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# POLY-L-LYSINE-*GRAFT*-PEG COMB-TYPE POLYCATION COPOLYMERS FOR GENE DELIVERY

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Key Words: Poly-L-lysine, PEG, Copolymer, Graft, Delivery, DN

## ABSTRACT

Polycations have been used for gene delivery *in vitro* quite successfully, however, *in vivo* applications suffered from serum effects that lower the overall gene drug efficiency. PEG polymers have been used extensively to minimize serum effects and create "stealth liposomes", biocompatible materials, and proteins with extended circulation. Here, we report our efforts towards creating "stealth polyplexes". A comb-type polycation, poly-L-lysine-*graft*-PEG copolymers were successfully prepared by ring opening of PEG-epoxide with ε-amino lysine groups of linear poly-L-lysine. The ratios of PEG-epoxide to poly-L-lysine, PEG-epoxide size (2K, 3K, and 5K), and poly-L-lysine size (10K, 26K, and 38K) were varied. Copolymers with as little as 2% grafted PEG chains sterically stabilized DNA/copolymer complexes (polyplexes) even at charge neutrality. These polyplexes, formed with copolymers with various size of PEG chains grafted on various lenghths of poly-L-lysine backbone, remained

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relatively small, approximately 100 nm in saline With higher degree of grafting, the binding was significantly diminished. In addition, the morphology of polyplexes changed from thoroidal to more elongated, worm-like forms. Some globular structures were detected in cases of a lower degree of grafting. Finally, DNA release form polyplexes when exposed to negatively charged macromolecules, poly-L-aspartic acid sodium salt, is very structure dependant. Enhanced levels of luciferase expression observed with PLL-PEG polyplexes versus either free DNA or PLL polyplexes are encouraging and warrant further optimization of the polymeric gene delivery system.

## INTRODUCTION



Gene Therapy, a medical treatment with the potential to cure disease at the molecular level, is making steady progress through preclinical development. The development work is mainly focused on two areas. One area is engineering of genes that can correct a disease state when delivered efficiently. The second more difficult area is development of efficient, selective, and safe gene delivery systems. Nonviral gene delivery systems currently under development are naked DNA, cationic liposomes, cationic polymers, and combinations of both; cationic lipids with cationic polymers [1-4]. In general, these delivery systems suffer from, simply termed, "serum effects" and show low levels of gene expression *in vivo* [5]. For example, DNA poly-L-lysine complexes (polyplexes) are cleared quickly from the vascular compartment and extensive DNA degradation is detected [6]. In addition, these polyplexes exhibit colloidal instability *in vitro*, aggregating and increasing in size over time [5, 7-9]. This colloidal instability and other "serum effects" prompted us to look for a means of polyplex stabilization in the vascular compartment. PEG polymers have been used extensively to minimize serum effects and create "stealth" liposomes, biocompatible materials, and proteins with extended circulation [10]. We considered creating "stealth" polyplexes; borrowing the term "stealth" from the liposome field. Our strategy was to introduce PEG chains onto a cationic polymer and thereby provide means for evasion of the reticuloendothelial system. PEG polymers seemed the best candidates because of the abundance of well-characterized and commercially available derivatives ready for grafting on poly-L-lysine ε-amino groups.

Here, we report our efforts towards creating "stealth" polyplexes. We report synthesis, characterization, and properties of comb-type polycations consisting of linear poly-L-lysine backbone with PEG grafts. The size of poly-L-

lysine and PEG moieties, as well as graft density on a polylysine were varied. Finally, these polymers were complexed with plasmid DNA and studied *in vivo* and *in vitro* [11].

## EXPERIMENTAL

### Materials and Methods

Poly-L-lysine (PLL) 10K [DP (Vis) 48, MW (Vis) 10,000; DP (LALLS) 32, MW (LALLS) 6,700, Mw/Mn (SEC-LALLS) 1.20], 26K [DP (Vis) 123, MW (Vis) 25,700; DP (LALLS) 120, Mw (LALLS) 25,000, Mw/Mn (SEC-LALLS) 1.20], 38K [DP(Vis) 184, Mw (Vis) 38,500; DP (LALLS) 172, Mw (LALLS) 35,900; Mw/Mn (SEC-LALLS) 1.10], Poly-L-aspartic acid (P(Asp)) sodium salt 10K [DP (Vis) 76, Mw 10,400 (Vis); DP (LALLS) 57, Mw (LALLS) 7,800] and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, MO. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP•HCL) was purchased from Pierce Chemical Co., Rockford, IL. PD 10 Sepadex G-25M (pre-packed) and phenyl sepharose high performance (hydrophobic interaction column [HIC]) columns and G-25M resin were purchased from Pharmacia Biotech, Inc., Piscataway, NJ. The CM/M Poros column (CM) was purchased from PerSeptive Biosystems, Inc., Farmington, MA. Synthetic polylysine, $(Lys)_{48}Cys$, was purchased from Dr. Schwabe (Protein Chemistry Facility at the Medical University of South Carolina). Polyethylene glycol (PEG) epoxides 2K ($M_n$ 1554; $M_w/M_n$ 1.044 (GPC)), 3K ($M_n$ 2696; $M_w/M_n$ 1.035 (GPC)), and 5K ($M_n$ 5231; $M_w/M_n$ 1.017 (GPC)) were purchased from Shear-Water Polymers, Inc., Huntsville, AL. $LiOH•H_2O$ was purchased from Aldrich Chemical Co., Milwaukee, WI. Plasmid DNA (pCMVβ, Clontech, Palo Alto, CA), and pCMV-Luciferase was prepared by BIO 101 (San Diego, CA). Plasmid DNA preparation contained more than 90% covalently closed circular DNA as determined by agarose gel electrophoresis.

### Synthesis

*Poly-L-lysine-graft-PEG Polymers*

Poly-L-lysine-*graft*-PEG polymers were prepared by reacting PEG-epoxide with ε-NH2 lysine groups under basic conditions [12]. For individual co-polymers, the ratios of PEG-epoxide to poly-L-lysine, PEG-epoxide size, and poly-L-lysine size were varied. The conditions of the syntheses are summarized in Table 1 and the general procedure is described below.

