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Addition of poly (propylene glycol) to multiblock copolymer to optimize siRNA delivery

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Keywords: RNAi, siRNA delivery, copolymer, poly(ethylene glycol), poly(propylene glycol), poly(L-lysine)

Previous studies have examined different strategies for siRNA delivery with varying degrees of success. These include use of viral vectors, cationic liposomes, and polymers. Several copolymers were designed and synthesized based on blocks of poly(ethylene glycol) PEG, poly(propylene glycol) PPG, and poly(L-lysine). These were designated as P1, P2, and P3. We studied the copolymer self-assembly, siRNA binding, particle size, surface potential, architecture of the complexes, and siRNA delivery. Silencing of GFP using copolymer P3 to deliver GFP-specific siRNA to Neuro-2a cells expressing GFP was almost as effective as using Lipofectamine 2000, with minimal cytotoxicity. Thus, we have provided a new copolymer platform for siRNA delivery that we can continue to modify for improved delivery of siRNA in vitro and eventually in vivo.

Introduction

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) is an exciting, new technology with many potential biomedical applications. siRNAs are generated by cleavage of long, double-stranded RNAs (dsRNAs) by the cytoplasmic enzyme Dicer in plants and worms,¹ but can also be synthetically produced and introduced into mammalian cells to achieve gene silencing.^{1,2} Inside the cells, siRNA targets specific mRNA (mRNA) for enzymatic degradation via association with the RNA-induced silencing complex (RISC). Exploiting this system, siRNAs have been designed as promising therapies to target human diseases including those caused by viruses^{3–6} and cancers.^{7–11}

A major challenge of using therapeutic siRNAs in vivo is effective and safe delivery. Typically, siRNAs are 7 nm in length with an approximate molecular weight of 13 KDa¹ and have a high net negative charge. Thus, size and charge make siRNAs unable to readily penetrate cellular membranes. Furthermore, naked siRNAs are quickly degraded by nucleases in the bloodstream, and have a short half-life of minutes in the plasma.¹² Therefore, different methods have been proposed and examined to protect and deliver siRNAs with varying degrees of success. These include use of viral vectors, cationic liposomes, and polymers.

While viral vectors have proven effective in delivering siRNAs processed from short hairpin-RNAs or micro-RNA mimics, concerns arise over the immunogenic potential and possibility of mutation of these viruses.^{2,13} Although cationic liposomes

can protect siRNA from nucleases and easily penetrate cells membranes, they are considered too toxic for systemic delivery.¹ However, cationic polymers are an appealing alternative for nucleic acid delivery as they can bind and condense nucleic acids into stable nanoparticles.¹ Furthermore, cationic polymers allow for synthetic modification of structures to enhance transfection efficiencies and reduce cytotoxicity.^{14–16}

Assembly of multiblock copolymer structures is a recent and popular approach for nucleic acid delivery. Common cationic polymers used for this purpose include poly-L-lysine (PLL)^{15,17–20} and polyethylenimine (PEI).^{21–24} In addition, polyethylene glycol (PEG)^{15,17–21,23,24} is often introduced to improve solubility of the nanoparticle complex, increase biocompatibility, and reduce toxicity to cells.^{14,15} Of note, micelle formation of copolymer blocks with nucleic acids is viewed favorably as it has been met with some success for nucleic acid delivery.^{17,18,22,23,25,26} In a micelle-siRNA complex, the hydrophobic polymer segments form the particle core, while the cationic polymer segments complex with nucleic acid chains to form the particle shell.¹⁸

Herein, we describe the design, synthesis, and evaluation of several copolymers based on PEG, poly(propylene glycol) PPG, and PLL blocks for siRNA delivery. It was hypothesized that the amphiphilic nature of particles formed by block combinations of these polymers would readily allow for micelle-complex formation with siRNA. We report on the structural and functional characterization of these polymers when complexed with siRNAs, and the feasibility of using these copolymers for safe and efficient siRNA delivery.

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Results

Synthesis of P1, P2, and P3

In this study, we designed and produced a new series of hybrid, cationic polypeptide block copolymers based on PEG, PPG, and PLL. These block copolymers were tailored for siRNA binding via amine groups in the branched side of the PLL and subsequent delivery. In this study, PEG-NH₂, H₂N-PEG-NH₂, and H₂N-PPG-PEG-PPG-NH₂ were used as initiators in the synthesis of the P1, P2, and P3 polypeptide copolymers. The synthesis of the P1, P2, and P3 copolymers involved three steps as shown in **Figure 1**. First, LL(Z)-NCA was prepared by intramolecular ring closure of LL(Z). The hybrid copolymers, protected by Z groups, were then synthesized using successive ring opening polymerization. After removal of the protective Z-groups on P1-Z, P2-Z, and P3-Z by HBr/HAc, target copolymers P1, P2, and P3 were obtained.

