. PEG-PLLD was synthesized as previously described.[14] Briefly, mPEG-amine was used as the polymeric supporter and the poly(L-lysine) dendrimer was prepared by repeated liquid phase peptide synthesis 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 6 equivalents of HOBt, HBTU, *N*-α-*N*-ε-di-Fmoc-L-lysine, and DIPEA, respectively in anhydrous DMF. After the coupling reaction reaches to completion, the mixture was precipitated with 10-fold excess of cold ether and further washed 2 times with ether. 30 % piperidine was used for deprotection of Fmoc groups of lysine residues. The reaction mixture was precipitated and washed with cold ether as mentioned above. The precipitates were dried *in vacuo* and prepared for further coupling reaction. The coupling and deprotection reactions were repeated 4 times. Each reaction progress was monitored by ninhydrin test and ^{1}H NMR until completed. The copolymer was dialyzed for 1 day against water using Spectra/Por dialysis membrane (molecular weight cut-off = 6000-8000, Spectrum, Los Angeles, CA) and collected by freeze-drying. This linear polymer/dendrimer block copolymer was further characterized by MALDI-TOF mass spectrum (PerSeptive Biosystems, Inc., Framingham, MA). The M_w and M_n value of the 4th generation copolymer were 7594 and 7553, respectively (M_w/M_n = 1.01). The M_w and M_n values of the product based on its structural formula were calculated to be 7678 and 7618, respectively. ^{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_2O-), 4.25 (br m, COCH-NH).

Plasmid preparation. Plasmid pSV-β-gal which expresses the β-galactosidase reporter gene was amplified in *Escherichia coli* and amplified as reported previously.[15]

Agarose gel electrophoresis studies. Complexes were formed at different charge ratios between the polymer and pSV-β-gal plasmid by incubating in HEPES buffer (25 mM, pH 7.4, 10 mM $MgCl_2$) at room temperature for 30 min. Herring testes DNA was sheared and used as described by Choy *et al.*.[16] Each sample was then analyzed by electrophoresis on a 0.7% agarose gel containing ethidium bromide (0.5 μg/mL of gel).

Dynamic light scattering. The Z-averaged particle size of the complexes were determined by the Malvern 4700 system using a 25-mW He-Ne laser (λ = 633 nm) as a incident beam at a scattering angle of 90^o and Automeasure software version 3.2 was used for analysis (Malvern Instruent LTd. UK).

DNase I protection assay. The copolymer was mixed with pSV-β-gal DNA at a charge ratio of 4 in 15 mM HEPES buffer (0.15 M NaCl, pH 7.4) containing 5 mM $MgSO_4$ for 30 min at room temperature. The absorbance change at 260 nm was observed to estimate plasmid degradation after the addition of 18 units of DNase I (Sigma, St. Louis, MO).

Solubility test of the polyplex. Each polymer was mixed with 26 μg of plasmid DNA at a charge ratio of 4 in 0.5 mL of 20 mM HEPES buffer (0.15 M NaCl, pH 7.4).[17] After incubation for 30 min at room temperature, each solution was centrifuged for 5 min at 13000 rpm, 10 ^{o}C. The absorbance of each supernatant was measured at 260 nm. The absorbance was calculated as percentage in comparison with that of DNA only solution.

Cell culture. Human embryonic kidney 293 cells (ATCC, Rockville, MD) were grown in DMEM with 10% FBS. The cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37 ^{o}C in a humidified 5% CO_2/95% air containing atmosphere.

***In vitro* cytotoxicity test.** 293 cells were seeded in 96 well (10,000 cells per well) and incubated for a day prior to the incubation with polymers. Poly(L-lysine) and PEG-PLLD were introduced to the cells and incubated for 48 hours, respectively. The cytotoxicity was determined by comparing the amount of MTT reduced by cells treated with carriers to that reduced by control cells.[18]

Transfection procedure. The experiments were performed as reported previously with some modifications.[17] For transfection, 1×10^4 cells per well were seeded in 96 well plates one day prior to transfection experiments, and grown in the appropriated medium with 10% FBS. The cell lines were 60-70% confluent at the time of transfection. Complexes were prepared by mixing each reagent with plasmid DNA (1 μg per well) in FBS-free media. Each complex solution was further incubated for 30 min at room temperature and added to the cells. Transfection was performed in serum-free media for 4 hours in the presence of 100 μM chloroquine for all the reagents. Media was replaced with fresh complete media and gene expression was assayed after 48 hours post transfection. Control transfections were performed by using commercially available reagents, poly-(L-lysine).