TABLE 1. Synthesis of PLL-*graft*-PEG Comb-Type Copolymers

| Copolymer | PLL $M_n$/ $10^{4\,a}$ | PLL [mg] | PEG Epoxide $M_n$ / $10^{3\,b}$ | PEG Epoxide [mg] | PEG Epoxide [wt%] | $^1$H NMR PEG/PLL Ratio | Copolymer $M_n$ / $10^{4\,c}$ | Copolymer PEG [mol %] | Yield [%] |
|---|---|---|---|---|---|---|---|---|---|
| PL10k-*graft*-PEG2K (1:8.8) | 1.00 | 801 | 1.55 | 354 | 29 | 8.8 | 23.5 | 18.2 | 24.7 |
| PL10k-*graft*-PEG2K (1:13.3) | 1.00 | 606 | 1.55 | 660 | 50 | 13.3 | 30.6 | 27.7 | 31.8 |
| PL10k-*graft*-PEG2K (1:21.2) | 1.00 | 405 | 1.55 | 882 | 67 | 21.2 | 42.9 | 44.0 | 52.3 |
| PL10k-*graft*-PEG3K (1:5.9) | 1.00 | 706 | 2.70 | 425 | 38 | 5.9 | 26 | 12.4 | 46.5 |
| PL10k-*graft*-PEG3K (1:10.7) | 1.00 | 504 | 2.70 | 750 | 60 | 10.7 | 39 | 22.3 | 39.4 |
| PL10k-*graft*-PEG3K (1:13.1) | 1.00 | 300 | 2.70 | 900 | 75 | 13.1 | 45.3 | 27.3 | 70.8 |
| $Lys_{48}$Cys-S-PEG5K (1:1) | 1.00 | 25 | 5.23 | 58 | 70 | 1.0 | 15.1 | 2.1 | 43.0 |
| PL10k-*graft*-PEG5K (1:2.9) | 1.00 | 600 | 5.23 | 600 | 50 | 2.9 | 25.3 | 6.1 | 47.0 |
| PL10k-*graft*-PEG5K (1:6.6) | 1.00 | 350 | 5.23 | 875 | 71 | 6.6 | 44.3 | 13.6 | 51.0 |
| PL10k-*graft*-PEG5K (1:12.9) | 1.00 | 200 | 5.23 | 1000 | 83 | 12.9 | 77.2 | 26.8 | 75.0 |
| PL10k-*graft*-PEG5K (1:31.2) | 0.96 | 42 | 5.23 | 759 | 95 | 31.2 | 172.8 | 67.8 | 96.0 |
| PL10k-*graft*-PEG5K (1:13.2) | 0.94 | 200 | 5.23 | 1000 | 83 | 13.2 | 74.5 | 28.7 | 62.0 |
| PL26k-*graft*-PEG5K (1:2.2) | 2.57 | 162.2 | 5.23 | 62.2 | 28 | 2.2 | 37.5 | 1.8 | 48.0 |
| PL26k-*graft*-PEG5K (1:5.6) | 2.57 | 152.2 | 5.23 | 152.8 | 50 | 5.6 | 55.2 | 4.6 | 63.0 |
| PL26k-*graft*-PEG5K (1:10.4) | 2.57 | 156.6 | 5.23 | 302.2 | 66 | 10.4 | 79.0 | 8.4 | 79.0 |
| PL26k-*graft*-PEG5K (1:18.9) | 2.57 | 200 | 5.23 | 778 | 80 | 18.9 | 124.6 | 15.4 | 42.9 |
| PL38k-*graft*-PEG5K (1:2.3) | 3.85 | 500 | 5.23 | 139.2 | 22 | 2.3 | 50.5 | 1.3 | 21.0 |
| PL38k-*graft*-PEG5K (1:5.6) | 3.85 | 526 | 5.23 | 349.9 | 40 | 5.6 | 67.8 | 3 | 23.7 |
| PL38k-*graft*-PEG5K (1:10.4) | 3.85 | 519 | 5.23 | 704.3 | 58 | 10.4 | 92.9 | 5.7 | 31.2 |

a) Provided by Sigma and based on viscosity
b) Provided by Shearwater Polymers, Inc. and based on GPC
c) Calculated using PEG/PLL $^1$H NMR ratio

Poly-L-lysine 10K (600 mg, 0.06 mmol) and lithium hydroxide monohydrate (41 mg, 2.9 mmol) were dissolved in water (2 ml) and methanol (6 ml) in a siliconized glass flask. Solid PEG5K-epoxide (600 mg, 0.12 mmol) was added, the flask was then sealed, and the solution incubated at 65°C for 48 hours. After incubation, the solvent was removed *in vacuo* and the product was re-dissolved in a minimum volume of water. The pH of the solution was adjusted to 4 with glacial acetic acid. The resulting solution was eluted over a G-25 column with 0.01 N acetic acid. The macromolecular fractions were pooled and lyophilized. The ratio of PEG chains to poly-L-lysine chains was determined by $^1$H-NMR [13].

*$Lys_{48}Cys$-S-PEG*

Buffers used in this procedure were degassed, stored, and used under an argon atmosphere. A 500 µL of TCEP•HCl solution (3.0 mg, 11 µmol) in phosphate buffer pH 8 (20 mM sodium phosphate, 5 mM disodium edetate) was added to 500 µl of $Lys_{48}Cys$ solution (25 mg, 2.3 µmol) in the same buffer and stirred under argon for 30 minutes at ambient temperature. After 30 minutes, the reaction mixture was eluted over a Pd-10 column with phosphate buffer. The macromolecular fractions were pooled, pH adjusted to 7 with 0.1 N HCl, and the solution added to solid PEG5K-vinyl sulfone (57.5 mg, 11.5 µmol) and stirred overnight. The product was purified by hydrophobic interaction column (HIC). The reaction mixture was loaded with 1 M ammonium sulfate 50 mM sodium phosphate pH 7.0 on a 10 mL phenyl sepharose HP column (1.6 x 5 cm). The product was eluted with a blend of two buffers, 33% buffer A (1.5 M ammonium sulfate 50 mM sodium phosphate pH 7.0) and 67% buffer B (50 mM sodium phosphate pH 7.0). The eluted fractions were pooled, lyophilized, re-dissolved in 0.05 M acetic acid and desalted on a G-25 column (0.05 M acetic acid). The fractions containing product were pooled and lyophilized. Yield 15.5 mg. 1:1 ratio of PEG to $Lys_{48}Cys$ was confirmed by $^1$H-NMR ($D_2O$).

## $^1$H-NMR Spectroscopy

Each polymer was first freeze-dried from $D_2O$, and then redissolved in $D_2O$ for spectral analysis. $^1$H-NMR spectra were recorded on 300 MHz ARX-300 Bruker spectrometer. The chemical shifts are expressed in parts per million and referenced to the HDO signal at 4.7 ppm. The ratio of PEG signal to $C_\alpha$-H of poly-L-lysine was used to determine the composition of the resulting copolymer. The structural formulas of the comb-type copolymers are shown in Figure

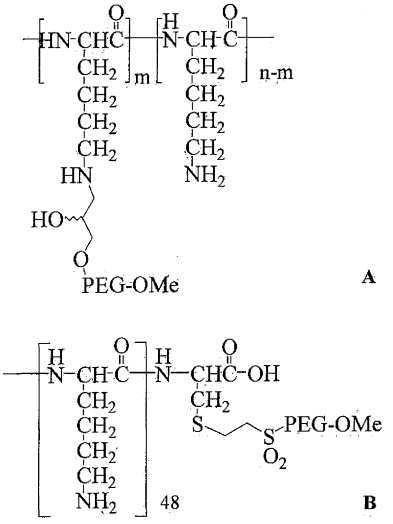


**Figure 1.** Structural formulas of copolymers. (A) PLL-*graft*-PEG; (B) ($Lys_{48}$Cys-S-PEG).



1 and a typical $^1$H-NMR spectrum in Figure 2. We have designated these copolymers as PLLxK-PEGyK(1:Z), where x represents molecular weight of poly-L-lysine; Y, molecular weight of PEG; 1:Z, ratio of PEG chains per poly-L-lysine chains; and K is thousands.

## Polyplex Formation

Polyplexes were prepared by rapidly adding an equal volume of plasmid DNA to a volume of the copolymer. DNA (2x) was prepared in water and copolymers were dissolved in the 2x diluent before mixing. Polyplex concentrations are reported by DNA content and were 10 μg/ml unless otherwise noted.

## Exchange Reaction

Polyplexes were formulated at room temperature by rapidly mixing 500 μL of DNA (2x) and 500 μL of copolymer stock solution. Final DNA concentration was 50 μg/mL at a charge ratio of 1.0 (+/-) in 150 mM NaCl. Each polyplex solution was divided into five 200 μL aliquots and incubated at room temperature for 30 minutes. Poly-L-aspartic acid sodium salt was added to the polyplex aliquots in increasing amounts (charge ratio 1, 4, 7, 10, and 100 per phosphate group). The samples were then incubated for 24 hours and analyzed on agarose gel (0.6%).