¹HNMR results verified the successful synthesis of P1-Z, P2-Z, and P3-Z as shown in **Figure 2**. The peak which appears at δ 3.50–3.51 ppm in **Figure 2** corresponds to the protons of the -O-CH₂- group in the PEG or PPG unit. The peaks that appear at δ = 4.98 ppm and 7.22 ppm represent the protons of the -CH₂- group and benzene group in the Cbz unit, respectively. The ratio of LL(Z) unit and the PEG or PPG unit can be obtained from ¹HNMR data. As the molecular weights of the commercial PEG-NH₂, NH₂-PEG-NH₂, and NH₂-PPG-PPG-NH₂ are known, molecular weights of P1, P2, and P3 can be deduced from ¹HNMR data^{18,27} and are shown in **Table 1**.

The CMC is defined as the concentration of surfactants above which micelles form, and almost all additional surfactants added to the system go to micelles. The CMC of P1, P2, and P3 copolymers were analyzed by fluorescent spectroscopy using pyrene as a probe. From the plot of fluorescence intensity vs. copolymer concentration (**Fig. 3**), the CMC of the P1, P2, and P3 obtained in this study are 556, 459, and 264 mg/l, respectively. The CMC of P3 is much less than that of P1 or P2 because of the highly hydrophobic PPG blocks in P3, and this is in agreement with previously reported research.²⁴

Self-assembly of cationic copolymers with siRNA

The water-soluble and cationic P1, P2, and P3 copolymers formed polyplexes with siRNA via electrostatic interactions as shown by agarose gel electrophoresis. In the gel retardation assay, when siRNA is efficiently bound to its carrier and shielded from the environment, migration through the gel is completely retarded. Polyplexes were formed at N/P ratio of 0 (naked siRNA) to 4. Cationic copolymers P1 and P2 condense siRNA at an N/P ratio of 4. However, complete retardation by cationic copolymer

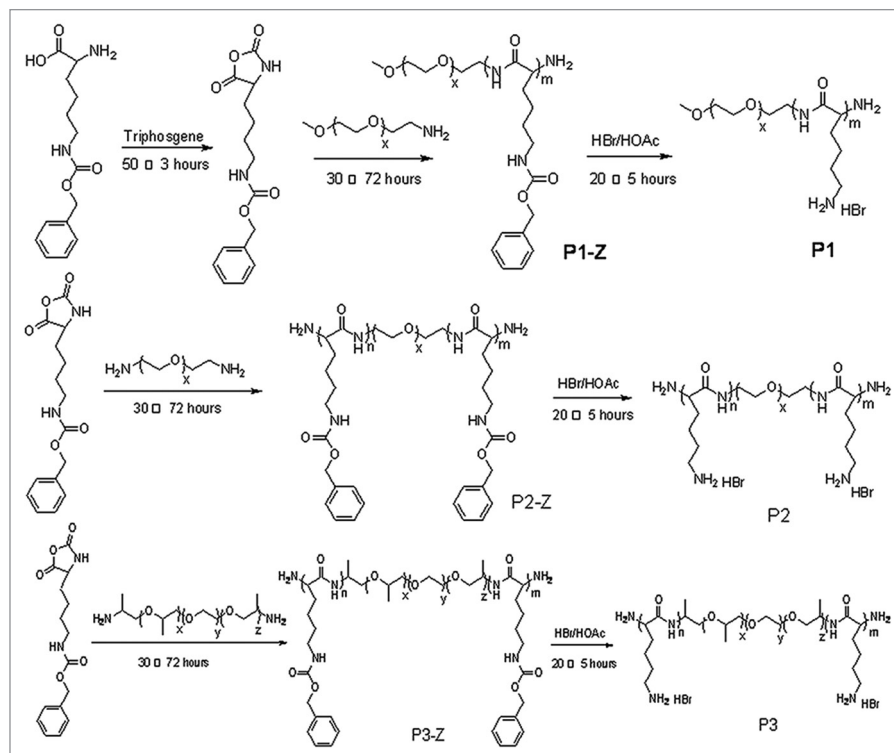


Figure 1. Synthesis of copolymers P1, P2, and P3. Synthesis of the copolymers began with preparation of LL(Z)-NCA by intramolecular ring closure of LL(Z). The hybrid copolymers were then synthesized using successive ring opening polymerization. Finally, Z-groups on P1-Z, P2-Z, and P3-Z were removed using HBr/HAc, and target copolymers P1, P2, and P3 were obtained. Reaction temperatures (°C) and times are indicated at each step shown in the figure.

Table 1. Characterization of the copolymers P1, P2, and P3 synthesized in this study.^a

Polymer	P1	P2	P3
Mn (kD)	10.2	7.9	6.5

^aDetermined by ¹H NMR.

P3 was achieved at N/P ratio of 2, indicating that the hydrophobic PPG blocks improve siRNA-binding (data not shown). The increased condensation associated with the PPG blocks of P3 copolymer can be explained by the increased charge density of the polymer.

