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Melittin-grafted HPMA-oligolysine based copolymers for gene delivery

Joan G. Schellinger [a], Joshuel A. Pahang [a], Russell N. Johnson [a], David S.H. Chu [a], Drew L. Sellers [b], Don O. Maris [b], Anthony J. Convertine [a,c], Patrick S. Stayton [a,c], Philip J. Horner [b], Suzie H. Pun [a,c,*]

[a] Department of Bioengineering, Univeristy of Washington, Box 355061, 3720 15th Ave NE, Seattle, WA 98195, USA
[b] Department of Neurological Surgery, University of Washington, Seattle, WA 98195, USA
[c] Molecular Engineering and Science Institute, University of Washington, Seattle, WA 98195, USA

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ABSTRACT

Non-viral gene delivery systems capable of transfecting cells in the brain are critical in realizing the potential impact of nucleic acid therapeutics for diseases of the central nervous system. In this study, the membrane-lytic peptide melittin was incorporated into block copolymers synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. The first block, designed for melittin conjugation, was composed of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and pyridyl disulfide methacrylamide (PDSMA) and the second block, designed for DNA binding, was composed of oligo-L-lysine (K10) and HPMA. Melittin modified with cysteine at the C-terminus was conjugated to the polymers through the pyridyl disulfide pendent groups via disulfide exchange. The resulting pHgMel*b*HK10 copolymers are more membrane-lytic than melittin-free control polymers, and efficiently condensed plasmid DNA into salt-stable particles (~100–200 nm). The melittin-modified polymers transfected both HeLa and neuron-like PC-12 cells more efficiently than melittin-free polymers although toxicity associated with the melittin peptide was observed. Optimized formulations containing the luciferase reporter gene were delivered to mouse brain by intraventricular brain injections. Melittin-containing polyplexes produced about 35-fold higher luciferase activity in the brain compared to polyplexes without melittin. Thus, the melittin-containing block copolymers described in this work are promising materials for gene delivery to the brain.

1. Introduction

Gene delivery to the central nervous system (CNS) is a promising approach for treating a broad range of disorders that currently have few treatment options. For example, delivery of trophic factors can mitigate cell deterioration that accompanies spinal cord injury, stroke, or neurodegenerative diseases like Alzheimer's and Parkinson's disease [1–3]. However, technologies that safely and effectively deliver genes to the CNS are still needed. Adeno-associated virus (AAV) is the vector of choice for current clinical trials due to its efficiency in neuron infection [4], but potential safety problems and immunogenicity remain a concern [5]. Synthetic vectors such as cationic polymers offer versatility, safety and relatively low cost for large-scale manufacturing compared to their viral counterparts but are hampered by low delivery efficiencies *in vivo* [6–9].

There are a number of polymeric-based carriers targeted to CNS [7,10–12] but the lack of material uniformity and the overall inefficiency of transfection are barriers to translation of these materials. To address these issues, numerous strategies have been developed such as improved chemical synthesis of polymeric materials and incorporation of components to address specific intracellular and physiological transport barriers [13,14]. Recently we reported the synthesis of well-defined, narrowly dispersed peptide-based polycationic polymers utilizing RAFT polymerization. By utilizing peptide monomers and HPMA, a panel of HPMA-co-oligolysine copolymers was prepared. Polymers incorporating the optimal length of the nucleic acid binding domain (K_{10}) was shown to form salt-stable polyplexes and provided efficient plasmid DNA delivery [15]. This class of biomaterials can be engineered to include specific peptide sequences that can condense DNA, disrupt and escape the endosomal membrane, facilitate nuclear transport, and target specific cell-types and tissues [16–18]. Intracellular trafficking studies using neurons and neuron-like cells have demonstrated that inefficient endosomal release, leading to lysosomal degradation of polyplexes is a primary limitation in non-viral gene delivery [19,20]. Strategies to increase endosomal release of polymeric gene

* Corresponding author. Department of Bioengineering, Univeristy of Washington, Box 355061, 3720 15th Ave NE, Seattle, WA 98195, USA. Tel.: +1 206 685 3488; fax: +1 206 616 3928.
E-mail addresses: spun@u.washington.edu, spun@uw.edu (S.H. Pun).

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carriers include pH-sensitive polymers and membrane-active peptides [21,22].

Melittin is a 26 amino acid (NH2-GIGAVLKVLTTGLPALISWIKR KRQQ-CONH2) membrane-lytic peptide whose sequence is derived from the venom of honey bee, *Apis mellifera*. This peptide has an amphipatic character in which the N-terminus is predominantly hydrophobic while the C-terminus is hydrophilic [23,24]. The peptide is relatively water-soluble but adopts an alpha-helical conformation when in contact with membranes. Its cytolytic activity is based upon its ability to insert into the lipid membrane and induce pore formation [25–28]. Melittin or melittin analogs have been incorporated into polyplex formulations to significantly increase transfection efficiencies *in vitro* [29–33], although there is to our knowledge only one report of *in vivo* application of melittin-functionalized polyplexes [34].

