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Cationic, amphiphilic copolymer micelles as nucleic acid carriers for enhanced transfection in rat spinal cord

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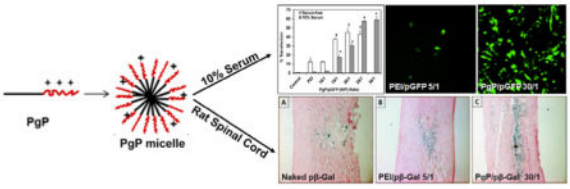
Abstract

Spinal cord injury commonly leads to permanent motor and sensory deficits due to the limited regenerative capacity of the adult central nervous system (CNS). Nucleic acid-based therapy is a promising strategy to deliver bioactive molecules capable of promoting axonal regeneration. Branched polyethylenimine (bPEI: 25kDa) is one of the most widely studied nonviral vectors, but its clinical application has been limited due to its cytotoxicity and low transfection efficiency in the presence of serum proteins. In this study, we synthesized cationic amphiphilic copolymers, poly (lactide-co-glycolide)-graft-polyethylenimine (PgP), by grafting low molecular weight PLGA (4kDa) to bPEI (25kDa) at approximately a 3:1 ratio as an efficient nonviral vector. We show that PgP micelle is capable of efficiently transfecting plasmid DNA (pDNA) and siRNA in the presence of 10% serum in neuroglioma (C6) cells, neuroblastoma (B35) cells, and primary E8 chick forebrain neurons (CFN) with pDNA transfection efficiencies of 58.8%, 75.1 %, and 8.1 %, respectively. We also show that PgP provides high-level transgene expression in the rat spinal cord *in vivo* that is substantially greater than that attained with bPEI. The combination of improved transfection and reduced cytotoxicity *in vitro* in the presence of serum and *in vivo* transfection of neural cells relative to conventional bPEI suggests that PgP may be a promising nonviral vector for therapeutic nucleic acid delivery for neural regeneration.

Graphical abstract



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Keywords

Polymeric micelle; nucleic acid delivery; combinatorial therapy; CNS regeneration

1. Introduction

Spinal cord injury (SCI) damages ascending and descending axons that are unable to regenerate and re-establish functional connections with their targets. In addition to paralysis and loss of sensory function below the level of the lesion, SCI may also lead to chronic pain, spasticity, respiratory impairment, loss of bowel or bladder control, and sexual dysfunction. A wide range of therapeutic strategies are being developed to promote axonal regeneration, including cell transplantation[1], neurotrophin delivery[2], removal of growth inhibition[3–7], manipulation of intracellular signaling[8, 9], immune modulation [10], and use of bridging scaffolds for axonal guidance [11–15]. However, there is no clinically effective therapy currently available. Relative to the peripheral nervous system and the developing central nervous system (CNS), one of the major mechanisms responsible for regenerative failure in the adult CNS is insufficiency of growth-promoting adhesion molecules and neurotrophic factors and abundance of growth-inhibitory molecules such as myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs)[16]. Delivery of therapeutic nucleic acids capable of changing gene expression levels offers a promising approach to overcome these barriers.[17, 18]

Gene therapy involves the intracellular delivery of a vector containing genetic material capable of expressing a therapeutic gene. Gene therapy providing overexpression of growth-promoting molecules has achieved increased axonal regeneration/plasticity and functional improvement in small animal models of SCI[19, 20]. Gene silencing approaches such as RNA interference have also demonstrated potential for the treatment of SCI in animal models[21, 22]. While viral vectors are most commonly used to achieve efficient transfection, they often lack specificity and evoke immune reactions and inflammation[23]. To overcome these safety concerns, nonviral vectors such as plasmid DNA or siRNA, usually formulated with cationic lipids or polymer carriers, are being developed and offer improved safety, reduced immunogenicity, and ease of large-scale manufacturing[24].

Polyethylenimine (PEI) was one of the first synthetic polymers investigated for nonviral gene delivery and early studies demonstrated its ability to achieve transfection of pDNA vectors in the CNS [25–27]. Branched PEI (bPEI, 25kDa) has been considered a gold standard material for nonviral gene delivery, providing efficient transfection in serum-free conditions due to its ability to form stable polyplexes and its buffering capacity, which facilitates endosomal escape by the proton sponge effect [26, 28]. However, important limitations of bPEI (25kDa) and other nonviral carriers are low transfection efficiency in the presence of serum, cytotoxicity, and aggregation with serum proteins or erythrocytes in blood[29], which may lead to rapid clearance by non-target cells of the reticuloendothelial system (RES) in systemic applications. A number of studies have sought to increase the transfection efficiency and lower cytotoxicity of bPEI in serum conditions by chemical modification of the polymer's primary amine groups. One of the most widely studied and

effective modifications of bPEI has been conjugation of low molecular weight hydrophobic groups [30].

Our long-term goal is to develop a bPEI derivative with hydrophobic polymer chains capable of forming polymeric micelles for combinatorial drug/gene delivery to the injured spinal cord. A micellar carrier can provide targeting through neuron-specific antibodies conjugated to the micelle surface, loading of rolipram in the hydrophobic core to prevent injury-induced reductions in cAMP levels, and complexation of siRNAs to the bPEI shell targeting intracellular signaling pathways activated by MAIs and CSPGs. Toward this end, here we report the synthesis and characterization of cationic amphiphilic copolymers, poly (lactide-co-glycolide)-graft-polyethylenimine (PgP) as an efficient vector for stable complexation, protection, and intracellular delivery of nucleic acids. We show that PgP micelles are capable of efficiently transfecting reporter genes and siRNA both in the presence of 10% serum *in vitro* and in the rat spinal cord *in vivo*. The combination of improved transfection and reduced cytotoxicity in the presence of serum relative to conventional bPEI (25 kDa) control as well as transfection of neural cells *in vivo* suggests PgP may be a promising nucleic acid carrier for *in vivo* gene delivery.

2. Materials and Methods

2.1. Materials

Poly (lactide-co-glycolide) (PLGA 4 kDa, 50:50) with a carboxylic end group was purchased from Durect Corporation (Pelham, AL). Branched poly (ethylenimine) (bPEI) (Mw 25 kDa), dicyclohexylcarbodiimide (DCC), and N-hydroxysuccinimide (NHS) were purchased from Sigma (Milwaukee, WI). Dialysis tubing (MWCO=50,000) was purchased from Spectrum (Houston, TX). QIAgen maxi plasmid purification kit was purchased from QIAgen (Valencia, CA). Plasmid DNA encoding the Monster Green Fluorescent Protein (phMGFP Vector: pGFP), plasmid DNA encoding beta-galactosidase (pSV40-β-gal, p β-gal), and marker dye for gel electrophoresis (Blue/Orange 6X Loading Dye) were purchased from Promega (Madison, WI). Albumin standard and BCA protein assay kit were obtained from Pierce (Rockford, IL). A molecular weight ladder of pDNA (1kb DNA Ladder) was purchased from Gibco BRL (Grand Island, NY). Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 mix with L-glutamine (DMEM/F12), 100X stock solution of penicillin/streptomycin, and 0.05% trypsin/0.53 mM EDTA in Hank's Balanced Salt Solution were purchased from Mediatech Inc (Manassas, VA). Bovine growth serum (BGS) was obtained from Hyclone (Logan, UT). Basal Medium Eagle (BME) was obtained from Life Technologies (Grand Island, NY). Other reagents were commercial special-grade, used without further purification.