Polyplexes were further characterized by size and Zeta-potential measurements. Because an N/P ratio of at least 4 was needed to achieve complete siRNA condensation by all of our copolymers, all experiments discussed hereafter were performed using greater N/P ratios of 6, 12, and 24. Size and surface charge measurements are presented in **Figure 4** and **Figure 5**, respectively. The diameters of P1 complexes were 128 nm, 130 nm, and 150 nm for respective N/P ratios of 6, 12, and 24. The diameters of P2 complexes were 190 nm, 200 nm, and 210 nm for respective N/P ratios of 6, 12, and 24. The diameters of P3 complexes were 160 nm, 152 nm, and 130 nm for respective N/P ratios of 6, 12, and 24 (**Fig. 4**). The diameters of P2 complexes were larger than those of P1 and P3. The two sides of PLL may contribute to making P2 complexes less compact by entrapping many water molecules in the complexes and thus, contributing to the larger

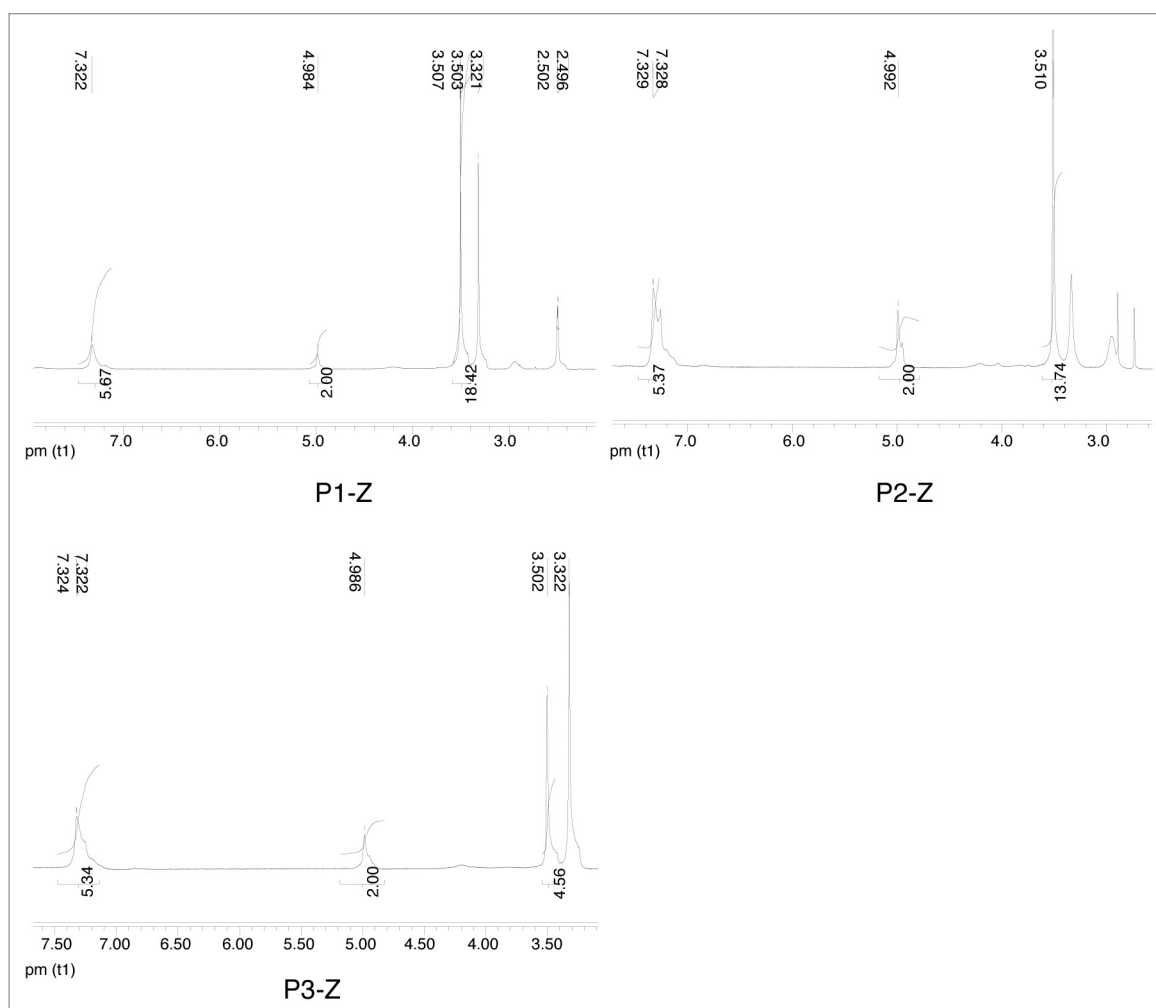


Figure 2. ^1H NMR spectra of P1-Z, P2-Z, and P3-Z. Characterizations by ^1H NMR using DMSO- d_6 as the solvent for measurements are shown.