Here, we report the synthesis and evaluation of a peptide-based copolymer that includes an oligolysine DNA binding domain, melittin as an endosomal release peptide and HPMA as a hydrophilic polyplex stabilizer. The membrane-lytic activity of the resulting copolymer was assessed using hemolysis assay. Size and zeta potential measurements, including gel retardation assay of the polymer/DNA complexes were conducted and compared to parent polymer without the melittin. Luciferase and GFP reporter genes were used to study the *in vitro* gene transfection efficiency of the melittin-functionalized complexes in comparison to the parent polymer and PEI. And lastly, transgene delivery in the mouse brain after intraventricular polyplex delivery was evaluated.

2. Materials and methods

2.1. Materials

All chemical reagents obtained from commercial sources were used without further purification. Solvents in capped DriSolv™ bottles were purchased and used directly without further purification and stored under argon. All glassware was utilized flame-dried or oven dried prior to use. N-(2-hydroxypropyl)methacrylamide (HPMA) was purchased from Polysciences (Warrington, PA). The initiator VA-044 was purchased from Wako Chemicals USA (Richmond, VA). Solid phase peptide synthesis (SPPS) reagents which includes HBTU and Fmoc-protected amino acid were purchased from AAPPTec (Louisville, KY), N-succinimidyl methacrylate from TCI America (Portland, Oregon), and Rink Amide Resin from EMD Biosciences (Darmstad, 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 manufacturer's recommendations. NMR experiments were conducted on a 500 MHz instrument using D_2O and MeOD (99.9% D) as a solvent.

2.2. Cell lines

HeLa cells (Human cervical carcinoma, ATCC CCL-2) were cultured in MEM medium supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic solution. PC-12 cells were obtained from ATCC (CRL-1721) and were maintained in growth medium (F-12K medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and antibiotics) in a 37 °C, 5% CO_2 environment. For differentiation to a neuron-like phenotype, cells were suspended in differentiation medium (F-12 K medium supplemented with 1% horse serum, 100 ng/mL nerve growth factor, and antibiotics). Medium was replaced every 2–3 days and cells were passaged when 60–80% confluent.

2.3. Synthesis of peptides and peptide monomers

Cysteine-modified melittin (Mel-cys; NH_2-GIGAVLKVLTTGLPALISWIKRKRQQC-$CONH_2$) and methacrylamido-functionalized oligolysine monomer ($MaAhxK_{10}$) were synthesized on a solid support with Rink amide linker following standard Fmoc/tBu chemistry on an automated PS3 peptide synthesizer (Protein Technologies, Phoenix, AZ). $MaAhxK_{10}$ was synthesized as reported previously [35]. Mel-cys was cleaved from resin by treating the solid support with TFA/dimethoxybenzene/TIPS/EDT (90:5:2.5:2.5, v/v/v/v). Cleaved peptides were then precipitated in cold ether, dissolved in methanol and reprecipitated in cold ether. Peptides were analyzed by RP-HPLC and MALDI-TOF MS and further purified by semi-preparative RP-HPLC using a *Jupiter* 5 μm C18 300A column 250 × 10.0 mm (Phenomenex, Torrance, CA) to attain purity greater than 95%. MALDI-TOF MS calculated for $MaAhxK_{10}$ $[M + H]^+$ 1479.98, found 1479.85. MALDI-TOF MS calculated for Mel-Cys $[M + H]^+$ 2949.775, found 2949.759.

2.4. Synthesis of polymers

The synthesis and characterization of a statistical copolymer of HPMA and MaAhxK10 via RAFT polymerization were reported in previous work [15]. Ethyl cyanovaleric trithiocarbonate (ECT) and pyridyl disulfide methacrylamide (PDSMA) were synthesized according to previous literature [36].

2.4.1. Preparation of macroCTA poly(HPMA-co-PDSMA), ***1 (pHPDS)***

The RAFT polymerization of N-(2-hydroxypropyl) methacrylamide (HPMA) and PDSMA was conducted at 70 °C for 4 h under nitrogen in septa-sealed vials. The monomer to CTA to initiator ratio used was 100:1:0.1. Briefly, HPMA (1.29 g, 9 mmol) and PDSMA (0.25 g, 1 mmol) monomers were added to ECT (26.4 mg, 100 μmol) and 4,4′-azobis-4-cyanopentanoic acid (VA-501, 2.8 mg, 10 μmol) in 9.67 mL water/ethanol (2:1) mixture. The polymerization solution was then transferred to a septa-sealed vial and purged with nitrogen for 30 min. The vial was then transferred to a preheated oil bath at 70 °C and allowed to react for 4 h. Purification was achieved by dialysis against ultrapure deionized water at 5 °C followed by lyophilization. Theoretical feed ratio was 90 mol% HPMA and 10 mol% PDSMA and was found to have an actual ratio of 89.5% HPMA, 10.5% PDSMA. The M_n and PDI of the resulting copolymer was determined by static light scattering to be 13,000 g/mol and 1.13 respectively.