2.2. Synthesis and characterization of poly (lactide-co-glycolide)-g-poly (ethylenimine)

2.2.1. Synthesis of poly (lactide-co-glycolide)-g-poly (ethylenimine) (PgP)—

PLGA (800 mg, 200 μmole) was dissolved in 20 ml dried anhydrous DMF. *N*-hydroxysuccinimide (NHS, 27.6 mg, 240 μmole) and *N,N'*-Dicyclohexylcarbodiimide (DCC, 49.6 mg, 240μmole) were added to the reaction solution and this mixture was stirred for 2 hrs to activate the carboxylic end group of PLGA. The resulting precipitate,

dicyclohexyl urea (DCU), was removed by filtration. bPEI (1.25 gm, 50 μmole) was dissolved in 20 ml dried DMF. The activated PLGA solution was added dropwise to the bPEI solution over 30 min, and then the mixture was allowed to react for 24 hrs at room temperature with stirring. Poly (lactide-co-glycolide)-g-poly (ethylenimine) (PgP) was purified by dialysis against deionized water using a membrane filter (MWCO=50,000), centrifuged at 5,000 rpm for 10 minutes to remove unreacted PLGA precipitate, and lyophilized. The structure of PgP was determined by FT-IR and ^{1}H- NMR (300 MHz, Bruker) using D_2O as a solvent. The molecular weight was determined by gel permeation chromatography (GPC, Waters, Milford, MA) using an Ultrahydrogel 250 column (7.8×300 mm) and guard column- 6×40 mm with water as the mobile phase. PgP solution (3 mg/ml, 20 μl) was injected by auto-injector and the flow rate was 0.7 ml/minute. A Waters 1525 HPLC pump and Waters 2414 Refractive Index Detector were used. Dextrans at molecular weights of 5, 12, 25, 50, and 80kDa were used as standards.

2.2.2. Critical micelle concentration—The critical micelle concentration (CMC) was determined using a dye solubilization method. Ten μl of 0.4 mM DPH (1, 6-diphenyl-1,3,5-hexatriene) was added to 1 ml solutions of various PgP concentration and incubated in the dark at room temperature for 6 hrs. Absorbance at 356 nm was plotted against the polymer concentration and the CMC was determined as the point of intersection between linear extrapolations of the absorbance in low- and high-concentration regions.

2.3. Plasmid amplification and purification

Plasmids encoding the Monster Green Fluorescent Protein (pGFP) and beta-galactosidase (pβGal) were transformed into *Escherichia coli* DH5α and amplified in LB medium at 37°C overnight with shaking at 250 rpm. pGFP and pβGal were purified using the Endofree Maxi Plasmid purification kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of pGFP and pβGal were determined using Biotek Take 3 microplate reader (BioTek, Synergy HT).

2.4. Preparation and characterization of PgP/nucleic acid polyplexes

PgP/pDNA polyplexes were prepared at various N/P (nitrogen atoms of polymer/phosphorus atoms of pDNA) ratios ranging from 5 to 30. DNA (20 μg pDNA) and varying amounts of PgP were separately diluted in 500 μl of deionized water. After 10 min, solutions were mixed and incubated for 30 min at 37 °C. bPEI/pDNA at N/P ratio 5/1 was prepared for comparison. PgP/siRNA complex at N/P ratio of 30/1 and PEI/siRNA at N/P ratio of 5/1 were prepared in nuclease-free water.

Polyplexes were initially characterized immediately after preparation. Particle size (PS) was determined by dynamic laser light scattering (DLS) using Zeta PALS (Brookhaven Instruments Corp, Holtsville, NY) and reported as effective mean diameter. ζ-potential (ZP) was measured electrophoretically using the same apparatus. In order to evaluate the effect of serum on particle size, PgP/pDNA and PEI/pDNA prepared at N/P ratio of 30/1 and 5/1, respectively, were diluted in 10% serum-containing media and PS measured by DLS. PgP/pDNA and PgP/siRNA prepared at N/P ratio of 30/1 were imaged by transmission electron microscopy (TEM, Hitachi H-7600, Tokyo, Japan). After polyplex formation, a small drop

(5 μl) of sample solution was placed onto a carbon coated copper grid (CF300-CU, Electron Microscopy Sciences, PA), blotted with filter paper to remove excessive water, and dried at room temperature. The specimens were vapor-stained with 0.5% Ruthenium tetroxide (RuO_4) solution (Electron Microscopy Sciences, PA) to improve the contrast, and imaged by TEM at an acceleration voltage of 100 kV and magnifications of ×50K and ×200K. Particle size of PgP/pDNA and PgP/siRNA was measured (n=16/group) from TEM micrographs using Adobe Photoshop.

2.5. Gel retardation assay

Gel retardation assays were performed to confirm the ability of PgP to neutralize the negative charge of pDNA and provide protection from degradation. First, PgP/pDNA polyplexes were prepared at various N/P ratios in deionized water and incubated for 30 min at 37 °C.. In the second study, PgP/pDNA at N/P ratio of 30/1 was prepared and incubated in 10% serum-containing media for 3 days at 37°C. Naked DNA was included in both studies for comparison. The samples were electrophoresed on a 1% (w/v) agarose gel for 90 min at 80 V. The gel was stained with ethidium bromide (0.5 μg/ml) for 30 min and imaged on a UV illuminator (Alpha Innotech FluorChem SP imager) to visualize the migration of polyplexes and control pDNA.

2.6. Transfection efficiency and cytotoxicity of polyplexes *in vitro*

2.6.1. Cell culture—C6 (rat glioblastoma) and B35 (rat neuroblastoma) cells were grown in DMEM/F12 supplemented with 10% FBS and 100 IU/ml penicillin/100 μg/ml streptomycin at 37 °C under 5 % CO_2. Cells were trypsinized and plated in 12-well plates ($0.9\sim1.1 \times 10^5$ cells/well).

Chicken eggs were obtained from Clemson University's Morgan Poultry Center and incubated at 37 °C with light rocking. Primary E8 chick forebrain neurons (CFNs) were prepared as described[31]. Briefly, after removal of the meninges, the forebrains were isolated and incubated in 0.25% trypsin for 5 minutes at 37 °C. The trypsin was then aspirated and the tissue was triturated; centrifuged; resuspended in BME supplemented with 10% FBS, 6mg/mL D-glucose, 2mM L-glutamine, and 1% antibiotic; and plated in 12-well plates ($0.9\sim1.1 \times 10^6$ cells/well) pre-coated with 0.01 % Poly-L-lysine.

2.6.2. Transfection efficiency and cytotoxicity of PgP/pGFP polyplexes in serum-free and 10% serum conditions—C6, B35, or CFN cells were plated in 12-well plates and cultured overnight. PgP/pGFP polyplexes (2 μg of pGFP) were prepared at charge (N/P) ratios ranging from 5/1 to 30/1. For the serum-free condition, the cells were transfected in media without serum for 4 hrs and then the media were removed and replaced by fresh media containing 10% FBS. The cells were incubated for an additional 44 hrs. For the serum condition, the cells were transfected in media containing 10% FBS for 24 hrs; then the media were removed and replaced by fresh media containing 10 % FBS. The cells were incubated an additional 24 hrs. GFP expression was measured by flow cytometry (Guava easyCyte, Millipore) and the results were expressed as % transfected cells. Transfection efficiency of PgP/pGFP polyplexes at different N/P ratios was compared with that of bPEI/pGFP at an N/P ratio of 5/1 as a positive control and non-transfected cells were

used as a control. Cytotoxicity relative to non-transfected control was analyzed in parallel experiments by MTT assay. At 48 hours post-transfection, media were replaced with 1 ml of fresh DMEM without serum containing 240 μl of MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) solution in PBS (2 mg/ml). Plates were incubated an additional 4 hours at 37 °C. MTT-containing medium was removed, and 1.5 ml of DMSO was added to dissolve the formazan crystals formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability } (\%) = (\text{OD}_{570\,(\text{sample})} / \text{OD}_{570\,(\text{control})}) \times 100\%$$

2.6.3. Neuron-specific beta-3-tubulin staining—To confirm neuronal transfection, transfected CFN cultures were fixed with 4% paraformaldehyde after 48 hrs transfection, stained by immunocytochemistry using a monoclonal beta-III-tubulin (Abcam) primary antibody and Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody, and digitally imaged using an inverted epifluorescent microscope (Zeiss Axiovert 200,Göttingen, Germany).