complex sizes measured. It was interesting to find that increasing the N/P ratio of P1 or P2 polymer complexes resulted in larger particles, while increasing the N/P ratio P3 polymer complexes resulted in smaller particles. Because the PPG segments are chemically attached to the PLL segments, formation of a PPG core causes dense packaging of P3-siRNA complexes. The Zeta potential analyses are provided in **Figure 5**. The Zeta-potentials of P1 complexes were 28 mv, 29 mv, and 31 mv for respective N/P ratios of 6, 12, and 24. The Zeta-potentials of P2 complexes range were 44 mv, 46 mv, and 52 mv for respective N/P ratios of 6, 12, and 24. Finally, the Zeta-potentials of P3 complexes were 19 mv, 20 mv, and 22 mv for respective N/P ratios of 6, 12, and 24. The Zeta-potentials of P2 complexes were greater than those of P1 and P3. It was interesting that P2 complex has greater potential but lower siRNA binding ability. A possible reason is that the larger surface areas coupled with lower siRNA binding efficiency would induce more charge leave on their surfaces. Taken together, the size and Zeta potential measurements have provided useful information on the architecture of the P1, P2, and P3 complexes: (1) P1 and P2 complexes were less dense, although they still appeared to form micelles, (2) a

well-defined boundary between PLL/siRNA complexes and PEG segments was not apparent, (3) PLL segments increasingly localized to the PEG corona, and (4) P3 complex is small in size and has a relatively defined layer structure because of the PPG core. It is possible that the small Zeta potential of P3 (~20 mV) may improve its affinity to the cells and internalization by the cells as compared with the other copolymers tested. The possible micelle architecture of complexes of copolymer with siRNA is shown in **Figure 6**.

Evaluation of cytotoxicity by P1, P2, and P3 copolymers

The cytotoxicity of cationic copolymers P1, P2, and P3 was evaluated by MTT viability assay in Neuro-2a cells. The cells were incubated with various concentrations of copolymers for 48 h. The results in **Figure 7** show that P1 copolymers were the least toxic, while P2 copolymers were the most toxic. When treated with any of the copolymers at about 60 $\mu\text{g/ml}$, the cells maintained over 80% viability. Cells treated at concentrations of 80 $\mu\text{g/ml}$ maintained over 85% viability when treated with copolymers P1 and P3, but less than 75% when treated with P2. Toxicity has been shown to be related to charge density of the polymer,²⁸ and may provide an explanation for the different results observed

using the different copolymers. Another explanation is that toxicity might be primarily caused by addition of an excess of polymer.²⁹

Cellular uptake of copolymer-siRNA complexes

Cellular uptake is a key step for siRNA delivery. To assess the ability of the various polymers to facilitate siRNA internalization, green fluorescent protein (GFP)-targeted siRNA labeled with a Cy3 fluorescent tag was used to form polyplexes. Fluorescence microscopy was used to monitor uptake of Cy3-tagged siRNA (red) and knock-down of GFP expression (green) in Neuro-2a/GFP cells (Fig. 8). The cells shown were transfected with polyplexes composed of the cationic copolymers P1, P2, and P3 with siRNA at N/P ratios of 6, 12, and 24. At the lowest N/P ratio of 6, red fluorescent signal was not detected inside the Neuro 2a cells and GFP expression was still very strong, indicating poor uptake of polyplexes. However, when the N/P ratio was increased to 24, labeled siRNA was detected inside the cells and GFP expression decreased. Furthermore, Cy3 signal increased while GFP expression decreased in cells treated with P2 and P3 complexes as compared with cells treated with P1 complex. Thus, at a high N/P ratio of 24, copolymers P2 and P3 demonstrated higher efficiencies for cellular uptake and gene silencing than P1.

Assessment of siRNA delivery and targeted gene silencing mediated by P1, P2, and P3 copolymers by flow cytometry

Although fluorescent microscopy results qualitatively showed major differences in siRNA delivery by the three copolymers, the ability of different polymers to enhance siRNA internalization was also quantitatively determined. Neuro-2a/GFP cells were transfected at an equivalent concentration of 80 nM siRNA complexed with the copolymers at N/P ratios of 6, 12, and 24. Lipofectamine™ 2000 was used to deliver siRNA as a positive control. The cells were treated with the complexes for 4 h and then cultured for 24 h as shown in Figure 9. For all polymers, increasing the N/P ratio resulted in decreased expression of GFP after transfection. Knockdown of GFP was not apparent when P1, P2, and P3 copolymers were complexed at N/P ratio of 6. Furthermore, P1-siRNA complexes exhibited little gene knockdown at any N/P ratio. Remarkably, the copolymers P2 and P3 coupled with siRNA led to greater gene knockdown than copolymer P1 when used at the N/P ratios of 12 and 24. The knockdown efficiencies when using copolymers P2 and P3 to deliver siRNA were similar to efficiencies observed using Lipofectamine™ 2000 lipopolyplexes, a standard reagent used for siRNA delivery in vitro.