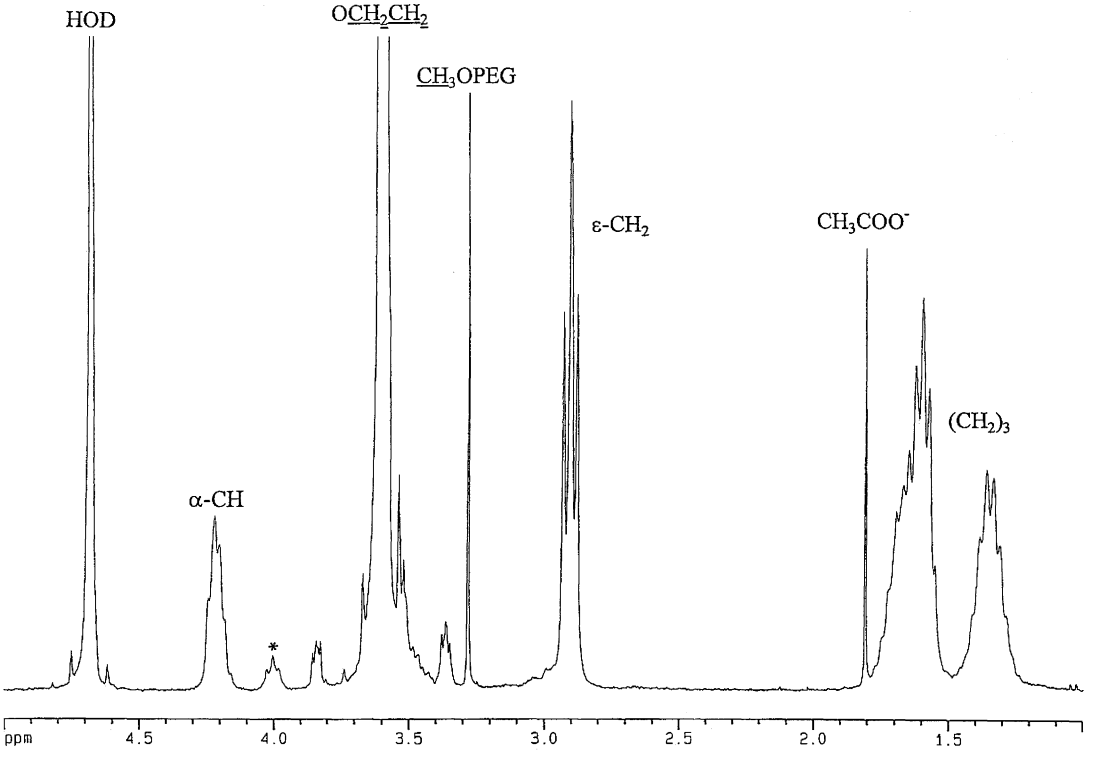


**Figure 2.** $^1$H-NMR spectrum of PLL10K-PEG3K(1:5.9) in $D_2O$.



## Fluorescence Quenching Assay

The relative binding efficiencies of polycationic polymers were examined using an ethidium bromide-based quenching assay. Solutions were prepared containing 2.5 μg/ml EtBr and 10 μg/ml DNA (1:5 EtBr:DNA phosphates molar ratio) in a total volume of 1 ml. The polycation was added incrementally with fluorescence readings taken at each point using a Sequoia-Turner 450 fluorometer with excitation and emission wavelengths at 540 nm and 585 nm, respectively. Fluorescence readings were adjusted to compensate for the change in volume due to addition of polycation which never exceeded 3% of the original volume. Results are reported as the percentage of fluorescence relative to that of uncomplexed plasmid DNA (no polycation).

## Estimation of Polyplex Size

Light scattering measurements were determined on a Brookhaven Instruments Corporation 90 Plus particle size analyzer equipped with a 50 mW

laser which emits light at a wavelength of 532 nm. Reagents were passed through a Nalgene 200 nm surfactant-free cellulose acetate filter prior to polyplex formation. Results are reported as effective diameter defined as the average diameter which is weighted by the intensity of light scattered by each particle (Table 2).

It should be noted that the equations used to determine the effective diameter assume the particles being measured are spherical. No correction was made to account for nonspherical particles, and since DNA condensed with PLL forms toroidal or rod-shaped particles, the measured effective diameters should be considered an approximation of the actual size of the polyplexes.

## Gel Electrophoresis

Tris-borate EDTA urea gels were obtained through Novex, San Diego, CA. The gels were run in 1X TBE buffer. The samples were mixed with an



TABLE 2. Size of Polyplexes in Water and Saline[a]

| Polyplex | Effective Diameter (nm) $H_2O$ | Effective Diameter (nm) 0.15 M NaCl | Charge Ratio at Maximum Size (+/-) | Percent of Lysine Residues Modified |
|---|---|---|---|---|
| PL10K | 46.0 ± 3.1 | 254.7 ± 5.5 | 0.9 | - |
| PL26K | 50.7 ± 6.9 | 238.2 ± 23.2 | 1.0 | - |
| PL10K-PEG2K(1:8.8) | 92.5 ± 7.3 | 88.3[b] | no maximum | 18.2 |
| PL10K-PEG2K(1:13.3) | 109.2 ± 8.6 | 110.6[b] | no maximum | 27.7 |
| PL10K-PEG2K(1:21.2) | 109.7 ± 16.3 | 115.5 | no maximum | 44.0 |
| PL10K-PEG3K(1:5.9) | 101.2 ± 7.5 | 100[b] | no maximum | 12.4 |
| PL10K-PEG3K(1:10.7) | 116.8 ± 11.8 | 115.4[b] | no maximum | 22.3 |
| PL10K-PEG3K(1:13.1) | 109.7 ± 16.3 | 116.3[b] | no maximum | 27.3 |
| $Lys_{48}$Cys-S-PEG5K(1:1) | 82.3 ± 14.0 | 79.8 ± 9.4 | 1.0 | 2.1 |
| PL10K-PEG5K(1:2.9) | 59.5 ± 7.0 | ND | no maximum | 6.1 |
| PL10K-PEG5K(1:6.6) | 113.0 ± 9.4 | ND | no maximum | 13.6 |
| PL10K-PEG5K(1:12.9) | 148.7 ± 27.4 | ND | no maximum | 26.8 |
| PL10K-PEG5K(1:31.2) | 132.8[c] | ND | no maximum | 67.8 |
| PL26K-PEG5K(1:2.2) | 55.0 ± 1.7 | 65.2 ± 3.2 | no maximum | 1.8 |
| PL26K-PEG5K(1:5.6) | 65.3 ± 3.5 | 66.7 ± 3.2 | no maximum | 4.6 |
| PL26K-PEG5K(1:10.4) | 88.9 ± 1.4 | 71.0 ± 4.1 | no maximum | 8.4 |
| PL38K-PEG5K(1:2.3) | 54.8 | ND | no maximum | 1.3 |
| PL38K-PEG5K(1:5.6) | 63.2 | ND | no maximum | 3.0 |
| PL38K-PEG5K(1:10.4) | 86.3 | ND | no maximum | 5.7 |

[a] Size determined at charge ratios (+/-) greater than 1.4
[b] Size determined at a charge ratio of 2.5
[c] Size determined at a charge ratio of 1.5
ND - Not Determined.

equal volume of sample buffer containing 40% sucrose, 0.1% methyl green dye (Sigma Chemical Co., St. Louis, MO) 7.2 M urea in 1 x TBE. The gels were run at 180 volts with polarity reversed for approximately 2 hours, stained with coomassie brilliant blue, and photographed.

### Electron Microscopy

Carbon-coated copper grids with formvar support film (Ted Pella, Inc., Redding, CA) were glow-discharged for 30 seconds just prior to sample preparation. Samples were negatively stained with uranyl acetate by one of the following methods: 1) the grid was floated on a 15 μl droplet containing polyplexes (at 10 μg/ml unless otherwise stated) for 3 minutes, then wicked to filter paper. The grid was washed 2x by placing on a distilled water droplet for 15 seconds followed by thoroughly removing liquid by wicking to filter. 2) The grid was floated on a droplet containing equal volumes of sample and 1.5% uranyl acid stain (1 minute) followed by 2 washes with water. The grids were examined under a Zeiss EM 10b microscope at 10,000x and 40,000x magnification.



