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Optimization of Tet1 ligand density in HPMA-*co*-oligolysine copolymers for targeted neuronal gene delivery

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ABSTRACT

Targeted gene delivery vectors can enhance cellular specificity and transfection efficiency. We demonstrated previously that conjugation of Tet1, a peptide that binds to the GT1b ganglioside, to polyethylenimine results in preferential transfection of neural progenitor cells *in vivo*. In this work, we investigate the effect of Tet1 ligand density on gene delivery to neuron-like, differentiated PC-12 cells. A series of statistical, cationic peptide-based polymers containing various amounts (1–5 mol%) of Tet1 were synthesized via one-pot reversible addition-fragmentation chain transfer (RAFT) polymerization by copolymerization of Tet1 and oligo-L-lysine macromonomers with *N*-(2-hydroxypropyl)methacrylamide (HPMA). When complexed with plasmid DNA, the resulting panel of Tet1-functionalized polymers formed particles with similar particle size as particles formed with untargeted HPMA–oligolysine copolymers. The highest cellular uptake in neuron-like differentiated PC-12 cells was observed using polymers with intermediate Tet1 peptide incorporation. Compared to untargeted polymers, polymers with optimal incorporation of Tet1 increased gene delivery to neuron-like PC-12 cells by over an order of magnitude but had no effect compared to control polymers in transfecting NIH/3T3 control cells.

1. Introduction

Gene delivery can potentially treat a range of neurological diseases inadequately addressed by current therapeutics. Delivery of genes expressing various neurotropic factors have been studied for neuroprotection and axonal regeneration following central nervous system (CNS) injury or for delayed progression of amyotrophic lateral sclerosis, Huntington's and Parkinson's [1–3]. Of the available delivery technologies, adeno-associated viral vectors have been most extensively explored for CNS gene delivery applications due to their high transduction efficiency and innate neural tropism [4], but immunogenicity, long-term safety, and cost of manufacturing remain significant concerns. Non-viral delivery systems, such as cationic polymers or liposomes, can potentially overcome such limiting barriers; however, relatively poor transfection efficiency and high cytotoxicity remain challenges [5].

The incorporation of targeting ligands into non-viral gene delivery vehicles has been shown to both increase gene delivery efficiency and specificity. Various ligands, such as folate [6], transferrin [7], and RGD sequences [8], have been used to mediate cellular binding and internalization. It has been postulated that to achieve specificity *in vivo*, the density of targeting ligands must be controlled [9]. Recent work reported that intermediate levels of ligand density in folate [10], transferrin [11], and antibody-targeted nanoparticles [12] conferred the highest level of tissue specificity *in vivo*.

Current approaches towards multivalent decoration of polymeric nanoparticles typically involve grafting of ligands onto the polymer carrier. However, control and reproducibility of synthesis remain a challenge for these approaches, leading to incomplete coverage or varied surface functionalization of nanoparticles [13]. We have developed an approach to controllably incorporate ligands into a polymeric construct through direct copolymerization of functionalized ligand monomers, allowing direct control over material properties. This method allows for control over ligand density, orientation of display, and architectural display in the final polymeric construct, overcoming some limitations of grafting approaches.

We recently reported the synthesis and optimization of well-defined, narrowly disperse oligo-L-lysine-HPMA cationic polymers utilizing reverse addition-fragmentation chain transfer (RAFT) polymerization to copolymerize HPMA with methacrylamido-functionalized peptide macromonomers [14]. Polymers displaying multiple peptide entities can be easily synthesized using this approach, and incorporation of water-soluble peptides can be

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controlled based on feed ratios [15,16]. This platform can therefore be used to probe the effect of targeting ligand density on polymer gene transfer efficiency. Tet1, a peptide identified by *in vitro* phage display, binds to GT1b gangliosides, sphingophospholipids highly expressed on neuronal cell types [17]. Tet1 has been used in applications such as peripheral neuron labeling and in the delivery of PLGA nanoparticles and polymersomes to neuronal targets [18–20]. Our group has previously shown neuronal transfection *in vitro* using Tet1-modified poly(ethylenimine) (PEI) polyplexes and specific gene delivery to neural stem and progenitor cells *in vivo* upon intraventricular administration using PEGylated versions of these materials [21,22].