Transfection assay. The expressed β-galactosidase activity was measured by standard method as recommended by manufacturer.[19] Briefly, each cell in a 96 well plate was washed with DPBS and lysed with Reporter lysis buffer. The cell lysates were analyzed using the colorimetric ONPG assay in a 96-well plate format.

Results and Discussion

Synthesis of PEG-PLLD. Recently, numerous PEG-

containing polycationic copolymers were synthesized and tested for gene delivery research.[5,6,8,17] Among them, PLL is one of the most widely used materials and is usually coupled to PEG by the ring opening polymerization method.[7,20] Instead of linear PLL, we conjugated poly(L-lysine) dendrimer (PLLD) to PEG. As in the synthesis scheme outlined in Figure 1, PLLD was coupled to methoxy-PEG-amine in a step-wise divergent method.[14] This method has two points of merit in comparison to the ring opening method. First, this method does not require a hazardous reagent such as triphosgene that is routinely used in the ring-opening polymerization method. Second, in the case of ring-opening polymerization, it is hard to control the number of repeating units but it is possible to exactly control the poly(L-lysine) residues by the liquid phase method.

Polyionic complex formation with DNA. Interestingly, when PEG-PLLD (generation 3 and 4) was mixed with plasmid DNA, generation 3 copolymer could not form completely complexed polyplexes (Fig. 2A). This phenomenon was also observed even at higher charge ratios (data not