## RESULTS AND DISCUSSION

### Synthesis

Poly-L-lysine-*graft*-PEG copolymers were successfully synthesized through epoxide chemistry. PEG-epoxides were reacted with ε-amino groups of poly-L-lysine chains under relatively mild, basic conditions. Lithium hydroxide monohydrate was used as a base in a water/methanol mixture solvent system to minimize degradation of poly-L-lysine and hydrolysis of PEG-epoxide. Yields were generally better for PEG-epoxides with higher molecular weights. The structure of poly-L-lysine-*graft*-PEG is shown in Figure 1A. In addition to graft copolymers, a polymer with a well-defined cationic domain was synthesized by coupling the PEG vinyl sulfone with the terminal cysteine of L-$lys_{48}$Cys-SH at pH 8. The synthetic peptide was first reduced with TCEP to maximize the amount of available thiol and then coupled. The product shown in Figure 1B is best described as an AB block copolymer in which A block is L-$lys_{48}$Cys-S- and B block is PEG5K. Table 1 contains a summary of synthesized polymers.

Figure 2 shows typical $^1$H NMR spectrum of PLL10K-*graft*-PEG3K (1:5.9) in $D_2O$. From the peak intensity ratio of methylene protons of PEG ($OCH_2CH_2$: $\delta$ = 3.7 ppm) [13] and α-methine protons of PLL ($C_\alpha$-*H*: $\delta$ = 4.2

ppm), the number of grafted PEG chains was calculated. Molecular weight of each copolymer was calculated from polymer molecular weights provided by manufacturers and the calculated number of grafted PEG chains. Experimentally determined PEG-PLL ratios after purification were close to stoichiometric ratios used in the syntheses.

## Gel Electrophoresis

Poly-L-lysine-*graft*-PEG polymers ran as sharper bands that did not migrate as far into gels as underivatized PLL (Figure 3, lanes 5-8). As the number of PEG molecules per PLL was increased, the number of bands migrating near the top of the gels increased, while the lower PLL band became less intense and practically undetectable when 5 or more equivalents of PEG-epoxide per PLL were used in the synthesis. At high PEG-epoxide to PLL ratios, bands near the top of the gel became a smear (lane 8). The PLL-PEG copolymers stained less intensely with coomassie blue as the ratio of PEG-epoxide per PLL increased. At PEG-epoxide to PLL ratios lower than 5:1, some unreacted PLL was detected in copolymer preparations.

Migration of PLL-PEG copolymers during electrophoresis is determined by the molecular weight of the PEG moiety, as well as the number of PEG grafts per PLL. The size of the PLL moiety does not influence copolymer migration as much as the molecular weight of the PEG moiety and the extent of derivatization. These two latter aspects alter the net molecular weight per unit positive charge, thus, decreasing the migratory tendency of the resulting copolymer. Gel migration patterns of PLL-PEG copolymers with increasing PEG size or with increasing PLL chain length are shown in panels B and C, respectively.

## DNA Binding Characteristics of PLL-PEGs

Binding of the various PLL-PEG copolymers to plasmid DNA was compared to the DNA binding to PLL using a fluorescence quenching assay and the results are presented in Figure 4. Briefly, addition of increasing amounts of PLL-PEG to a solution of ethidium bromide/DNA results in a rapid decrease in fluorescence. Relative DNA binding affinity of the PLL-PEG copolymers can be inferred from the residual fluorescence of ethidium bromide (EtBr)/DNA in excess PLL-PEG. Figure 4A shows fluorescence quenching observed in water with PLL10K-PEG5K copolymers grafted with different amounts of PEG. There was no difference in the residual fluorescence observed between PLL10K and PLL10K-PEG5K copolymers at ratios of 1:2.9, 1:6.6 or 1:12.9. The

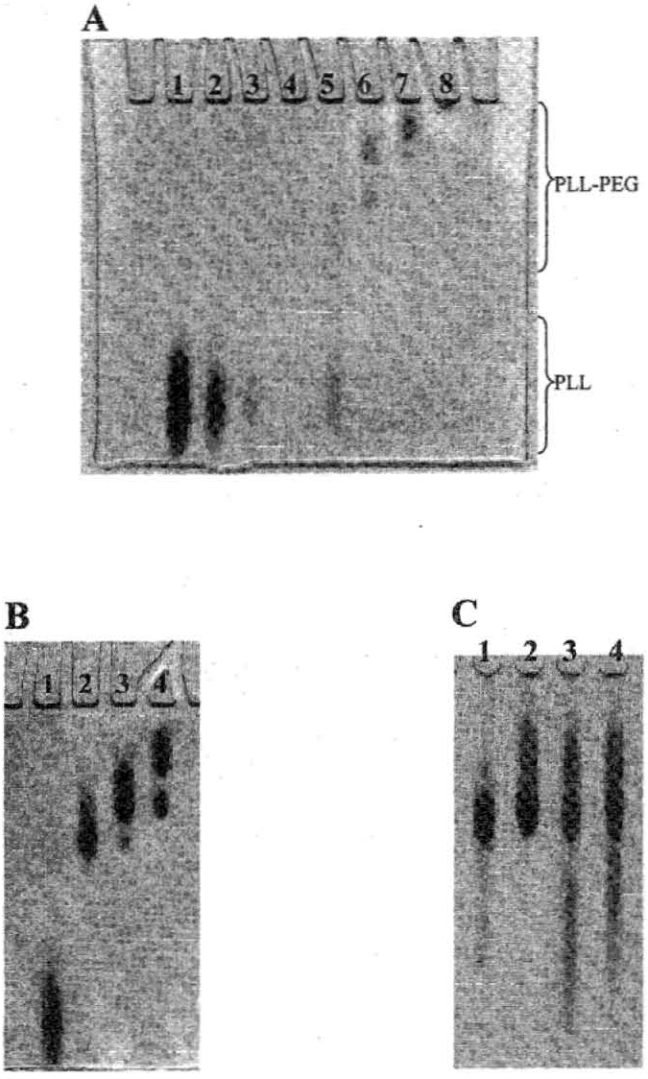


**Figure 3.** Comparison of PLL and PLL-PEG copolymers (epoxide chemistry) by gel electrophoresis on tris-borate EDTA/urea gels using reverse polarity. (A) 1, PLL10K, 5 μg; 2, PLL10K, 3 μg; 3, PLL10K, 1 μg; 4, blank; 5, PLL10K-PEG5K (1:2.9), 6 μg; 6, PLL10K-PEG5K (1:6.6), 10.5 μg; 7, PLL10K-PEG5K (1:12.9), 18 μg; 8, PLL10K-PEG5K (1:31.2), 57 μg. (B) 1, PLL10K 22.4 μg; 2, PLL10K-PEG2K (1:8.8), 5.7 μg; 3, PLL10K-PEG3K (1:5.9), 6.1 μg; 4, PLL10K-PEG5K (1:6.6), 11.1 μg. (C) 1, PLL9.4K-PEG5K (1:2.3); 2, $Lys_{48}$Cys-S-PEG5K (1:1); 3, PLL26K-PEG5K (1:2.2); 4, PLL38K-PEG5K (1:2.3). Copolymers were loaded with the equivalent of 2.5 μg PLL per lane for panels B and C.