We report here the synthesis and evaluation of a series of peptide-based polymers containing varying amounts of Tet1 for targeted gene delivery to neuron-like cells. We utilize RAFT polymerization for one-pot synthesis of three component peptide–polymers using Tet1 as a targeting sequence, oligolysine for DNA binding and condensation, and HPMA for colloidal stability. Polyplexes were formed by self-assembly of polycations with plasmid DNA, and characterized by YOYO-1 DNA packaging assay and particle sizing. Cellular uptake as a function of Tet1 modification as well as gene transfection efficiency was studied in cultured neuron-like cells.

2. Materials and methods

2.1. Materials

N-(2-Hydroxypropyl)methacrylamide (HPMA) was purchased from Polysciences (Warrington, PA). The initiator VA-044 was purchased from Wako Chemicals (Richmond, VA). Fmoc-protected amino acids and HBTU were purchased from AAPPTec (Louisville, KY), N-succinimidyl methacrylate from TCI America (Portland, OR), and Rink Amide Resin from EMD Biosciences (Darmstadt, Germany). All other materials were reagent grade or better and were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Endotoxin-free plasmid pCMV-Luc2 was prepared using the Qiagen Plasmid Giga kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

2.2. Synthesis of peptide monomers

Two peptide sequences were synthesized on solid support: K_3-Tet1 (KKKHLNILSTLWKYR) and the oligolysine K_{10} (KKKKKKKKKK). Peptides were synthesized via solid phase peptide synthesis following standard Fmoc chemistry using an automated PS3 Peptide Synthesizer (Protein Technologies, Phoenix, AZ). 6-Aminohexanoic acid (Ahx) was added to the N-terminus of the peptide sequences. Prior to peptide cleavage from resin, the N-termini of the peptides were deprotected and coupled with N-succinimidyl methacrylate. These methacrylamido-functionalized peptides were cleaved from resin by treatment with trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/1,3-dimethoxybenzene/ddH_2O (90:2.5:5:2.5, *v*/*v*/*v*/*v*) for 3 h under gentle mixing. Cleaved peptide monomers were precipitated in ice-cold ether, dissolved in methanol and re-precipitated twice in ice-cold ether. Peptide monomers were analyzed by RP-HPLC and MALDI-TOF MS and purified as necessary.

2.3. Polymer synthesis

A series of copolymers were synthesized with varying amounts of Tet1 peptide in the feed (0%, 1%, 3%, 5%) while holding K_{10} peptide constant at 20%. Monomers were dissolved in acetate buffer (1 M, pH 5.1) with 10% ethanol (*v*/*v*) such that the final monomer concentration of the solution was 0.5 M. The RAFT chain transfer agent (CTA) used was ethyl cyanovaleric trithiocarbonate (ECT, molecular weight 263.4 g/mol) and the initiator (I) used was VA-044. The molar ratios of total monomer:CTA:I at the start of the polymerization were 190:1:0.1. The reaction solutions were transferred to round bottom flasks, capped with a rubber septa, purged with argon for 10 min, and then submerged in a 44 °C oil bath to initiate polymerization. The polymerization was allowed to proceed for 48 h. The flasks were removed from the oil bath and polymers dialyzed against distilled H_2O to remove unreacted monomers and buffer salts. The dialyzed products were lyophilized dry.

2.4. Size exclusion chromatography

Molecular weight analysis was carried out by size exclusion chromatography. The copolymers were dissolved at 2 mg/mL in running buffer (1:1 MeOH:300 mM acetate buffer, pH 4.4) for analysis by size exclusion chromatography – multiangle laser light scattering (SEC–MALLS). Analysis was carried out on an OHpak SB-804 HQ column (Shodex, Kawasaki, Japan) in line with a miniDAWN TREOS multiangle laser light scattering detector (Wyatt, Santa Barbara, CA) and an OptiLab rEX refractive index detector (Wyatt). Absolute molecular weight averages (M_n, M_w) were calculated using ASTRA software (Wyatt).