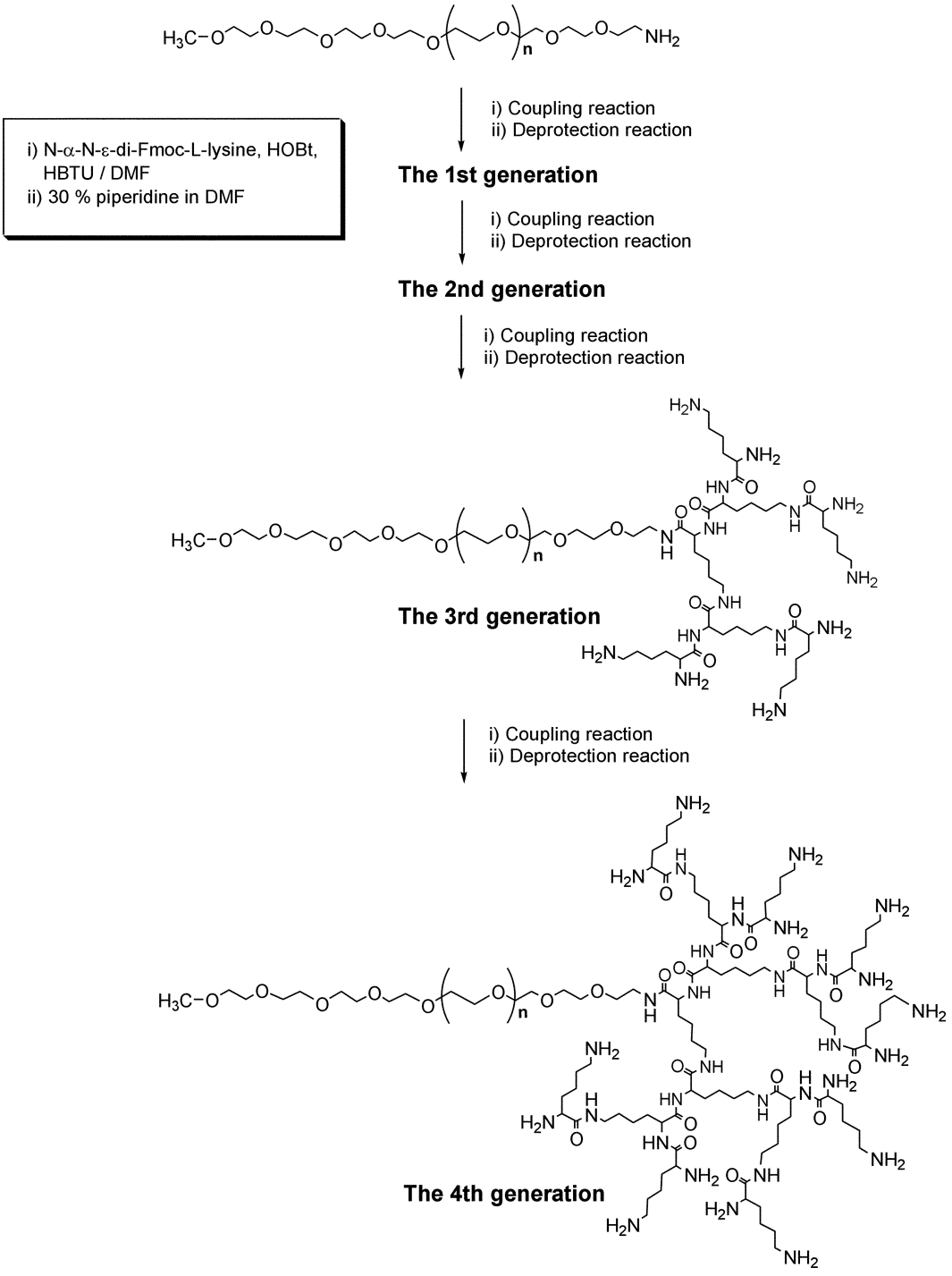

Figure 1. Outline of step-wise liquid phase synthesis of poly(ethylene glycol)-*block*-poly(L-lysine) dendrimer (PEG-PLLD).

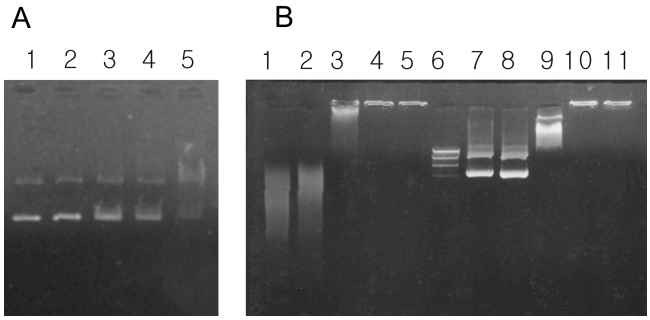

Figure 2. Analysis of complex formation at various charge ratios by agarose gel electrophoresis. (A) PEG-PLLD (generation 3) 1.0 μg of pSV-β-gal plasmid DNA only (Lane 1), charge ratio of copolymer/DNA = 0.5, 1, 2, and 4 (Lane 2, 3, 4, and 5, respectively). (B) PEG-PLLD (generation 4) 1.0 μg of sheared Herring testes DNA only (Lane 1), charge ratio = 0.5, 1, 2, and 4 (Lane 2, 3, 4, and 5, respectively). Lane 6 is λ/*Hin*dIII cut DNA marker (23.1, 9.4, 6.5, 4.3 kbp). 1.0 μg of pSV-β-gal plasmid DNA only (Lane 7), charge ratio of copolymer/DNA = 0.5, 1, 2, and 4 (Lane 8, 9, 10, and 11, respectively).

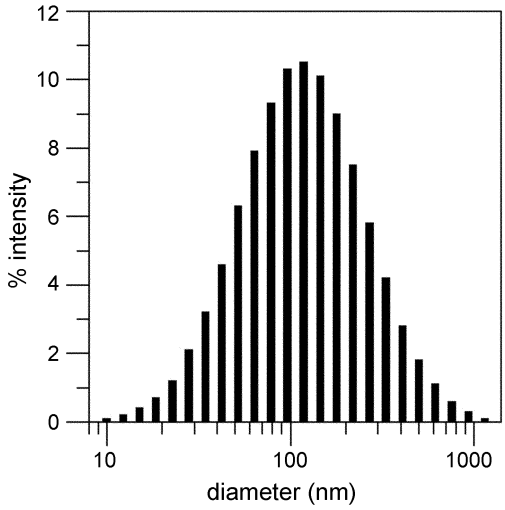

Figure 3. Dynamic light scattering of PEG-PLLD/pSV-β-gal plasmid DNA complexes.