2.6.4. Effect of polymer concentration on cytotoxicity—To characterize the dose-dependent cytotoxicity of PgP, B35 and C6 cells were transfected using various concentrations of PgP/pGFP prepared at N/P ratio of 30/1. At 48 hours post-transfection, cell viability was evaluated as described above.

2.6.5. Time course study of polyplex stability and transfection efficiency—PgP/pGFP was prepared at N/P ratio of 30/1 and stored at 4°C for up to 7 days. At predetermined time points, the stability of polyelectrolyte complexes was evaluated by gel retardation assay and transfection efficiency measured in B35 cells in 10 % serum condition.

2.7. Transfection efficiency and cytotoxicity of PgP/siRNA polyplexes *in vitro*

2.7.1. Transfection efficiency using siGLO Red—The siGLO Red transfection indicator (siGLO®, Thermo Fisher Scientific), consisting of a fluorescently labeled siRNA duplex with a chemical modification for nuclear localization, was used to evaluate siRNA transfection efficiency. PgP/siGLO Red complexes (1 μg siGLO Red) at various N/P ratio were transfected in B35 cells in 10% serum condition. bPEI/siGLO at N/P 5/1 and RNAiMAX (Life Technologies) were used as positive controls. The cells were incubated at 37 °C for 24 hrs and then the media were replaced with fresh media containing 10 % FBS. At 48 hours post-transfection, siGLO Red-transfected cells were counted by flow cytometry and the results expressed as % transfected cells. Cytotoxicity relative to untransfected cells control was analyzed in parallel experiments by MTT assay.

2.7.2. Silencing efficiency of PgP/GFP siRNA after co-transfection of bPEI/pGFP complexes—To evaluate PgP as a siRNA delivery carrier, B35 cells were co-transfected with bPEI/pGFP at an N/P ratio of 5/1 and PgP/GFP siRNA (Ambion) complexes at N/P ratio of 30/1. The cells were first transfected with bPEI/pGFP (2 μg of pGFP) in serum-free condition as described above. At 4 hrs post-transfection, the media were removed and replaced by fresh media containing 10% FBS. PgP/GFP siRNA

complexes at N/P ratio 30/1 (1 μg of siRNA) were co-transfected in the bPEI/pGFP-transfected B35 cells in 10% serum condition and then incubated for 24 hrs. RNAiMAX/GFP siRNA polyplexes prepared according to the manufacturer's protocol and bPEI/GFP siRNA (N/P ratio of 5/1) were used as controls. The level of GFP fluorescence was measured by flow cytometry. GFP-silencing efficiency of PgP/GFP siRNA polyplexes was calculated relative to the level of GFP fluorescence obtained from bPEI/pGFP (N/P: 5/1) transfection.

2.8. Transfection efficiency of PgP/pβ-Gal polyplexes in 10% serum media condition *in vitro*

To evaluate PgP as a nucleic acid carrier *in vivo*, we used plasmid-β-Galactosidase (pβ-Gal) to avoid potential interference of tissue autofluoresecence with GFP analysis. Polyplexes at an N/P ratio of 30/1 were prepared using both pGFP and pβ-Gal in three different conditions to test the effects of the polyplex preparation volume and injection through a Hamilton syringe (G 26) on transfection. The three preparation conditions were 1) mixing 50 μl of pDNA(2 μg) with 50 μl of PgP (Low conc: used above for *in vitro* studies), 2) mixing 10 μl of pDNA(10 μg) with 10 μl of PgP (High Conc.: planned for *in vivo* studies) and then diluting to 500 μl, and 3) mixing 10 μl of pDNA(10 μg) with 10 μl of PgP and passing it through a Hamilton syringe (G 26) (Hamilton syringe: simulation of polyplex injection *in vivo*), and then diluted to 500 μl. C6 cells were trypsinized and plated in 12-well plates. Transfection was performed in 10% serum condition as described above (Section 2.6.2). One hundred μl (2 μg pDNA) of each of three polyplexes were transfected in C6 cells. GFP expression was evaluated by flow cytometry and β-Gal expression was evaluated by staining β-Gal+ cells using a β-Gal staining kit (Life Technologies) at 48 days post-transfection.

2.9. Transfection efficiency of PgP/pβ-Gal polyplexes in rat spinal cord *in vivo*

All surgical procedures and postoperative care will be conducted according to NIH guidelines for the care and use of laboratory animal (NIH publication No. 86-23, revised 1996) and under the supervision of the Clemson University Animal Research Committee. Sprague Dawley rats (male, 200 gm) were anesthetized with isoflurane gas. Their backs were shaved and prepared with betadine solution, chlorhexidine, and sterile water. A 4 cm longitudinal incision was made over the dorsal mid-thoracic region and the T9 spinous process was identified and removed using an orthopedic bone cutter and then the ligamentum flavum was removed to expose the intervertebral space. PgP/pβ-gal complexes (10 μg pβ-gal, 20 μl) were prepared at an N/P ratio of 30/1 as described above (Section 2.8) and injected into the exposed dorsal T9 spinal cord using a 26-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA). bPEI/pβ-gal at an N/P ratio of 5/1 was used as positive control and naked pβ-gal was used as negative control. Following injection, the paraspinal muscles were closed with 4–0 vicryl suture and the skin was closed with 3-0 silk suture. At 7 days after polyplex injection, animals were anesthetized by isoflurane gas and sacrificed via cardiac perfusion with 4% paraformaldehyde solution. The retrieved spinal cords were fixed with 4% paraformaldehyde solution and 10 μm thick sections cut longitudinally and mounted on positively charged glass slides. To evaluate transfection efficiency, sections were stained using a β-Gal staining kit (Life Technologies) to detect β-Gal+ transfected cells.

2.10. Statistical analysis

The results were analyzed using Student's t-test for pairwise comparisons and ANOVA for multiple comparisons (significance level: $p \leq 0.05$).

3. Results

3.1. Synthesis and characterization of Poly (lactide-co-glycolide)-g-polyethylenimine (PgP)

The amphiphilic graft copolymer PgP was synthesized by conjugating the carboxyl groups of PLGA to the amine groups of bPEI through amide bonds. The structure and grafting ratio of PLGA to bPEI were confirmed by ^{1}H-NMR (δ=2.4~3.5 (m, PEI backbone -CH_2), δ=1.4~1.6 (d, 3H, PLGA -CH_3), δ=4.3 (q, 1H, PLGA-CH), δ=3.9 (s, 2H, PLGA –CH_2)). The ratio of the integrals of the PEI backbone (δ=2.4~3.5) to the methylene of PLGA (δ=3.9) indicated that approximately three PLGA (MW: 4 kDa) were grafted to each PEI. The molecular weight of PgP was determined as approximately 38,168 by GPC using dextran standards (Fig. S1.). The critical micellar concentration (CMC) of PgP determined by dye dissolution method was 0.69 mg/ml (1.86 X 10^{-5} M).

3.2. Preparation and characterization of PgP/nucleic acid polyplexes

The particle size, polydispersity, and surface charge of PgP/pDNA and bPEI/pDNA polyplexes at various N/P ratios are shown in Table 1. The mean particle size of PgP/pDNA polyplexes at N/P ratio of 5/1 was ~340 nm and larger than the bPEI control (~160 nm) at the same N/P ratio. PgP/pDNA particle size significantly decreased relative to the N/P 5/1 group at ratios of 10/1 and higher, ranging between ~150–180 nm. The zeta potential at N/P ratio 2.5/1 was +14.07 mV for bPEI/pDNA polyplexes, while that of PgP/pDNA was −14.94 mV (Data not shown). At N/P ratios of 5/1 and higher, both bPEI/pDNA and PgP/pDNA polyplexes were positively charged, indicating that negatively charged pDNA was completely neutralized by polycationic polymers. We also measured the particle size of bPEI/pDNA (N/P ratio of 5/1) and PgP/pDNA (N/P ratio of 30/1) polyplexes after exposure to 10% serum-containing media. Particle size of bPEI/pDNA (317.8±13.6 nm, PDI: 0.281±0.009) and PgP/pDNA (258.7 ± 2.9 nm, PDI: 0.212±0.021) increased relative to previous measurements in water by approximately 101% and 50%, respectively. PgP/siRNA (N/P ratio of 30/1) and bPEI/siRNA (N/P ratio of 5/1) formed polyplexes of similar particle size (~200 nm) in water. Both PgP/siRNA and bPEI/siRNA were positively charged and the zeta potential of bPEI/siRNA at N/P ratio of 5/1 was significantly higher than PgP/siRNA at N/P ratio of 30/1 (Table I). TEM imaging of PgP/pDNA and PgP/siRNA prepared at 30/1 N/P ratio showed that both groups of polyplexes formed particles with spherical morphology (Figure 1) and the particle size of PgP/pDNA and PgP/siRNA was 176.1±7.2 nm and 193 ± 11.9 nm, respectively.