Discussion

The effectiveness of our siRNA-bound complexes may be explained by their physical, structural, and chemical

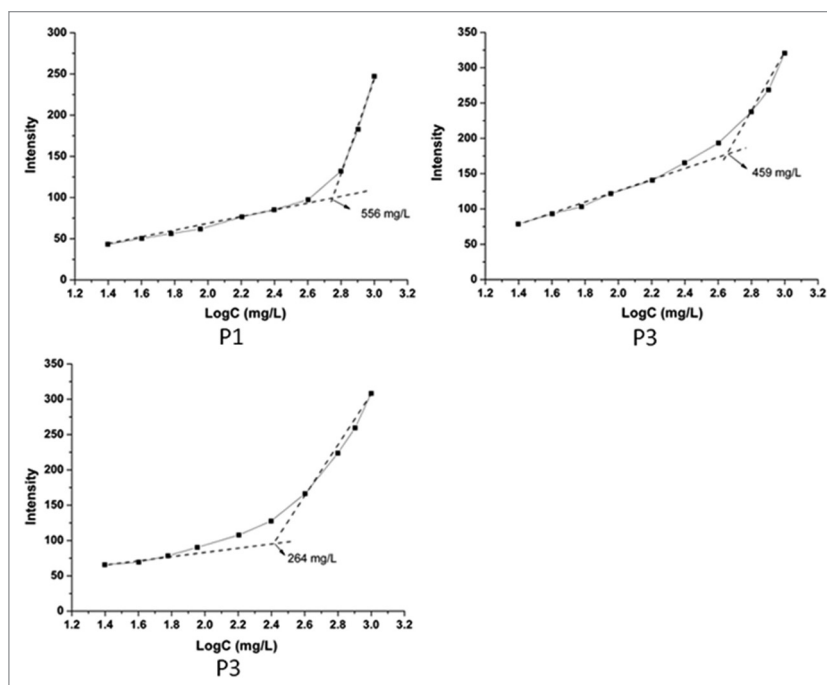


Figure 3. Intensity of the emission spectra at 393 nm as a function of the logarithm of the concentration of P1, P2 and P3. Different concentrations of each polymer were mixed with the pyrene fluorescent probe. Emission spectra were recorded at 393 nm with excitation at 337 nm and analyzed as a function of the polymer concentrations. The CMC value for each copolymer was then determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration as depicted.

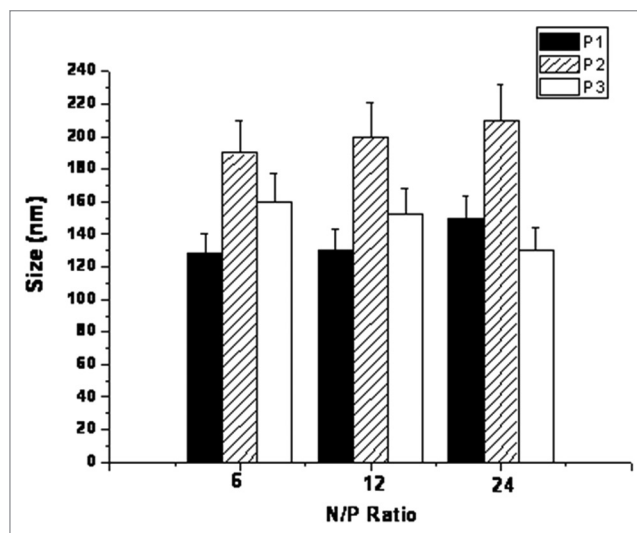


Figure 4. Particle size of complexes of copolymer P1, P2, and P3 with GFP siRNA at various N/P ratios. Copolymer-siRNA complexes were prepared in aqueous solution using multiple N/P ratios while keeping siRNA (200 pmol) constant. Sizes were determined at 25 °C using the Zetasizer Nano.

characteristics. P2-siRNA complex was larger in size and had a greater surface charge. A higher cationic charge in the complexes corresponds to higher transfection efficiency, and is favorable for

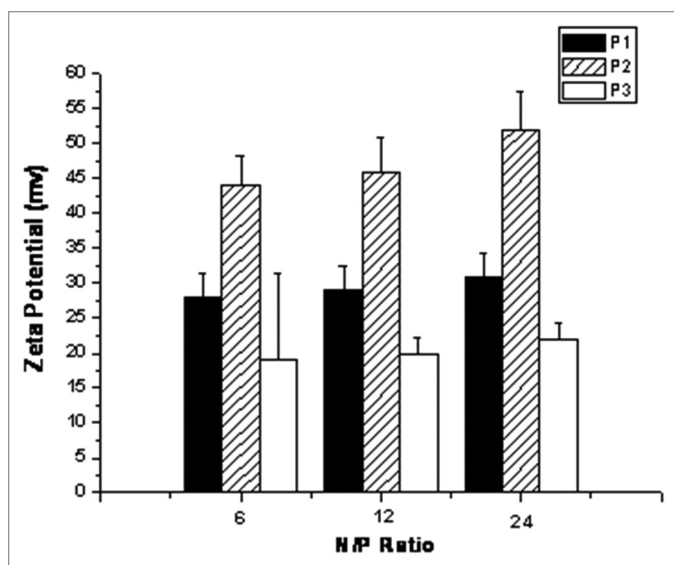


Figure 5. Zeta potentials of complexes of copolymer P1, P2, and P3 with GFP siRNA at various N/P ratios. Copolymer-siRNA complexes were prepared in aqueous solution using multiple N/P ratios while keeping siRNA (200 pmol) constant. Zeta potentials were determined at 25 °C using the Zetasizer Nano.