2.5. Amino acid analysis

The actual incorporated amount of peptide and HPMA in the final copolymers was determined through modified amino acid analysis as previously reported [23]. Briefly, hydrolyzed polymers were derivatized with o-phthalaldehyde/β-mercaptopropionic acid and run on a Zorbax Eclipse X-18 (Agilent Technologies, Santa Clara, CA) HPLC column with pre-column derivatization to label hydrolyzed amino acids and 1-amino-2-propanol (hydrolysis product of HPMA). Calibration curves were generated using serial dilutions of L-lysine, L-histidine, and reagent grade 1-amino-2-propanol.

2.6. Polyplex formation

pCMV-Luc2 plasmid was diluted in ddH_2O to a concentration of 0.1 mg/mL and mixed with an equal volume of polymer (in ddH_2O) at the desired amino to phosphate (N:P) ratio. After mixing, polyplexes were allowed to form for 10 min at room temperature.

2.7. Polyplex sizing by dynamic light scattering (DLS)

Polyplexes (0.5 μg DNA, 10 μL) were formed with polymers pHT1K10, pHT3K10, pHT5K10, and pHK10 at N:P ratios of 2, 3, and 4 and were mixed with either 90 μL of ddH2O or PBS such that the final salt concentration was 150 mM. Particle size was determined by dynamic light scattering (ZetaPLUS, Brookhaven Instruments Corp, Holtsville, NY).

2.8. DNA condensation using YOYO-1 fluorescence quenching assay

pCMV-Luc2 plasmid was mixed with the bis-intercalating dye YOYO-1 iodide (Invitrogen, Carlsbad, CA) at a dye/base pair ratio of 1:50 and incubated at room temperature for 1 h. Polyplexes were formed at N/P ratios of 0, 1, 2, 4, 6, and 10 by complexing YOYO-1-labeled DNA with pHT1K10, pHT3K10, pHT5K10, or pHK10. Ten microliters (containing 0.5 μg DNA) of polyplex was added to each well of a 96-well plate, followed by 90 μL of ddH_2O. The fluorescence from each well was measured on a Tecan Safire^2 plate reader with excitation at 491 nm and emission at 509 nm. The fluorescence signal for each N/P ratio was normalized to the N/P 0 (uncomplexed DNA) signal.

2.9. Cell culture

NIH/3T3 cells and PC-12 cells were grown according to the ATCC recommendations. For *in vitro* studies, PC-12 cells were seeded onto collagen-coated plates. PC-12 cells were differentiated in F12K media supplemented with 1% HS, 1% ABAM, and 100 ng/mL nerve growth factor. Media was changed every 2 days.

2.10. Polyplex uptake

pCMV-Luc2 plasmid was mixed with the bis-intercalating dye YOYO-1 iodide at a dye/base pair ratio of 1:50 and incubated at room temperature for 1 h. Polyplexes were formed at N/P 3 by complexing YOYO-1 labeled DNA with pHK10, pHT1K10, pHT3K10, and pHT5K10 polymers. Labeled polyplexes were added to cells for 2 h at 37 °C. Cells were washed 3 times with PBS, detached by treatment with collagenase, and then washed twice more before labeling with propidium iodide (PI) stain. Cells were analyzed for fluorescence intensity by flow cytometry using the MACSQuant Analyzer (Miltenyi Biotec, Cologne, Germany) and gated for PI- (live) cells.

2.11. In vitro transfection efficiency

PC-12 and NIH/3T3 cells were transfected as previously described [14]. Briefly, polyplexes (1 μg DNA) were formed at 2.5, 3, and 4 N:P, diluted to 200 μL in Opti-MEM (Invitrogen), and added to cells for 4 h. After an additional 44 h, luciferase expression was quantified using a luciferase assay kit (Promega, Fitchburg, WI) according to the manufacturer's instructions, except with an additional freeze–thaw cycle at −20 °C to ensure complete cell lysis. Luminescence intensity was measured on a Tecan Safire^2 plate reader (Männerdorf, Switzerland) with 1 s integration; total protein content was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions so luciferase activity could be normalized to the total protein content. Each sample was tested in triplicate.

2.12. Hemolysis assay

A hemolysis assay evaluating the membrane-lytic activity of the materials was performed as previously described [24]. Briefly, plasma was removed from freshly-drawn human blood via centrifugation. The erythrocyte layer was washed 3× with