Formation of polyelectrolyte complexes and neutralization of pDNA's negative charge was evaluated by gel retardation assay. Complete retardation of electrophoretic mobility was observed for bPEI/pDNA polyplexes prepared at 5/1 N/P ratio and PgP/pDNA polyplexes prepared at N/P ratios greater than or equal to 10/1 (Figure 2A). Polyplexes incubated in 10% serum-containing media remained detectable in gel retardation assays for up to 3 days (Figure 2B), while naked pDNA was undetectable after 30 minutes (data not shown).

3.3. Transfection efficiency and cytotoxicity of polyplexes *in vitro*

To evaluate the feasibility of PgP as a gene delivery carrier for CNS applications, we tested the transfection efficiency of PgP/pGFP polyplexes at different N/P ratios in various neural cell types including C6, B35, and primary E8 chick forebrain neurons (CFNs) in both serum-free and 10% serum condition. Transfection efficiency of PgP/pGFP polyplexes was compared with that of bPEI/pGFP at an N/P ratio of 5/1 as a positive control.

Figure 3 shows the transfection efficiency and cytotoxicity of PgP/pGFP polyplexes in C6 cells. Transfection efficiency in serum-free media increased with increasing N/P ratio, attaining 47 % at N/P ratio of 30/1 compared to 13% for bPEI control (Figure 3A). In the presence of 10% serum, transfection efficiency also increased with increasing N/P ratio and was significantly higher than the bPEI control at all N/P ratios. PgP achieved a maximum transfection efficiency of 59 % (N/P ratio 30/1) in the presence of serum, while that of the bPEI control (2%) was significantly reduced relative to serum-free conditions. Cell viability was modestly but significantly decreased after transfection in serum-free conditions with PgP/pGFP at N/P ratios of 15 and greater (Figure 3B). However, no significant differences in cell viability relative to non-transfected control were observed after transfection in the presence of serum, indicating that PgP/pDNA polyplexes were non-toxic under these more physiologically relevant conditions. Figure 3C shows representative images of C6 cells transfected at varying N/P ratio in the 10% serum condition.

In B35 cells, the transfection efficiency of PgP/pGFP polyplexes also increased with increasing N/P ratio in both serum-free and 10% serum condition and reached 68.3 % and 75.1 % at N/P ratio of 30/1, respectively, while the transfection efficiency of bPEI was significantly reduced from 35.3 % in the serum-free condition to 2.27% in the 10% serum condition (Figure 4A). PgP/pGFP polyplexes were also generally non-toxic in B35 cells, with significant changes in cell viability only observed at N/P ratios of 25/1 and 30/1 in the 10% serum condition (Figure 4B). While exposure to serum reduces the performance of most nonviral vectors, these results demonstrate that PgP achieved significantly higher transfection efficiency than bPEI in the 10% serum condition medium at all N/P ratios in both C6 and B35 cell lines.

We also evaluated the feasibility of PgP as a gene delivery carrier to non–dividing primary E8 chick forebrain neurons. In serum-free conditions, transfection efficiency of bPEI/pGFP at N/P ratio of 5/1 (11.8 %) was higher than that of PgP/pGFP complexes below 20/1 N/P ratio (9.62 %), but didn't show any significant difference above 25/1 N/P ratio (Figure 5A). In the 10% serum condition, transfection efficiency increased as the PgP/pGFP N/P ratio increased and the transfection efficiency was significantly higher at N/P ratios of 10/1 and above than the bPEI/pGFP control. The transfection efficiency of PgP/pGFP at N/P ratio of 30/1 (8.12 %) was 3 times higher than that of the bPEI control (2.73 %) in 10% serum condition ($P<0.05$). In both serum-free and 10% serum conditions, cytotoxicity increased as the PgP/pGFP N/P ratio increased (Figure 5B). In the case of bPEI/pGFP at N/P 5/1, the cytotoxicity was very high in non-serum condition while it was less toxic in 10% serum condition, likely a result of low transfection. Representative images of GFP-transfected (green), beta-III-tubulin+ (red) primary E8 CFNs are shown in Figure 5C.

3.4. Effect of the polymer concentration on cytotoxicity

We also evaluated the cytotoxicity of PgP/pDNA (N/P ratio of 30/1) and bPEI/pDNA (N/P ratio of 5/1) polyplexes as a function of polymer concentration (Figure 6). In both C6 cells and B35 cells, PgP/pDNA showed higher viability than bPEI/pDNA at all polymer concentrations and it was significantly different at higher concentrations (≥ 20 μg/ml in C6 cells and ≥ 10 μg/ml in B35 cells).

3.5. Time course study of polyplex stability and transfection efficiency

PgP/pGFP polyplexes prepared at N/P ratio of 30/1 and stored at 4°C were stable and retarded in the wells at all time points and no degraded or dissociated DNA was observed (Fig. S2-A). Stored polyplexes maintained high transfection efficiency in B35 cells in 10% serum condition that was not significantly different from freshly prepared polyplex controls at any time point (Fig. S2-B).

3.6. Gene knockdown efficiency of PgP/GFP siRNA *in vitro*

Intracellular delivery of siRNA was first evaluated by transfecting B35 cells with PgP/siGLO red transfection indicator at varying N/P ratios in 10% serum condition. PgP/siGLO red at an N/P ratio of 25/1 showed approximately 79 % transfection efficiency, which was not significantly different from RNAimax (94%), while naked siGLO showed very low transfection (17%) (Figure 7A). No significant differences in cell viability were observed relative to the non-transfected control (Figure 7B). In the next study, gene knockdown efficiency was evaluated by co-transfection of PgP/GFP siRNA, bPEI/GFP siRNA, and RNAiMAX/GFP siRNA in bPEI/pGFP-transfected B35 cells. The relative GFP knockdown efficiency of PgP/GFP siRNA polyplexes at N/P ratios of 30/1 was ~65 %, which was similar to that obtained with RNAiMAX/GFP siRNA (~70 %) (Figure 8A). Figure 8B shows representative images of GFP expression after serum-free transfection with bPEI/pGFP at N/P of 5/1 (Fig 8B-i) and after co-transfection with PgP/GFP siRNA at N/P ratio of 30/1 in 10% serum (Fig 8B-ii).

3.7. Transfection efficiency of PgP/pβ-Gal polyplexes in 10% serum media condition *in vitro*

In order to minimize the sample volume required for injection into the spinal cord, polyplexes for *in vi*vo studies were prepared at higher concentrations (10 μg pDNA/20 μl) than used for earlier *in vitro* studies (2μg pDNA/100 μl). Therefore, we first performed *in vitro* tests to compare the transfection efficiency of pβ-Gal and pGFP polyplexes prepared at increased concentration and after passage through a Hamilton syringe (26G) to our conventional preparation and transfection procedure. GFP transfection measured by flow cytometry showed no significant differences between polyplexes prepared at low concentration without syringe passage and those prepared at high concentration with and without passage through a Hamilton syringe (Fig S3-A). Figure 3S-B shows represenative images of C6 cells transfected with pβ-Gal (top) and pGFP (bottom) prepared under the different conditions. These results demonstrate the PgP/pDNA polyplexes can be prepared at increased concentration and injected through small diameter syringe for *in vivo* delivery without loss of bioactivity.