2.4.2. Preparation of diblock poly((HPMA-co-PDSMA)(HPMA-co-MaAhxK10)), ***2 (pHPDSbHK10)***

The block polymer was synthesized by dissolving HPMA (27.4 mg, 0.19 mmol) and MaAhxK10 (70.8 mg, 0.05 mmol) in 1 M acetate buffer pH 5.2 at final monomer concentration of 0.7 M. The solution was then added to the macroCTA poly(HPMA-co-PDSMA) (20.8 mg, 1.6 μmol) and VA-044 (0.05 mg, 0.16 μmol) into a nitrogen-purged septum-sealed pear-shaped flask. The molar ratios of total $monomer_o$:CTA_o:I_o at the start of polymerization were 150:1:0.1. The flask was then immediately capped with a rubber septum, purged with N_2 for 30 min and then submerged in an oil bath, equilibrated at 44 °C, to initiate copolymerization. The copolymerization reactions were allowed to proceed for 24 h. The flask was then removed from the oil bath and the polymers were purified using pre-packed PD-10 desalting columns (Sephadex™ G-25 Medium, GE Healthcare, Buckinghamshire, UK), followed by lyophilization producing a white, fluffy solid.

2.4.3. Preparation of melittin-grafted diblock copolymers poly[(HPMA-g-melittin)-b-(HPMA-MaAhxK10)]. ***3 (pHgMelbHK10)***

Peptide conjugation to the diblock copolymer followed the conditions reported by Wagner and co-workers with a few modifications [37]. In a 10 mL flame-dried pear-shaped flask was added 30 mg (0.41 umol polymer, 3.24 μmol PDS groups) of **2** dissolved in 1.3 mL of 0.5 M NaCl 20 mM HEPES buffer at pH 7.1. Then 19.1 mg (6.48 μmol, 2 equiv relative to diblock copolymer PDS groups) of Mel-cys dissolved in 1.1 mL of buffer was added into the flask and allowed to stir under argon at room temperature. After overnight reaction, released 2-thio-pyridine was measured at 343 nm to determine the extent of conjugation reaction. The reaction mixture was passed through a PD-10 column and lyophilized.

2.5. Characterization of polymers

2.5.1. Size exclusion chromatography

Molecular weight analysis of polymers was determined by size exclusion chromatography (SEC) as described by Jiang and co-workers [38]. Analysis was carried out on an OHpak SB-804 HQ column (Shodex) in line with a miniDAWN TREOS light scattering detector (Wyatt) and an Optilab rEX refractive index detector (Wyatt), with an eluent made up of 150 mM sodium acetate buffer at pH 4.4 and a flow rate at 0.5 mL/min. Absolute molecular weight averages (M_n and M_w), and d*n*/d*c* were calculated using ASTRA software (Wyatt).

2.5.2. Amino acid analysis

The relative ratio of peptide (K10 and Melittin) to HPMA in the final copolymers was determined through modified amino acid analysis following the method of Bidlingmeyer and co-workers [39]. In this procedure, hydrolyzed peptide and HPMA (which resulted in 1-amino-2-propanol) were derivatized with o-phthalaldehyde/β-mercaptopropionic acid and run on a ZORBAX Eclipse Plus C18, 3.5 μm 4.6 × 75 mm (Agilent Technologies, Santa Clara, CA) column with precolumn derivatization to label hydrolyzed lysine for K10, arginine for melittin and 1-amino-2-propanol for HPMA. Calibration curves were generated using serial dilutions of L-lysine, L-arginine and reagent grade 1-amino-2-propanol.

2.5.3. Hemolysis assay

A hemolysis assay was used to evaluate the membrane-lytic activity of the synthesized materials following the procedure described by Hoffman and co-workers [40]. Briefly, plasma from drawn human blood was removed by centrifugation. The supernatant containing the erythrocytes was washed three times with 150 mM NaCl and resuspended into phosphate buffer at pH 7.4. The polymers at various concentrations (0.01–0.08 mg/mL) and 1% Triton X-100 as control, were added to the erythrocyte suspensions in a 96-well conical plate and was allowed to