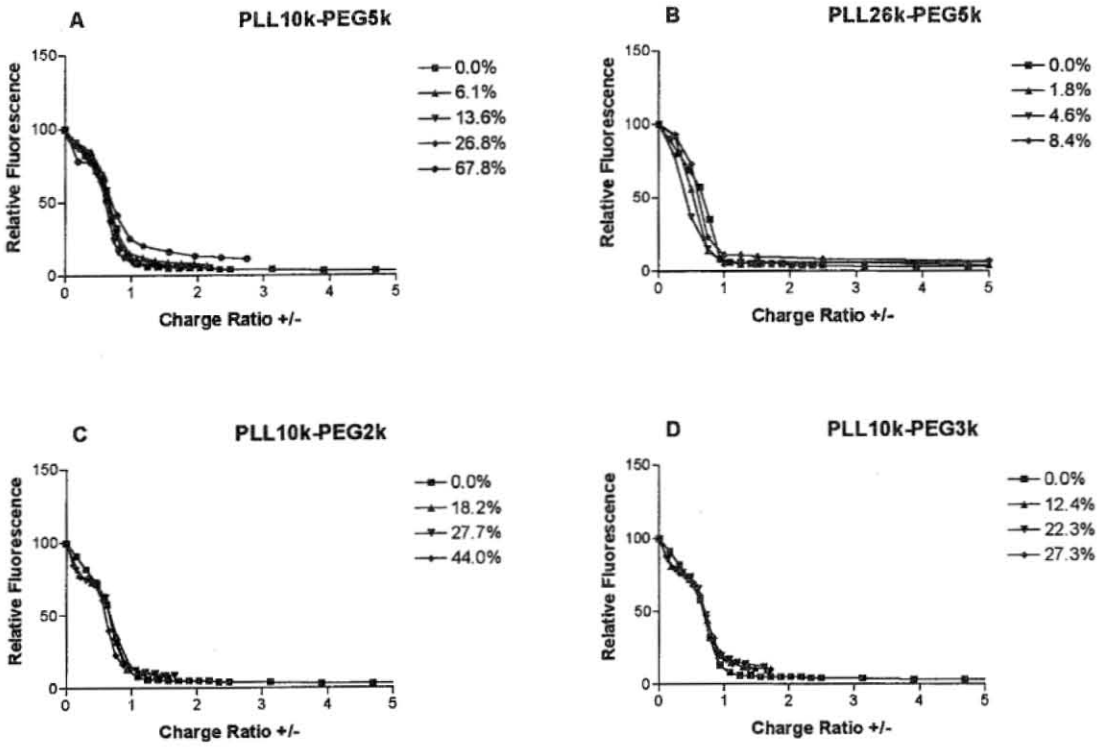


**Figure 4.** Fluorescence quenching assay to determine relative binding affinities of PLL-PEG copolymers in water compared to PLL controls. Relative fluorescence is the percentage of fluorescence compared to free DNA after background ethidium fluorescence has been subtracted. Titration curves of poly-L-lysine-graft-(PEG) copolymers with increasing percent of PEG grafts: (A) PLL10K-PEG5K; (B) PLL26K-PEG5K; (C) PLL10K-PEG2K; (D) PLL10K-PEG3K.

PLL10K-PEG5K copolymer with 31.2 PEG molecules per PLL10K polymer exhibited higher levels of fluorescence even at high charge ratios, indicating that very high levels of grafted PEG weakens DNA binding. Figure 4B shows the fluorescence quenching observed with PLL26K-PEG5K copolymers in water. DNA binding of PLL26K was not affected by grafted PEG for PLL26K-PEG5K copolymers prepared by epoxide chemistry at PLL-PEG ratios of 1:2.2, 1:5.6 and 1:10.4. There was also no significant difference between the fluorescence quenching curves of PLL10K and PLL10K-PEG2K at ratios of 1:8.8, 1:13.3, or 1:21.2 or PLL10K-PEG3K at ratios of 1:5.9, 1:10.7 or 1:13.1 (Figure 4C and D).

## Stabilization of Polyplexes with Poly-L-lysine-*graft*-PEG Copolymers

PLL polyplexes exhibit colloidal instability such that at a charge ratio of 1.0 (+/-) the effective diameter of the PLL polyplexes increases significantly in both water and saline due to aggregation [7, 8]. The effective diameters of PLL26K polyplexes at different charge ratios, determined by laser light scattering (LLS), are plotted in Figure 5A. The sharp increase in size at charge ratio 1 (+/-) is typical for PLL polyplexes. The increased size of PLL26K polyplexes in

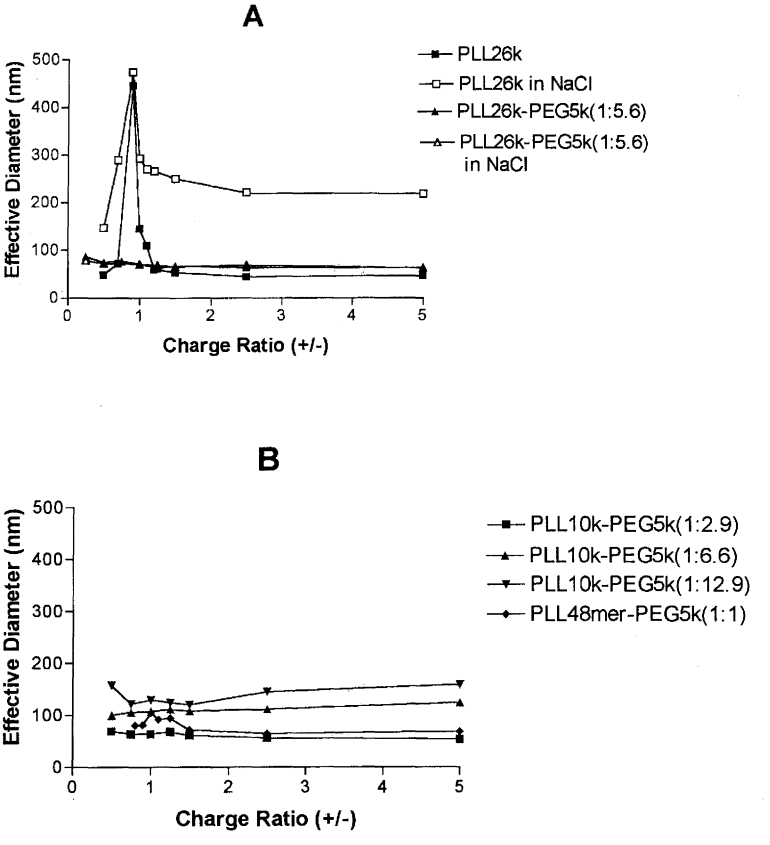


**Figure 5.** Particle sizing of polyplexes at different charge ratios in water or saline (0.15 M NaCl) by laser light scattering. (A) PLL26K polyplexes in water, PLL26K polyplexes in saline, PLL26K-PEG5K (1:5.6) polyplexes in water, and PLL26K-PEG5K (1:5.6) polyplelxes in saline. (B) $Lys_{48}$Cys-S-PEG5K polyplexes in water, PLL10K-PEG5K (1:2.9) polyplexes in water, PLL10K-PEG5K (1:6.6) polyplexes in water, and PLL10K-PEG5K (1:12.9) polyplexes in water.

saline at all charge ratios is also typical for PLL polyplexes [7]. In contrast, the effective diameter of PLL26K-PEG5K (1:5.6) polyplexes did not change with charge ratios ranging from 0.75 to 5.0 (+/-) in either water or 0.15 M NaCl, indicating that PEG sterically stabilized PLL-PEG polyplexes by preventing aggregation even near charge neutrality.

In general, the effective diameter of the PLL10K-PEG polyplexes increased slightly as the number of PEG grafts increased, Figure 5B. $Lys_{48}$Cys-S-PEG5K (1:1) polyplexes showed a detectable size maximum at charge ratio of 1.0 (+/-) suggesting steric instability. Thus, the minimum amount of PEG5K required to stabilize PLL-PEG polyplexes under our experimental conditions was determined to be greater than 1 PEG5K chain per 48 lysine residues, which corresponds to 2.1% grafting.