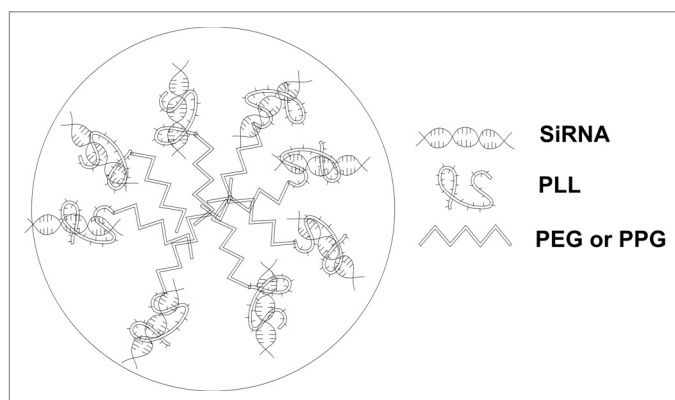


Figure 6. The expected micelle architecture of complexes of copolymer with siRNA.

intracellular release of siRNA. While this higher cationic charge of P2 complex may induce greater gene knockdown, it may also induce greater cytotoxicity. In contrast, for P3-siRNA complex, greater gene knockdown may be attributed to the PPG structure of copolymer P3 and the micelle-complex architecture of their aggregates. In addition, while copolymers P1 and P2 are water soluble block copolymers that can complex with siRNA to form micelle-like aggregates of larger size and lesser density, copolymer P3 has hydrophobic block PPG units. It is possible that the copolymer P3-siRNA complex entered the cells more readily because of its small volume and dense architecture as compared with P1- and P2-siRNA complexes.

Our results showed that copolymers P1, P2, and P3 as designed were minimally toxic up to a concentration of 60 µg/ml for copolymer P2 and 80 µg/ml for copolymers P1 and P3.

Furthermore, P3 was able to complex with siRNA at a low N/P ratio of 2. Compared with P1 and P2, P3 complexes are smaller and denser, and thus, may be internalized via endocytosis more readily than free siRNA, P1, or P2 complexes. P3 complexes are more efficient for siRNA delivery and gene silencing than P1. P3-siRNA complexes decreased GFP expression in GFP-expressing Neuro-2a cells to 28%. In addition to the amount of internalized amount of siRNA, the internalization pathway or endo/lysosomal escape of siRNA can also contribute to an effective RNAi effect. Of interest, siRNA delivery by P3 was similarly efficient as that by Lipofectamine™ 2000, making it a viable candidate for siRNA delivery pending further optimization.

While neither of our copolymers described outperformed the commercially available Lipofectamine™ 2000 reagent, we have provided a new copolymer platform for siRNA delivery that we can continue to modify for improved delivery of siRNA delivery in vitro and eventually in vivo.

Materials and Methods

Materials

Methoxypolyethylene glycol amine (PEG-NH₂, Mw 5000), Poly(ethylene glycol) bis(amine) (H₂N-PEG-NH₂, Mw 3400), O,O'-Bis(2-aminopropyl) polypropylene glycol-block-polyethylene glycol-block-polypropylene glycol (H₂N-PPG-PEG-PPG-NH₂, Mw 1900), N ϵ -benzyloxycarbonyl-L-lysine, bis(trichloromethyl) carbonate, hexane, diethyl ether, tetrahydrofuran (THF), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), hydrogen bromide (30% in acetic acid), and other chemical reagents were purchased from Sigma-Aldrich. Lipofectamine™ 2000 transfection reagent and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies. GFP-targeted siRNAs were synthesized by Integrated DNA Technologies and the sequence used was 5'-AAGCUGACCC UGAAGUUC AdTdT-3' for the sense strand and 5'-UGAACUUCAG GGUCAGCU UdTdT-3' for the anti-sense strand. For cellular uptake studies, the 5' end of the sense strand was modified with Cy3 fluorescent dye.

Synthesis and characterization of polymers

The following procedure to synthesize 3-benzyloxycarbonyl-L-lysine N-carboxyanhydride LL(Z)-NCA was adapted from Zhou et al.³⁰ Ten grams of N ϵ -benzyloxycarbonyl-L-lysine was suspended in 100 ml of THF, the mixture was heated to 50 °C, and an equivalent amount of triphosgene was added. Three hours later, the mixture was poured into 300 ml of hexane and was stored for 16–20 h at -20 °C. Recrystallization from THF/hexane generated NCA-monomer crystals. PEG-PLL(Z) P1-Z, PLL(Z)-PEG-PLL(Z) P2-Z, and PLL(Z)-PPG-PEG-PPG-PLL(Z) P3-Z copolymers were prepared by ring opening polymerization of LL(Z)-NCA as initiated by PEG-NH₂, NH₂-PEG-NH₂, and NH₂-PPG-PEG-PPG-NH₂. Briefly, PEG-NH₂ (200 mg) and LL(Z)-NCA (500 mg) were added to 10 ml dry DMF and were stirred at 30 °C for 72 h under N₂ atmosphere. Next, the reaction was poured into an excess of diethyl ether to precipitate the