3.8. Transfection of PgP/pβ-Gal polyplexes in rat spinal cord *in vivo*

Based on our *in vitro* studies in which transfection efficinecy generally increased with increasing N/P ratio without substantially increasing cytocoxity in the presence of serum, we used a 30/1 N/P ratio for the spinal cord injection model. pβ-Gal was used for the *in vivo* model in order to avoid tissue autofluorescence confounding the interpretation of GFP transfection. Transfection efficiency in the rat spinal cord was observed in the order of PgP/pβ-Gal ≫ bPEI/pβ-Gal > naked pβ-Gal (Figure 9). Figure 9 shows representative images of β-Gal staining 7 days after injection of naked pβ-Gal (A), bPEI/pβ-Gal polyplexes (B), and PgP/pβ-Gal polyplexes (C). These results demonstrate that PgP nonviral vectors can achieve efficient transfection of neural cells *in vivo*.

4. Discussion

The pathophysiology of SCI involves changes in both the neuronal microenvironment and intrinsic neuronal biochemistry that offer potential targets for therapeutic intervention by gene-based therapy to increase regenerative capability. Our long-term goal is to develop a bPEI derivative with hydrophobic polymer chains capable of forming polymeric micelles for application as a combinatorial drug/gene delivery carrier. In this study, we synthesized an amphiphilic copolymer, PgP, by grafting three PLGA (~4 kDa) polymers to primary amine groups of 25kDa bPEI and evaluated its feasibility as a nucleic acid-delivery carrier in neural cells. In aqueous solution, PgP spontaneously formed micelles with a CMC of 0.69 mg/ml (1.86×10^{-5} M), which is much lower than low molecular weight surfactants ($\sim 10^{-3}$–10^{-4}M), e.g. sodium dodecyl sulfate (8.2 mM). This low CMC value indicates greater thermodynamic stability of PgP micelles in solution, which is important because a thermodynamically or kinetically unstable delivery carrier might release drugs or genes by dissociation of the micelle structure in the blood stream. We expect this thermodynamic stability will allow PgP to be a systemic and local delivery carrier for drugs and nucleic acids.

Positively charged PgP efficiently condensed negatively charged plasmid DNA to form stable polyeletrolyte complexes with 150–180 nm size. Particle size is a critical physicochemical property for nonviral vectors, with size range less than 200 nm important to allow cellular uptake by endocytosis and avoid rapid clearance by the reticuloendothelial system (RES) in systemic delivery applications[32, 33]. At low N/P ratios, PgP/pDNA polyplexes exhibited less efficient charge neutralization (2.5/1) and lower stability (5/1) than bPEI/pDNA complex. Several previous studies have similarly reported that higher N/P ratios were required for stable pDNA condensation by PEI modified with hydrophobic groups relative to the parent polymer[30, 34]. While this may result from reduction in the number of primary amines for derivatives extensively modified with low molecular alkyl groups, in our case, this is more likely attributable to micellization and resulting steric hindrance reducing accessibility of some of the PgP primary amines to pDNA at low N/P ratios. Due to their net positive charge, polyplexes can electrostatically bind anionic proteins present in physiological fluids such as blood, leading to aggregation that increases particle size and can reduce bioactivity. Although exposure to serum increased the size of both PgP and bPEI-based polyplexes, the change in PgP/pDNA particle size was substantially lower, which may

provide a basis for improved retention of bioactivity and reduced rate of clearance. One limitation of this study is that 10% serum-containing medium does not have the same protein and salt concentrations as blood or cerebrospinal fluid and it will be important to evaluate particle size/aggregation under these conditions in future studies.

PgP/pDNA polyplexes showed very high transfection efficiency in the presence of serum without significant cytotoxicity in various cell types. In both C6 neuroglioma and B35 neuroblastoma cell lines, transfection efficiencies of approximately 60% were achieved without significant changes in cell viability relative to either the non-transfected or bPEI-transfected controls. Furthermore, in dose-dependent cytotoxicity testing, both C6 and B35 cells maintained significantly higher viability after transfection with PgP/pDNA relative to bPEI/pDNA at relatively higher vector concentrations. Transfection of primary neurons was substantially less efficient, but still significantly higher at all N/P ratios than the bPEI control in the presence of serum. Most nonviral gene-delivery carriers have limited efficacy in terminally-differentiated, post-mitotic cells such as neurons, while viral gene delivery carrier can efficiently transfect [35, 36]. Although relatively higher transfection efficiencies for CNS neurons using nonviral vectors, such as bPEI (14%) [27], dendrimers (35 %)[37], or lipofectamine (25–27%)[38], have been reported, these studies were performed in serum-free condition. In addition to the high transfection efficiency of freshly prepared polyplexes, we also show that PgP/pDNA pre-formed vectors can be stably stored for up to 7 days without significant reduction in transfection efficiency. Although longer-term studies are required, this is a potentially promising feature since stable formulation is important for commercialization and clinical application and has proven challenging for many nonviral vectors.

Several previous studies have demonstrated that bPEI modified with low molecular weight hydrophobic groups increases transfection efficiency in the presence of serum. For example, Thomas et al. reported that conjugation of alanine increased bPEI (25kDa) transfection efficiency approximately 2-fold relative to the parent polymer[39]. Liu et al. modified bPEI (10kDa) with 2,4,6-trimethoxybenzylidene-tris (hydroxymethyl)ethane containing an acid-degradable acetal linkage and attained 16-fold higher transfection than bPEI (25 kDa) control[40]. Aravindan et al. synthesized acetic and proprionic derivatives of bPEI (25kDa) that achieved up to 50% transfection efficiency[41]. Although the exact mechanism underlying increased transfection efficiency of hydrophobic PEI derivatives has not been elucidated, various studies have reported increased cellular uptake and reduced polyplex stability based on heparin dissociation assays and FRET analysis demonstrating increased intracellular polyplex dissocation[34, 40, 42]. Other groups have prepared bPEI derivatives with lower degree of substitution of higher molecular weight hydrophobic polymers to create micellar carriers. For example, Lee et al. prepared PEI-g-PLGA (bPEI:10 kDa and PLGA: 14kDa) and Mishra et al. synthesized $(PLGA)_2$-b-PEI (bPEI: 25kDa and PLGA: 36kDa) [43, 44]. Although both derivatives showed reduced cytotoxicity relative to the parent bPEI, neither showed increased transfection efficiency relative to bPEI 25kDa even under serum-free conditions. We hypothesize that a critical difference between these materials and PgP is the hydrophilic:lipophilic balance (HLB). Because PgP incorporates relatively lower molecular weight of PLGA, its HLB is 13.5, while the HLB values of the polymers prepared by Lee and Mishira were 8.33 and 8.2, respectively. Although the

mechanism still remains incompletely understood, previous studies have indicated that the bioactivity of amphiphilic Pluronic block copolymers towards membrane interactions, inhibition of drug efflux transporters, and increased gene expression from nonviral vectors is related to HLB and maximized at intermediate HLB values similar to that of PgP[45]. In our own previous work, we have found that a related Tetronic block copolymer of similar HLB to PgP can increase gene expression from plasmid vectors by as much as an order of magnitude by increasing steady-state transgene mRNA levels.[46]. To further characterize the effect of HLB on transfection efficiency and gain further insight into its mechanistic basis, we synthesized PgP using different molecular weights of PLGA (25kDA and 50 kDa) in ongoing studies.

We also investigated the potential efficacy of PgP as a siRNA delivery carrier using both siGLO red as a transfection indicator and GFP-targeting siRNA in 10% serum conditions. PgP was able to achieve transfection efficiencies above 80% without significantly increased cytotoxicity relative to non-transfected controls. PgP was substantially more effective than bPEI in co-transfection studies and achieved similar GFP knockdown to the RNAiMAX positive control. Previous studies have suggested that the electrostatic interaction of bPEI with siRNA is less stable than with higher molecular weight pDNA, requiring relatively high siRNA doses [47]. The efficacy of PgP for siRNA delivery observed in these studies may result from improved stability of the micellar carrier. In future studies, siRNA delivery and knockdown efficiency may be further improved by using oligomerization methods to increase the nucleic acid molecular weight [47–51].