Table 2 compares the effective diameters of various PLL-PEG polyplexes to PLL polyplexes in both water and 0.15 M NaCl at charge ratios greater than 1.4 (+/-). In water, $Lys_{48}$Cys-S-PEG5K polyplexes were slightly larger than PLL10K-PEG5K (1:2.9) polyplexes, although they had a lower PEG to PLL ratio. This may reflect either some aggregation of the $Lys_{48}$Cys-S-PEG5K polyplexes, the polydispersity of PLL10K-PEG5K copolymers or simply the difference between the architecture of the two copolymers; AB block vs random grafts. $Lys_{48}$Cys-S-PEG5K polyplexes at charge ratios $\geq$ 1.4 (+/-) remained small in 0.15 M NaCl compared with PLL polyplexes, even though at charge ratio 1.0 (+/-), they showed some residual colloidal instability. This is in contrast to PLL polyplexes which tend to aggregate in salt at all charge ratios, but similar to AP26K polyplexes which showed less instability than PLL polyplexes at a charge ratio of 1.0 (+/-) and remained small (90 nm) in 0.15 M NaCl at charge ratios $\geq$ 1.4 (+/-) [7]. With increasing density of PEG grafts on the PLL26K chain, PLL26K-PEG5K polyplexes increased in size in water but showed a lesser size increase under saline conditions (Figure 6). Thus, PEG may contribute steric bulk to polyplexes while it ameliorates colloidal instability.

## DNA Release from Polyplex

During *in vivo* DNA delivery, it is desirable that polyplexes remain intact while in vascular compartments, and release their DNA payload intracellularly, as viruses do. Therefore, binding of copolymer to pDNA should be strong, but reversible. DNA release in the vascular compartment results in a rapid degradation due to nuclease activity. Upon polyplex arrival at target site and subsequent internalization, DNA should be released since only uncomplexed DNA can result

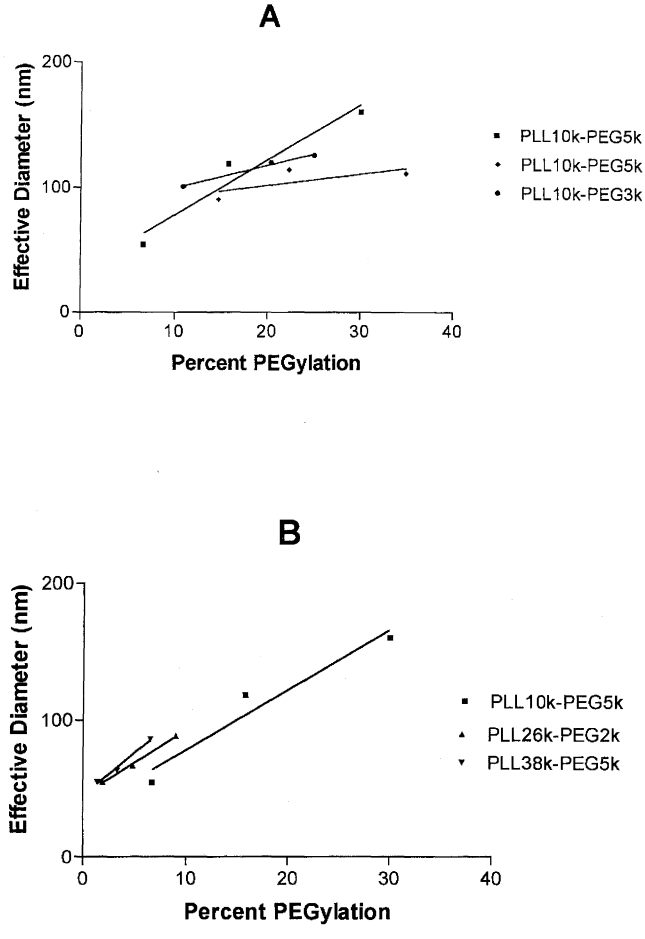


**Figure 6.** Effect of PEGylation on polyplex size in water. A) Effect of PEG size; B) Effect of PL size.

in protein expression. Thus, binding of copolymer to DNA must be sufficient to tightly bind and protect DNA, but also reversible enough to release it under appropriate conditions.

DNA binding was monitored by fluorescence quenching assay with DNA release from polyplex initiated by an exchange reaction with P(Asp). In this assay, DNA release is triggered by increasing amounts of P(Asp) [14c] and the reaction mixture analyzed on agarose gel. A representative gel is presented in Figure 7. Complexed and released DNA is fluorescently stained with ethidium bromide and appears as bright white bands on an agarose gel.

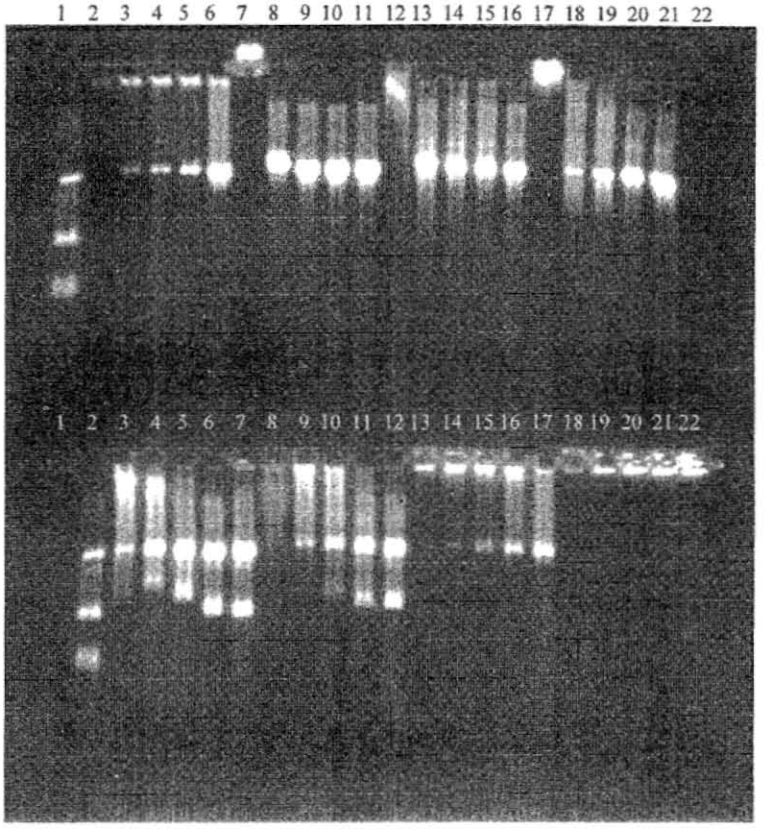


**Figure 7.** Effects of P(Asp) addition on the exchange reactions of copolymer/pDNA complexes at a charge ratio (+/-) of 1 as seen on a 0.6% agarose gel. Each lane was loaded with 0.5 mg pDNA. Five lanes were run for each type of copolymer/pDNA complex. P(Asp) was added at charge ratios of 1, 4, 7, 10, and 100 to the respective five samples in each complex group. Top Row: lane 1 free pDNA; lanes 2-6 PLL38K; lanes 7-11 PLL10K-PEG5K (1:12.9); lanes 12-16 PLL26K-PEG5K (1:10.4); lanes 17-21 PLL38K-PEG5K (1:10.4). Bottom Row: lane 2 free pDNA; lanes 3-7 Lys48Cys-S-PEG5K; lanes 8-12 PEG5K-Lys20; lanes 13-17 PEG5K-Lys120; lanes 18-22 PEG5K-(Cys-S-C18)10-Lys120.