PEG–PLL(Z) copolymer. The product was purified by repeated precipitation in diethyl ether, and then dried in a vacuum. We then proceeded with the synthesis of PEG–PLL P1, PLL–PEG–PLL P2, and PLL–PPG–PEG–PPG–PLL P3 copolymers using methods adapted from Zhou et al. with some modifications.³⁰ The PEG–PLL(Z) samples were first dissolved in 5 ml of TFA. HBr (30% in acetic acid) was then added in excess and the mixtures were stirred for 5 h at room temperature. Deprotected polymers were isolated by precipitation using diethyl ether. The precipitated peptides were subsequently washed with excess diethyl ether and acetone, followed by dialysis against deionized water. The product was dried in a vacuum at –40 °C. Characterization by ¹H NMR spectrum was recorded by Chemtos LLC. DMSO-d-6 was used as the solvent for ¹H NMR measurements. Molecular weight (Mw) was determined from the ¹H NMR data.

Determination of critical micelle concentration

Pyrene was used as a hydrophobic fluorescent probe. Different concentrations of each polymer in 0.2 ml aqueous solution were mixed with 6.7×10^{-7} M pyrene residue to measure the critical micelle concentration (CMC). The solutions were maintained at room temperature for 24 h to reach the equilibrated solubilization of pyrene in the aqueous phase. Emission spectra were recorded at 393 nm with excitation at 337 nm. These emission spectra were analyzed as a function of the polymer concentrations. The CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

Preparation and characterization of polymer-siRNA complexes

siRNA and copolymer solution were each diluted in RNase-free water or Opti-MEM (Life Technologies) at room temperature and mixed together to form complexes using different copolymer to siRNA N/P ratios or the molar ratio of cationic lipid nitrogen (N) to phosphate (P) from siRNA. The mixture was vortexed for 5 s and incubated for 30 min at room temperature before use. Lipofectamine™ 2000 reagent was used as a transfection control and Lipofectamine™ 2000: siRNA complexes were prepared as per manufacturer's protocol.

Gel retardation assay

The ability of the copolymers for binding siRNA was evaluated by electrophoresis through 2% agarose in TAE buffer (40 mM Tris–HCl, 1 v/v% acetic acid, and 1 mM EDTA) at 100 V for 20 min. The siRNA bands were stained with ethidium bromide and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories).

Particle size and surface Zeta potential

Copolymer-siRNA complexes were prepared in aqueous solution and contained 200 pmol siRNA. The size and the Zeta potential of the complexes were determined at 25 °C using the Zetasizer Nano (Malvern Instruments) Zeta potential analyzer.

Cell culture

Neuro 2a (ATCC) and Neuro 2a/GFP cells (kindly provided by Dr. N. Manjunath from Texas Tech University Health Sciences Center at El Paso) were cultured at 37 °C in 5% CO₂ and 95% humidity in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS),

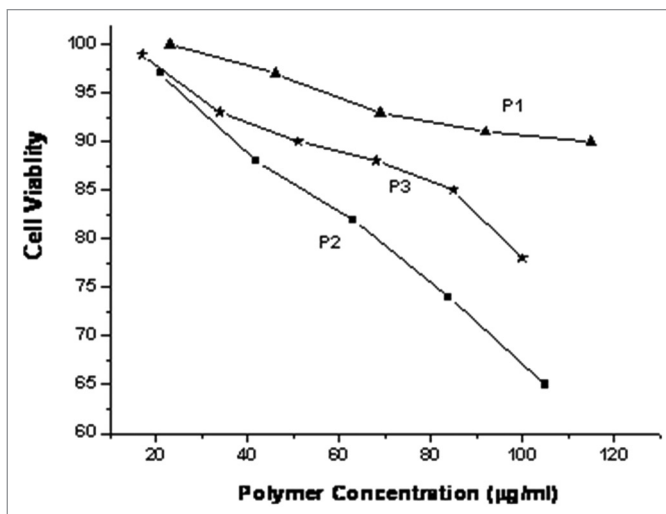


Figure 7. Cytotoxicity of copolymers P1, P2, and P3 in Neuro 2a cells. The toxicities of increasing concentrations of copolymers to Neuro 2a cell lines were assessed by an MTT viability assay.