The ability of PgP to achieve high transfection efficiency of pDNA and siRNA in 10% serum condition suggests it may be an effective nucleic acid delivery carrier. However, translation of promising *in vitro* results to *in vivo* models has been a major challenge in the development of nonviral vectors. Therefore, we used β-Galactosidase as a model gene to evaluate the transfection efficiency of PgP/pDNA polyplexes in the rat spinal cord. PgP/pβ-Gal achieved substantial transfection in the injection site and surrounding neural tissue that was substantially higher than bPEI/pβ-Gal or naked pβ-Gal controls. These *in vivo* gene transfer results correspond well with PgP's transfection efficiency in the presence of serum *in vitro* and suggest it provides an effective nucleic acid carrier upon which to build targeted, multi-functional delivery systems.

5. Conclusions

An amphiphilic, micelle-forming copolymer (PgP) was synthesized by grafting bPEI (25 kDa) with three low molecular weight PLGA (4 kDa) chains. PgP transfected pDNA and siRNA with efficiencies greater than 60% in neural cell lines and 8% in primary CNS neurons in 10% serum conditions that dramatically reduced the efficacy of the bPEI control. *In viv*o efficacy was demonstrated by robust transfection in the rat spinal cord that substantially exceeded pPEI or naked pDNA controls. These results suggest that PgP may be useful as a therapeutic nucleic acids delivery carrier for the treatment of spinal cord injury, as well as other forms of CNS trauma and neurodegenerative diseases. Future studies will extend the capabilities of PgP through conjugation of neuron-specific targeting ligands for

siRNA delivery, hydrophobic drug loading within the micelle core, and evaluation in spinal cord injury models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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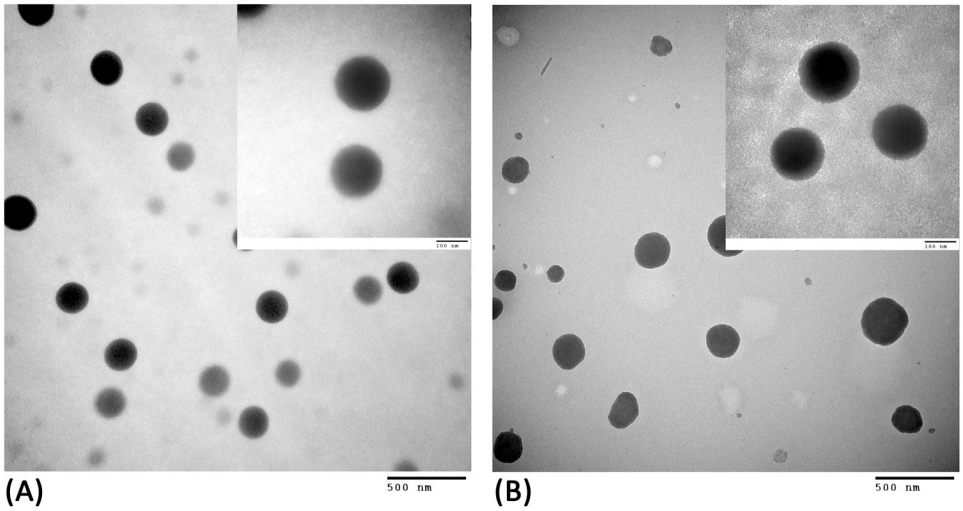

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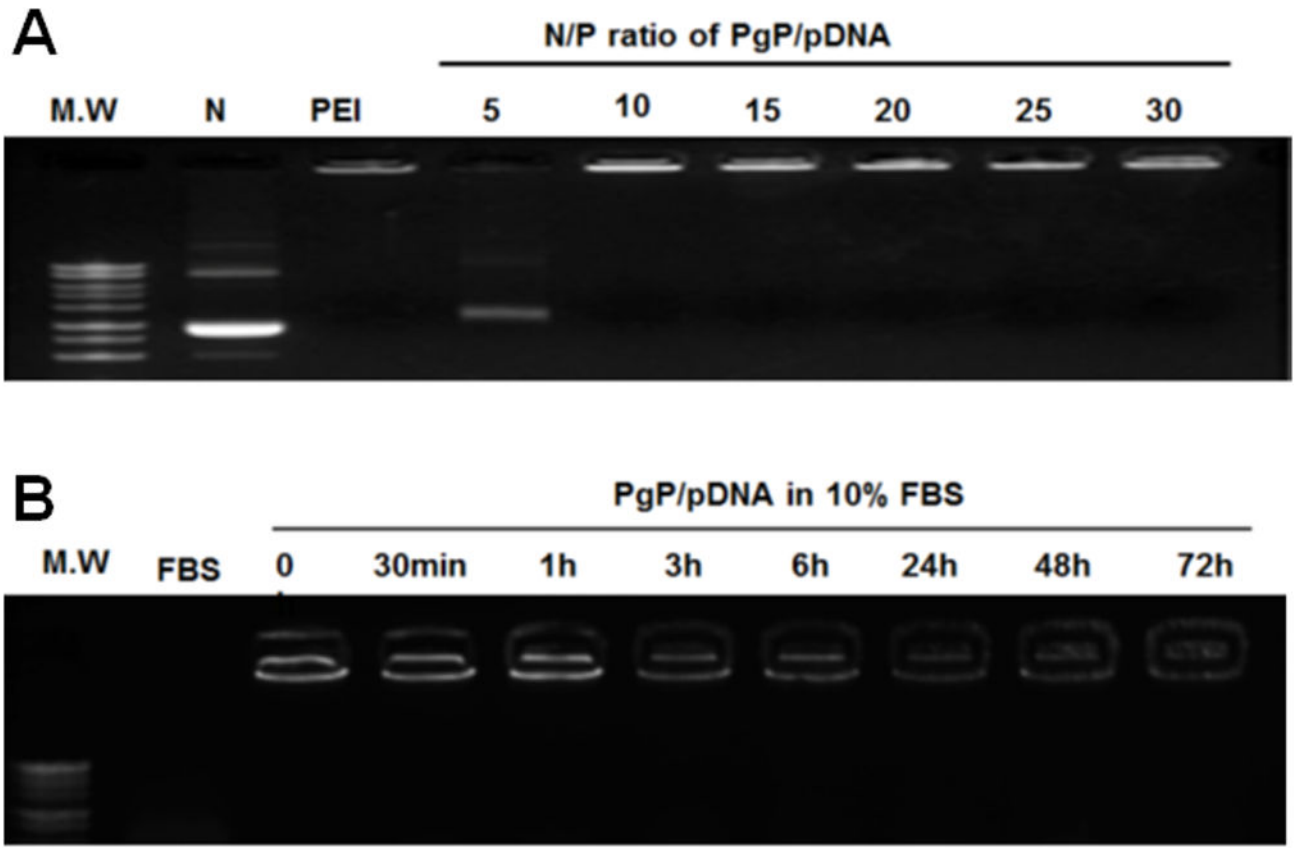