The release of DNA from complexes with unmodified poly-L-lysine, PLL38K/pDNA, was compared to that of complexes with poly-L-lysine-*graft*-PEG copolymers, and also, with AB and ABC block copolymers [3a, 14, 15]. DNA was not completely released from PLL38K/pDNA polyplex even in the presence of 100 equivalents of P(Asp) (Figure 7, top row, Lines 2-6). On the other hand, only 1 equivalent of P(Asp) was able to release DNA from PLL10K-

PEG5K (1:12.9), and considerably more of P(Asp) was needed to release DNA from complexes with longer cationic domains.

This observation suggests that grafting PEG chains on poly-L-lysine weakens DNA binding and makes polyplexes more kinetically labile than the ones formed with unmodified PLL (line 6 vs 21).

In addition, it was interesting to compare DNA release from complexes with polymers of different architectures. AB block copolymers, $(L\text{-lys})_{48}$Cys-S-PEG5K, PEG5K-$(L\text{-lys})_{20}$, PEG5K-$(L\text{-lys})_{120}$ [14, 15] were compared to poly-L-lysine-*graft*-PEG copolymers in DNA release assay (bottom row, lines 3-17 vs top row, lines 7-21). DNA was released from complexes formed with AB copolymers (bottom row, lines 3-17). PEG5K-$(L\text{-lys})_{20}$/DNA complex released DNA almost completely in the presence of 100 equivalents of P(Asp), while PEG5K-$(L\text{-Lys})_{120}$/DNA complex released only a small amount of DNA (bottom row, line 12 vs line 17).

In contrast, ABC triblock copolymer, PEG5K-$(L\text{-Cys-S-}C_{18})_{10}$-$(L\text{-Lys})_{120}$, when complexed with pDNA at a charge ratio of 1.0 (+/-), did not release DNA at all (bottom row, lines 18-22) [15]. DNA complexes formed with PEG-grafted copolymers seem to be the least kinetically stable, while AB copolymer-DNA complexes are more kinetically inert, and ABC copolymer-DNA-complexes are the most stable and totally inert.



## Size and Shape of PLL-PEG Polyplexes

It was shown previously that polyplexes prepared with AB block copolymer, PEG-PLL, are more rod-shaped and worm-like than polyplexes prepared with underivatized PLL, which are mostly globular or toroidal as determined by atomic force microscopy or electron microscopy (EM), respectively [2a]. Figure 8, panels A-C, F-H show that as the ratio of PEG to PLL increased, more rod-shaped or worm-like structures and fewer toroids were observed in electron micrographs of PLL-PEG polyplexes. The toroids that were observed had larger inner diameters than toroids detected with PLL polyplexes, panel K [7, 8]. The structures observed for PLL10K-PEG2K (1:13.3) (panel D) and PLL10K-PEG3K (1:10.7) (panel E) were more worm-like and less compact than those observed for PLL10K-PEG5K (1:6.6) (panel H). By laser light scattering, however, PLL10K-PEG5K (1:6.6) polyplexes were slightly larger than PLL10K-PEG2K (1:8.8) or PLL10K-PEG3K (1:5.9) polyplexes. As the number of PEG grafts increased, it became increasingly difficult to detect PLL-PEG polyplexes from background on EM grids. Apparently, PLL polyplexes with high ratios of

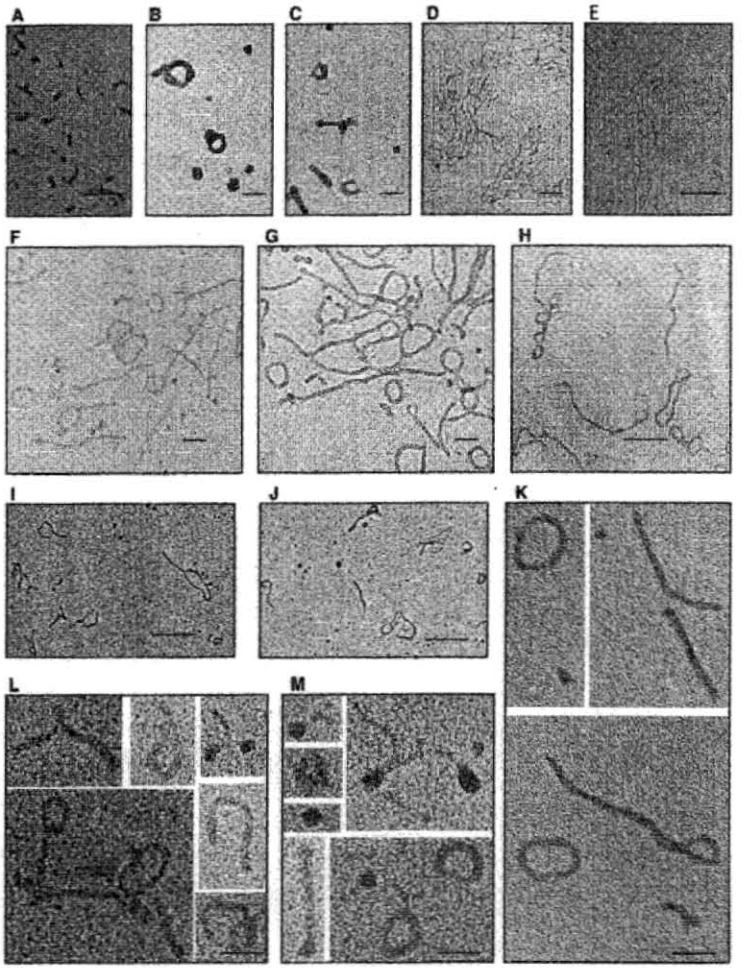


**Figure 8.** Electron micrographs of PLL-PEG polyplexes: (A) Lys48Cys-S-PEG5K polyplexes (+/- ratio = 5.0); (B) Lys48Cys-S-PEG5K polyplexes (+/- ratio = 2.5); (C) Lys48Cys-S-PEG5K polyplexes (+/- ratio = 5.0); (D) PLL10K-PEG2K (1:13.3) polyplexes (+/- ratio = 1.0); (E) PLL10K-PEG3K (1:10.7) polyplexes (+/- ratio = 1.25); (F) PLL10K-PEG5K (1:2.3) polyplexes (+/- ratio = 1.0) in water; (G) PLL10K-PEG5K (1:2.3) polyplexes (+/- ratio = 2.5) in 0.15 M NaCl; (H) PLL10K-PEG5K (1:6.6) polyplexes in water (+/- ratio = 2.5); (I) PLL26K-PEG5K (1:10.4) polyplexes in water (+/- ratio = 2.5); (J) PLL26K-PEG5K (1:10.4) polyplexes in 0.15 M NaCl (+/- ratio = 2.5); (K) PLL10K-PEG5K (1:2.3) polyplexes (+/- ratio = 2.5) in 0.15 M NaCl; (L) PLL26K-PEG5K (1:10.4) polyplelxes in water (+/- ratio = 2.5); (M) PLL26K-PEG5K (1:10.4) polyplexes in 0.15 M NaCl (+/- ratio = 2.5). Grids for panels A-E, and H were prepared by staining method 1; grids for panels F, G and I-M were prepared by staining method 2 described in Materials and Methods. Panels B, C, F, G, H, K, L, and M, bar = 50 nm. Panels A, D, E, I, J, bar = 200 nm.

grafted PEG do not stain well with uranyl acetate. However, we cannot rule out the possibility that the higher amounts of PEG per polyplex may prevent particles from adhering to grid surfaces.

Previously, we found that PLL polyplexes prepared in saline did not adhere to EM grids [7]. A comparison of panel F to G and I to J shows that PLL-PEG polyplexes in 0.15 M NaCl adhere as well to EM grids as polyplexes prepared in water. Some very small globular particles were observed in several electron micrographs of PLL-PEG5K polyplexes, panels B, C, K, L and M. Similar small compact polyplexes were observed for AsOR-PLL26K polyplexes when prepared in salt, and for polyplexes prepared with PLL-PEG copolymers synthesized by tresylate chemistry [7].

