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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: 25 kDa) 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 (4 kDa) to bPEI (25 kDa) 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.

Statement of Significance

Gene therapy is a promising strategy to overcome barriers to axonal regeneration in the injured central nervous system. Branched polyethylenimine (bPEI: 25 kDa) is one of the most widely studied nonviral vectors, but its clinical application has been limited due to cytotoxicity and low transfection efficiency in the presence of serum proteins. Here, we report cationic amphiphilic copolymers, poly (lactide-co-glycolide)-graft-polyethylenimine (PgP) that 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 as well as transfection of neural cells *in vivo* suggests PgP may be a promising nucleic acid carrier for CNS gene delivery.

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

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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, 25 kDa) 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 (25 kDa) 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 (1 kb 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), 100× 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 μmol) was dissolved in 20 ml dried anhydrous DMF. *N*-hydroxysuccinimide (NHS, 27.6 mg, 240 μmole) and *N,N′*-Dicyclohexylcarbodiimide (DCC, 49.6 mg, 240 μmol) were added to the reaction solution and this mixture was stirred for 2 h to activate the carboxylic end group of PLGA. The resulting precipitate, dicyclohexyl urea (DCU), was removed by filtration. bPEI (1.25 g, 50 μmol) 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 h 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 5000 rpm for 10 min 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/min. A Waters 1525 HPLC pump and Waters 2414 Refractive Index Detector were used. Dextrans at molecular weights of 5, 12, 25, 50, and 80 kDa were used as standards.

2.2.2. Critical micelle concentration

The critical micelle concentration (CMC) was determined using a dye solubilization method. Ten microliter 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 h. 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