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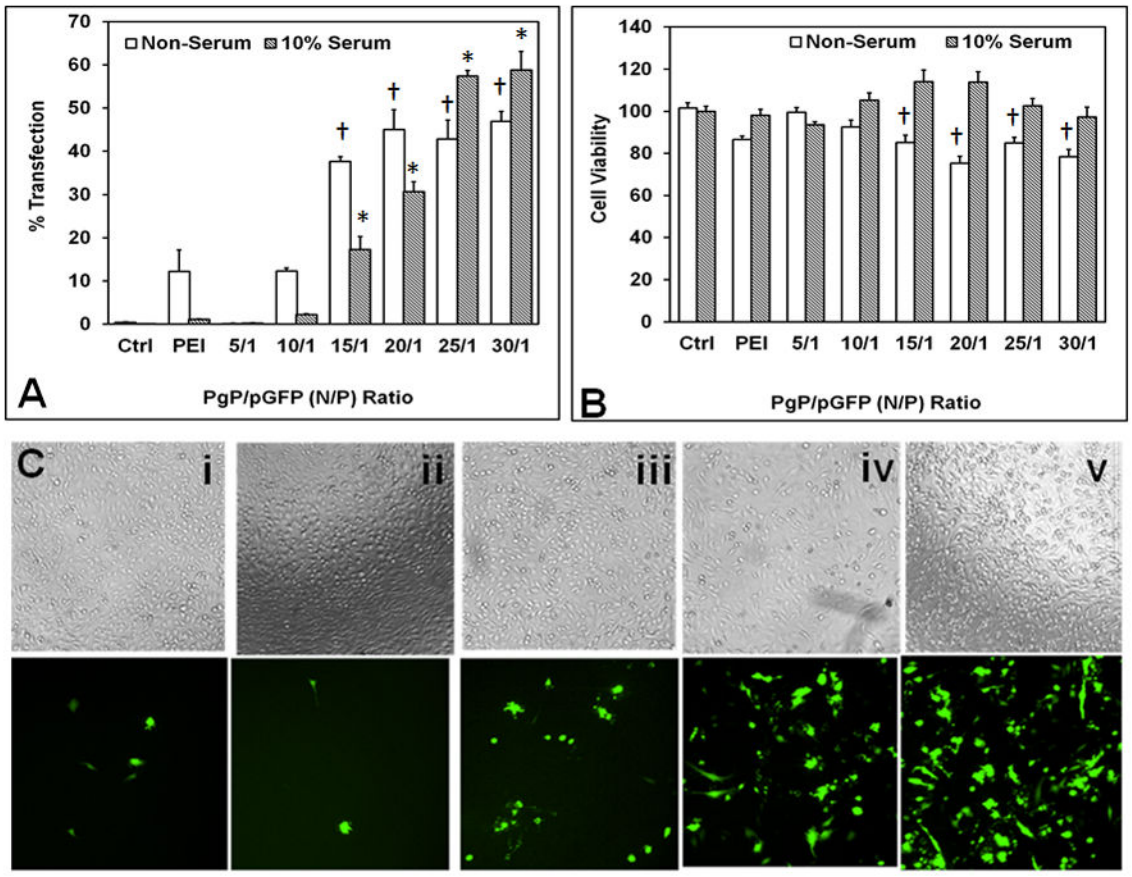

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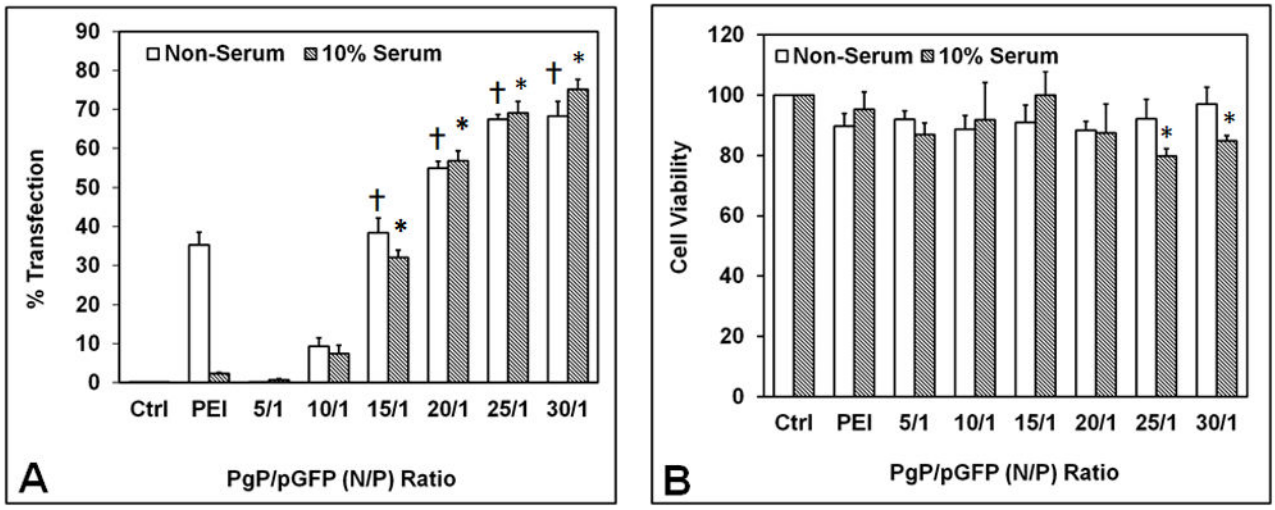

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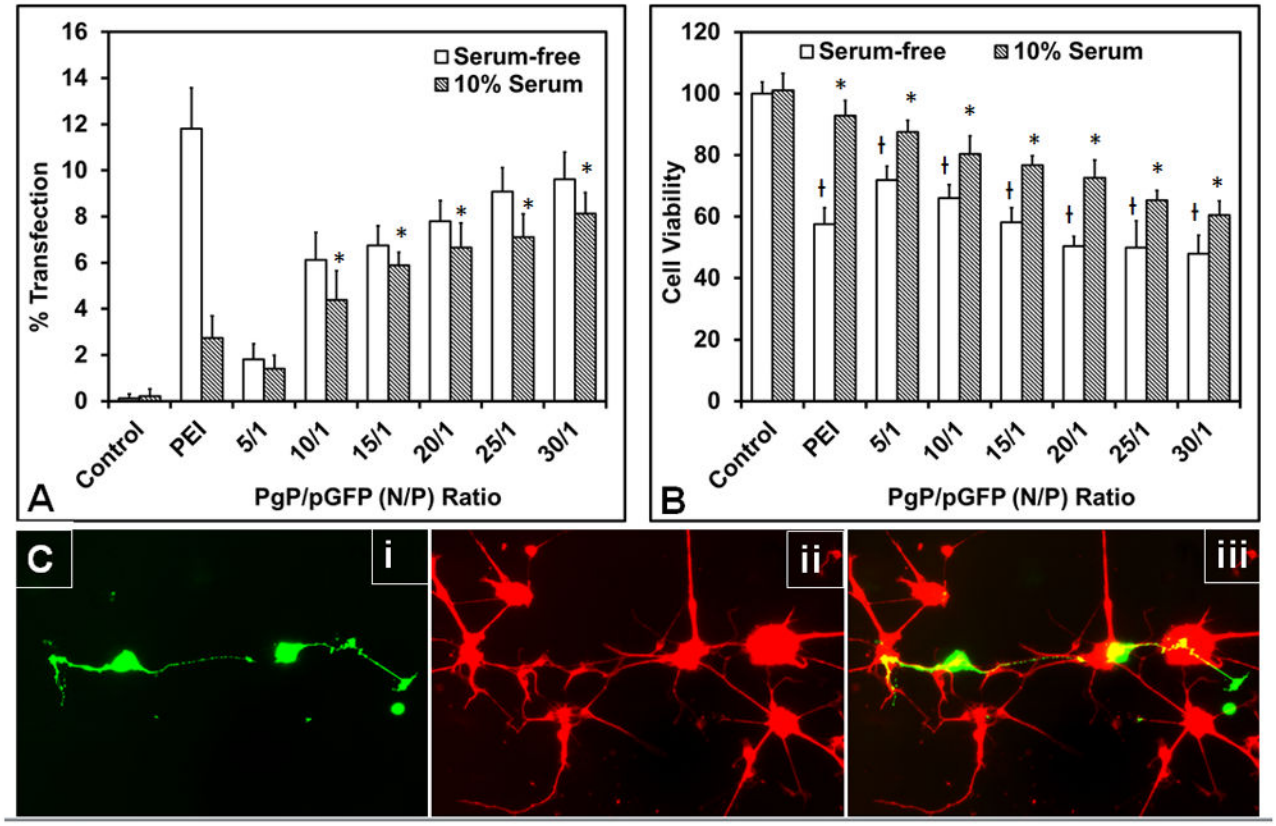