## Discussion

Synthetic polymer-based gene delivery vectors are attractive alternatives to viral vectors because they can be fully characterized and are easily prepared in bulk. In designing nonviral delivery vehicles, however, certain desirable features of virus particles should be maintained such as small size (DNA condensation), receptor-specific ligands, and elements that facilitate efficient delivery of DNA to the nucleus of the transfected cell. Maintaining the first of these viral features ensures that a majority of nonviral particles remain intact in the vasculature during delivery, and maximizes the chance that each particle will interact with targeted cells. Since nonviral gene therapy vectors will not replicate in host cells, higher efficiencies of delivery and cellular uptake will likely be required for successful transfection than are required for successful infection by replication-competent viruses.

PLL condenses DNA well and by covalently attaching a liver-specific ligand, asialoglycoprotein (AsOR), the resulting PLL-AsOR-DNA-polyplex should be capable of binding to the asialoglycoprotein receptor on hepatocytes. However, PLL and AsOR-PLL DNA polyplexes are unstable at physiologic ionic strength. We have shown previously that covalent attachment of PEG5K to PLL10K by tresylate chemistry could marginally stabilize the resulting PLL-PEG/DNA polyplexes in water at charge ratios near 1.0 (+/-) [7]. Tresylate chemistry, however, alters the overall charge on the PLL polymer and thereby alters significantly the DNA binding of PLL at high ratios of PEG to PLL. In this report, epoxide chemistry was used to graft PEG onto PLL polymers, leaving the overall charge of the copolymer the same as underivatized PLL. The physiochemical properties of PLL polymers grafted with various amounts of PEG have

been examined to determine their potential as DNA condensing and vascular compartment stabilizing components in a self-assembling, nonviral delivery vehicle.

Using epoxide chemistry, copolymers containing 44%, 27%, and 29% graft percentages of PEG-2K, 3K, and 5K, respectively, were prepared. The copolymers with as high as 44% PEG graft density did not show significantly lowered DNA binding in fluorescence quenching assay. The weak DNA binding of PLL-PEG copolymers may have a negative impact during delivery where polyplexes are exposed not only to physiologic ionic strength, but also to many polyanionic species in the blood, which in turn may facilitate DNA decomplexation followed by DNA degradation. On the other hand, weaker DNA binding may allow easier disassembly of polyplexes in intracellular compartments and thereby improve overall delivery of DNA to the nucleus of transfected cells. Clearly, copolymer-DNA binding will be a significant factor in this delivery system and will have to be optimized.

Increasing density of PEG grafts results in less compact polyplexes with fewer toroids and more worm-like or rod-shaped particles. The effective diameter of PLL-PEG polyplexes tended to increase with increasing grafting density. Because of the changing shapes of polyplexes with increasing PEG-grafting density, interpretation of the particle sizing results is not straightforward.

The effective diameter is determined by LLS using algorithms for spherical particles. Thus, changes in the effective diameter of PLL-PEG polyplexes may not necessarily represent changes in the average size of polyplexes, but instead, may indicate that the polyplexes are changing shape. Polyplexes, formed with copolymers with various sizes of grafted PEG chains grafted on various lengths of poly-L-lysine, remained relatively small (Table 2, Figure 6). Polyplexes were smaller than 150 nm and 100 nm when formulated in water and saline, respectively.

An important difference between PLL and PLL-PEG polyplexes is that PLL-PEG polyplexes are sterically stabilized; they minimize or eliminate aggregation of particles at charge ratios near 1.0 (+/-). PLL-PEG polyplexes are also more stable in saline. PEG stabilizes particles by preventing close contact between individual particles and by increasing the overall hydrophilicity of polyplexes [16, 17]. Complete stabilization of PLL-PEG polyplexes depends on the percent modification with PEG. PLL polyplexes with as little as 2% PEG do not show the characteristic increase in particle size near neutrality and remain small in saline. Wolfert and coworkers have shown that an AB block copolymer, PEG-PLL, lowered the zeta potential of net positive PLL-PEG polyplexes as com-

pared to similar PLL polyplexes [18]. Since positively charged polyplexes can activate the complement system [19, 20, 21], the use of PEG is likely to be helpful in this regard, as well.

Some other designs of polymeric gene delivery systems have appeared in the literature. Among those are poly-L-lysine-*graft*-dextran [22], polyethylene glycol with pendant amino groups [23], diblock and tri-block copolymers [3, 14, 15]. Although these polymers form water-soluble polyplexes, limited data on their size, stability, and morphology are available. DNA release was also not adequately addressed. In one example, however, DNA release from complex with diblock copolymer, PEG-L-$lys_{20}$, was demonstrated [14c]. In our hands, DNA release from polyplexes formed with either PEG-L-$lys_{20}$ or PEG-L-$lys_{120}$ was also established and shown to be dependent on size of cationic domain (20 mer vs 120 mer). In addition, DNA is released easily from polyplexes with poly-L-lysine-*graft*-PEG copolymers, while AB copolymer-DNA polyplexes require higher concentration of P(Asp) for DNA release. DNA is not released from ABC copolymer-DNA polyplex. DNA polyplexes formed with PEG-*grafted*-copolymers seem to be the least kinetically stable, while AB copolymer-DNA complexes are more kinetically inert, and ABC copolymer-DNA complexes are the most stable and totally inert. In summary, DNA release from polyplexes, when exposed to negatively charged macromolecules, is very structure dependent and may have an impact on efficiency of DNA delivery and expression.

Poly-L-lysine-*graft*-PEG copolymers seem to minimize aggregation and improve tissue penetration *in vivo*. Silver grains detected by cryoautoradiaography were evenly distributed over entire liver sections after injection of labeled PLL-PEG polyplexes [6, 11], while silver grains were clustered in liver sinusoids after injection of either PLL or AsOR-PLL polyplexes [7]. Furthermore, *in vivo*, higher levels of luciferase expression were determined when PLL-PEG polymers were used to deliver luciferase cDNA as compared to levels of luciferase expression when either naked DNA or PLL polyplex were administered by the same method [11]. These results indicate that the incorporation of PEG moieties to PLL polyplexes improves bioavailability, presumably by preventing aggregation, and possibly, by minimizing interactions with blood components and the reticuloendothelial system.

In summary, PEG grafts on PLL chains increase disperisty of polyplexes both *in vitro* and *in vivo*, and possibly, the bioavailability. The increased *in vivo* expression observed for PLL10K-PEG5K (1:12.9)/pCMV-Luc polyplexes over free DNA or PLL10K/pCMV-Luc is encouraging and warrants optimization of

the polymeric DNA delivery system. At this time, we assume that *in vivo* liver expression will improve further with the addition of a liver-specific ligand.

## CONCLUSION

Poly-L-lysine-*graft*-PEG copolymers form soluble DNA complexes and minimize aggregation *in vitro*, as well as *in vivo*. Copolymers with as little as 2% grafted PEG chains sterically stabilized polyplexes even at neutral charge. Although epoxide chemistry permits preservation of positive charges on poly-L-lysine chains, it still introduces some steric hindrance, which in turn weakens DNA binding marginally. Polyplexes, formed with copolymers with various sizes of grafted PEG chains, remained relatively small, approximately 100 nm in saline. With a higher degree of grafting, the binding is severely diminished. In addition, the morphology of polyplexes changed from toroidal to more elongated, worm-like forms. Some globular structures were detected in cases of lower degree of grafting. Finally, DNA release from polyplexes when exposed to negatively charged macromolecules is very structure dependent.

Enhanced levels of luciferase expression observed with PLL-PEG polyplexes, versus either free DNA or PLL polyplexes, are encouraging and warrant further optimization of the polymeric gene delivery system.



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