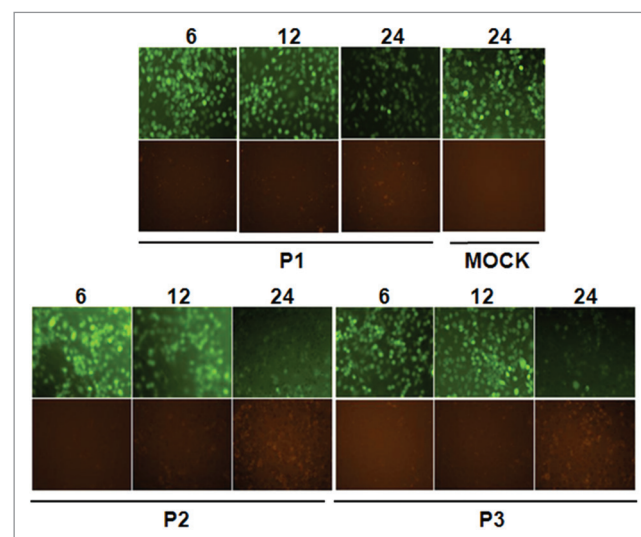


Figure 8. Fluorescent microscopy of Neuro 2a/GFP cells after treatment with complexes of copolymer P1, P2, and P3 with GFP siRNA at N/P ratio of 6, 12, and 24. Neuro 2a/GFP cells (green) were treated with different complexes containing a final concentration of 80 nM Cy3-labeled siRNA (red). Images were taken at 4 h post-transfection. Untransfected Neuro 2a/GFP cells are shown as an additional control.

100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. For transfection experiments, cells were cultured in complete medium with FBS, but without antibiotics.

Cytotoxicity assay

The cytotoxicity of the copolymers to Neuro 2a cell lines was assessed by an MTT viability assay adapted from Qi et al.¹⁸ The cells were seeded in 96-wellplates at 6000 cells per well in 100 µl of DMEM supplemented with 10% FBS and incubated at 37 °C and 5% CO₂ for 24 h. Copolymers were added at varying concentrations in a total volume of 100 µl in DMEM to the cells, which were then maintained in culture for 48 h. Next, 1 g/l of

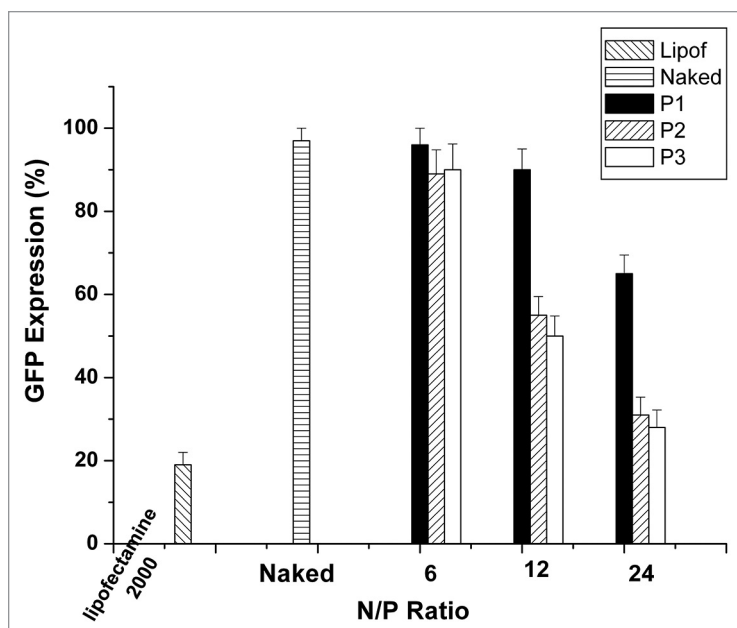


Figure 9. Gene silencing of GFP protein in Neuro 2a/GFP cells at a final concentration of 80 nM siRNA. Cells were treated with complexes of copolymer P1, P2, and P3 with GFP siRNA at N/P ratio of 6, 12, and 24. Cells were also treated with naked siRNA or Lipofectamine-complexed siRNAs as additional controls.

MTT was added to each well for an incubation time of 4 h. The culture medium was then removed and 150 μ l of DMSO per well was added and the samples were incubated 10 min with shaking. The absorbance was measured at 492 nm using a BioTek PowerWave XS2 microplate reader (Winooski, VT USA) with Gen5 software. Cell viability was normalized to Neuro 2a cells that were not treated with copolymers.

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Cellular uptake

Neuro 2a/GFP cells (8×10^3 cells per well) were seeded in 12-wellplates 48 h before transfection. Medium was replaced with serum-free medium, and cells were treated with 20 μ l of different complexes with a final concentration of 80 nM Cy3-labeled siRNA. At 4 h post-transfection, cells were imaged using a Nikon Ti Eclipse fluorescent microscope for GFP expression and uptake of Cy3-labeled siRNA.

Gene silencing and intracellular translocation

Neuro 2a/GFP cells were seeded in 12-well plates at 8×10^3 cells/well and cultured for 24 h. Then, cells were transfected with copolymer complexes containing GFP-siRNA at various N/P ratios. The final concentration of siRNA was 80 nM in each well. GFP-siRNA complexed with-Lipofectamine™ 2000 was used as a control. After a 4 h incubation period, the transfection medium was discarded and supplemented with fresh medium containing 10% FBS. The cells were incubated for another 24 h. The medium was removed, and the cells were washed twice with cold PBS and detached using 0.25% trypsin-EDTA. GFP expression was quantified via flow cytometry using a Gallios flow cytometer (Beckmann Coulter) and analyzed using FlowJo software (TreeStar). The parent Neuro 2a cell line not expressing GFP was used to control for background fluorescence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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