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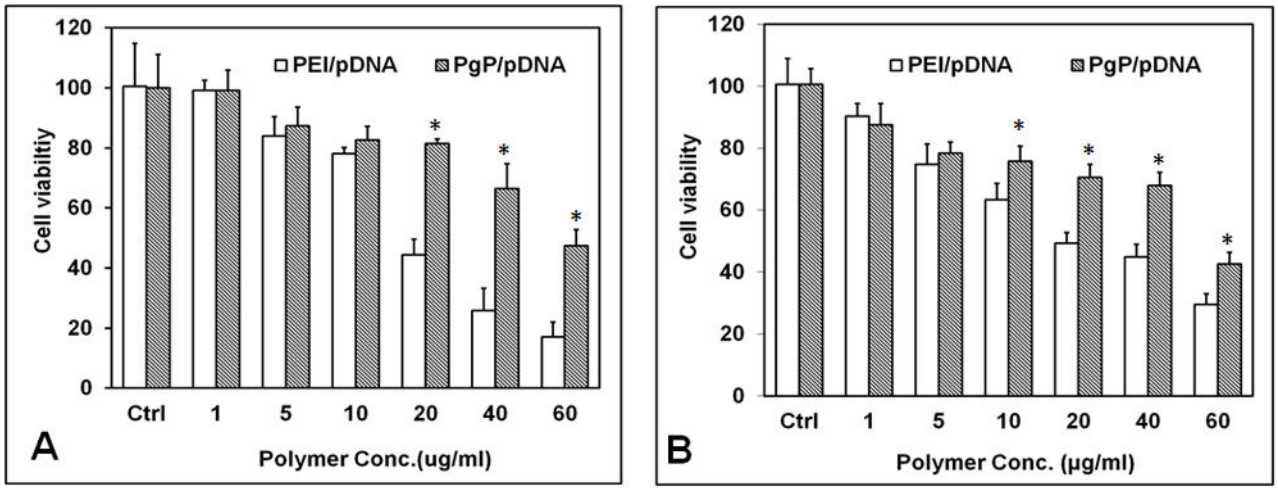

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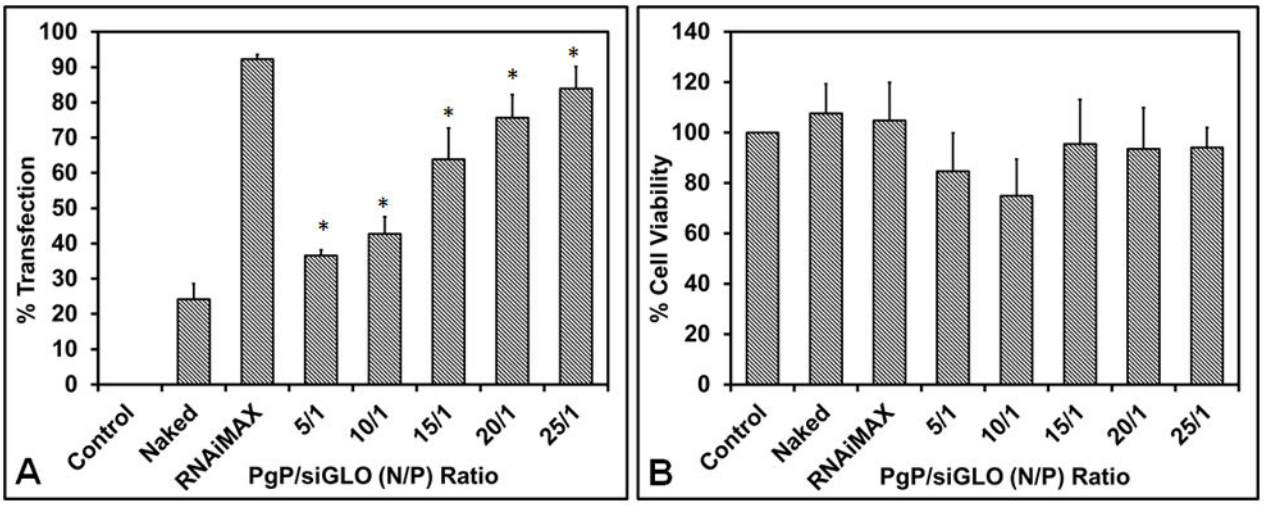

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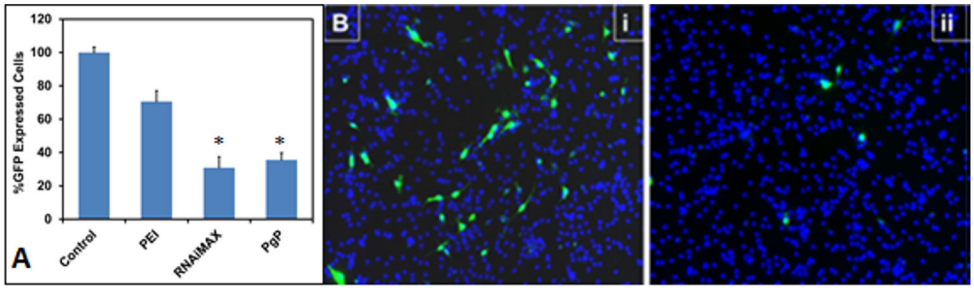

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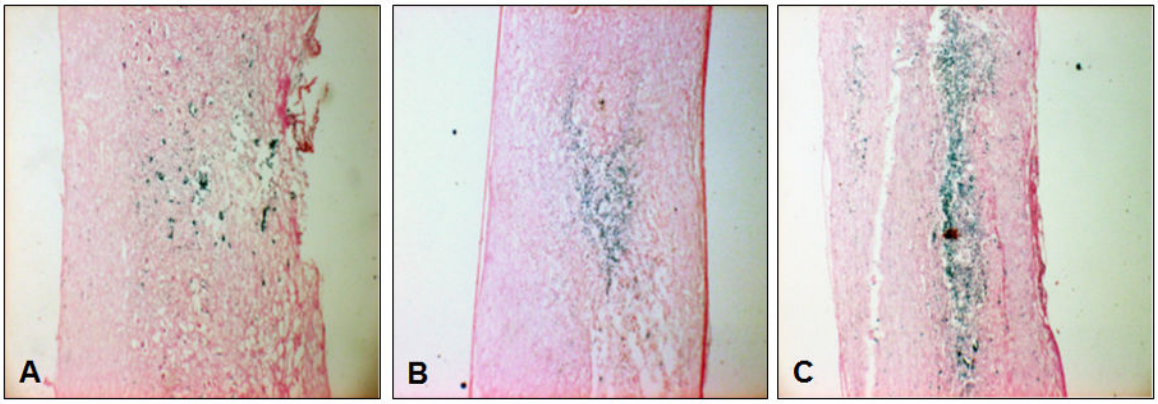

Figure 9.

Table 1

Mean particle size (PS), zeta potential (ZP), and polydispersity index (PDI) of PgP/nucleic acids polyplexes.

	PEI/pDNA	PgP/pDNA						PEI/siRNA	PgP/siRNA
N/P ratio	5	5	10	15	20	25	30	5	30
Particle Size (nm)	157.4 ± 8.1	340.6 ± 13.7	146.9 ± 2.5	150.9 ± 2.7	167.1 ± 3.1	177.2 ± 1.7	177.6 ± 2.0	213.9 ± 39.2	201.4 ± 4.2
Zeta Potential (mV)	40.2 ± 0.8	27.3 ± 9.5	44.1 ± 2.3	46.4 ± 1.8	48.0 ± 0.5	49.6 ± 1.3	48.3 ± 3.9	44.1 ± 1.3	30.6 ± 1.6
PDI	0.15 ± 0.01	0.18 ± 0.05	0.14 ± 0.01	0.14 ± 0.03	0.14 ± 0.02	0.13 ± 0.02	0.14 ± 0.03	0.23 ± 0.10	0.24 ± 0.03