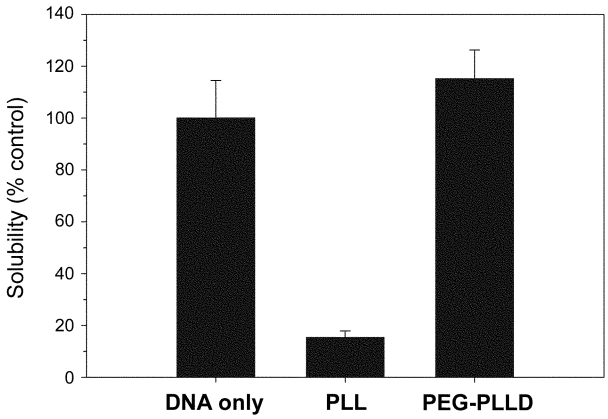

Figure 4. Water-solubility test of PEG-PLLD/DNA polyplex. Poly(L-lysine) was used as control reagent.

shown). As for generation 4, however, it efficiently constructed fully retarded complexes with various sizes of sheared herring testes DNA as well as plasmid DNA (Fig. 2 B). It is supposed that the complex formation with DNA is dependent on the generation of the copolymer and at least generation 4 is required for sufficient complexation.[21]

The reason why such a low generation copolymer forms partially complexed polyplexes could be explained as follows. As for lower generation copolymers, some part of the plasmid DNA may interact with the copolymer but more cationic charges are required to fully compensate for the excess negative phosphate anions of DNA backbones. However, at a certain concentration level, further copolymers could not be incorporated into the preformed polyplexes because of steric hindrance of poly(ethylene glycol) chains that are part of partially formed complexes. As for higher generation copolymers, the charge density per copolymer is considered to be high enough to form fully complexed polyplexes at a lower concentration level.

Formation of nanoparticles. The size distribution of PEG-PLLD/DNA complexes was analyzed by dynamic light scattering.[22] As shown in Figure 3, the copolymer formed nanoparticles with plasmid DNA with a mean diameter of 154.4 nm. Plasmid DNA with a diameter ranging from *ca.* 0.5 to 1 μm was efficiently condensed into nanometer sized particles. Complex formation at the nanometer scale level is generally considered to be important in polyplex-mediated gene delivery.

Solubility test in aqueous media. Water-solubility of polyplexes is one of the major problems encountered by many other cationic polymers currently used in gene transfer experiments. This is because if a polymer forms insoluble precipitates with DNA in aqueous media, it is not suitable for an injectable gene delivery system. So, a solubility test was perfomed for PEG-PLLD, and poly(L-lysine) was introduced as control reagent. As presented in Figure 4, the DNA complexes with PLL showed a very low level of solubility in water. On the other hand, PEG-PLLD/DNA showed much increased solubility, almost the same level as that of naked DNA itself. These phenomena also prove the core-shell forming characteristics of the copolymer with DNA. It is well known that conjugating linear PEG increases the solubility of many hydrophobic materials.[4]

Stability of the complexed DNA. DNase I was treated to the formed complex to investigate whether plasmid DNA was protected from the attack of the enzyme. After the addition of DNase I to the polymer/DNA mixture, hyperchromicity was measured by the absorbance difference at 260 nm as a function of time. As shown in Fig. 5, most of the plasmid DNA was degraded in less than 2 min. However, the degradation of plasmid DNA was totally prevented by treating the DNA with the copolymer. This result shows that the PEG groups of this copolymer form the outer shell of the polyionic complex hindering the action of DNase I. It is consistent with the previously described result concerning the block copolymer, poly(ethlene oxide)-*block*-poly(L-lysine).[20,23]

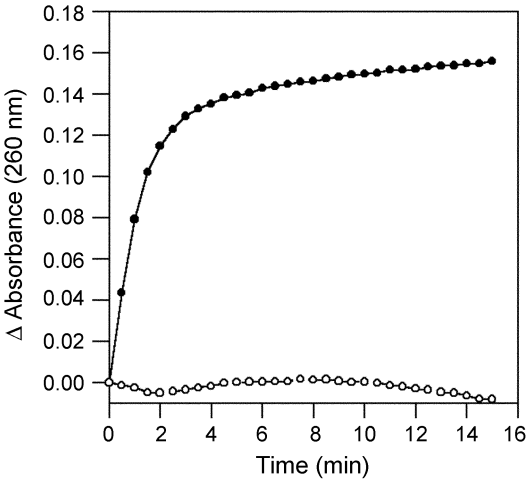

Figure 5. DNase I protection assay of PEG-*b*-poly(L-lysine) dendrimer/pSV-β-gal DNA complex. The difference in absorbance at 260 nm versus time was plotted. Plasmid DNA only (●), and 5.6 μg of copolymer per 1.0 μg of plasmid DNA (○).

Cytotoxicity test. A test of the time- and concentration-dependent cytotoxicity of PEG-PLLD was performed and the results are shown in Figure 6. The cells were exposed to each polymer for 4 hr or 24 hr and the toxicity for each condition was presented. For 4 hr incubation, PLL was a little toxic to the cells causing *ca*. 80% viability. However, when the time is prolonged to 24 hr, PLL caused significant toxicity to the cells even at the level of 10 μg/mL. But, the PEG-PLLD copolymer was shown not to have any influence on cell viability for either condition even at a higher concentration level of 200 μg/mL (Fig. 6). This is a quite outstanding characteristic of the copolymer, since there was no harm to the cells even at much elevated concentrations.

Transfection efficiency. The PEG chain of the polymer was considered to possess a putative fusogenic activity,

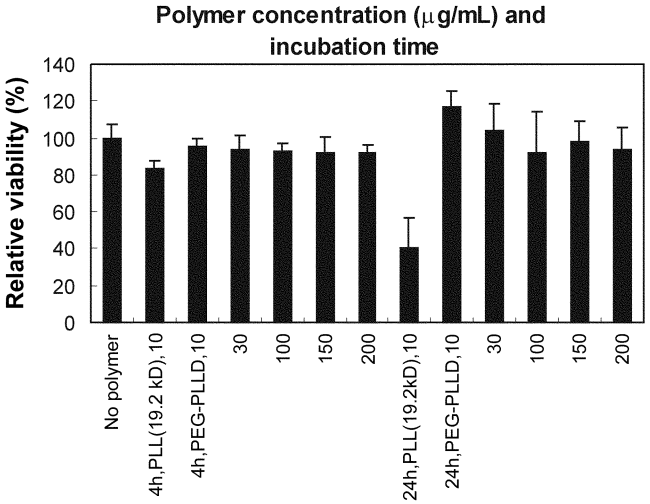

Figure 6. Effect of poly(L-lysine) and PEG-PLLD (G = 4) on the viability of 293 cells. Relative viability is expressed considering the absorbance at 570 nm of intact cells as 100%. Each data point is the average ± SD of six different experiments.

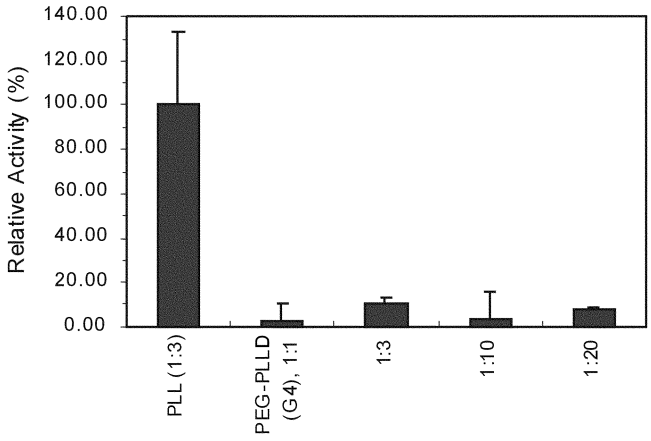

Figure 7. Transfection efficiency for 293 cell lines. The weight ratio of DNA and poly(L-lysine) was 1 μg and 3 μg per well, respectively (1 : 3). Various weight ratios of DNA : PEG-PLLD (generation 4) were tested as indicated.

which might help the complexes to interact with the cell membrane effectively leading to increased transfection efficiency. However, the transgene expression level was observed to be too low to be detectable. Transfection efficiency of PLL and PEG-PLLD was tested for 293 human embryonic kidney cells *in vitro*. The expressed β-galactosidase in cell lysates hydrolyzes ONPG and produces a yellow color. Absorbance at 405 nm was measured and presented in Figure 7. In comparison with PLL, the 4th generation of PEG-PLLD showed a very low level of transfection efficiency, which was almost comparable to the basal level of negative control. In considering the results, it is more likely that the large exclusion volume of PEG polymer and its high mobility in water might cause interference in the interaction between the polyionic complexes with the cell membranes. The results coincide with some previous reports.[8,24]

In summary, the PEG-PLLD block copolymer efficiently forms nanoparticles with plasmid DNA. The complexes showed remarkable water-solubility and biocompatibility toward cell lines. However, its *in vitro* transfection level was not significant and even much lower than that of PLL. In keeping with the physicochemical merits of the polymer, further trials to increase gene expression efficiency are being undertaken in our laboratory for future possible *in vivo* applications.

Acknowledgement. This work was supported by grants from the Center for Molecular Catalysis at Seoul National University, Brain Korea 21 program, and the Research Fund from the Ministry of Commerce, Industry and Energy.

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