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Melittin-grafted HPMA-Oligolysine Based Copolymers for Improved Gene Delivery

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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 pendant groups via disulfide exchange. The resulting pHgMelbHK10 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.

Keywords

gene delivery; melittin; peptide-based polymers; RAFT polymerization

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

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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 (K₁₀) 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 carriers include pH-sensitive polymers and membrane-active peptides.[21, 22]

Melittin is a 26 amino acid (NH₂-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH₂) membrane lytic peptide whose sequence is derived from the venom of honey bee, *Apis mellifera*. This peptide has an amphipathic 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. We demonstrate that inclusion of the melittin peptide increases membrane-lytic ability and *in vitro* transfection efficiency of the parent material. Most importantly, melittin functionalization of polymers significantly increases transgene delivery in the brain after intraventricular polyplex delivery.

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 were 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 (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 manufacturer's recommendations. NMR experiments were conducted on a 500 MHz instrument using D₂O 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% CO₂ 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; NH₂-GIGAVLKVLTTGLPALISWIKRKRQQC-CONH₂) and methacrylamido-functionalized oligolysine monomer (MaAhxK₁₀) 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). MaAhxK₁₀ 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 5μm C18 300A column 250 × 10.0 mm (Phenomenex, Torrance, CA) to attain purity greater than 95%. MALDI-TOF MS calculated for MaAhxK₁₀ [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 was 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 were conducted at 70 °C for 4 hours 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 minutes. The vial was then transferred to a preheated oil bath at 70 °C and allowed to react for 4 hours. 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 μ mol) and VA-044 (0.05 mg, 0.16 μ mol) into a nitrogen-purged septum-sealed pear-shaped flask. The molar ratios of total monomer₀:CTA₀:I₀ at the start of polymerization were 150:1:0.1. The flask was then immediately capped with a rubber septum, purged with N₂ for 30 minutes and then submerged in an oil bath, equilibrated at 44 °C, to initiate copolymerization. The copolymerization reactions were allowed to proceed for 24 hours. 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 flame-dried flask was added 30 mg (0.41 μ mol polymer, 3.24 μ mol 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 μ mol, 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 Hennink and coworkers.[38] Analysis was carried out on an OHPak SB-804 HQ column (Shodex) in line with a miniDAWN TREOS light scattering detector (Wyatt) and a 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 dn/dc 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 coworkers [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 μ m 4.6 $\times$ 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 coworkers.[40] Briefly, plasma from drawn human blood was removed by centrifugation. The supernatant containing the erythrocytes were 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 incubate for 1 hour at 37 °C. The plate was then centrifuged down which allows intact erythrocytes to settle at the bottom while released hemoglobin within the supernatant was measured spectrophotometrically at 541 nm absorbance. Percent hemolysis was calculated relative to Triton X-100. Experiments were performed in triplicates.

2.6. Characterization of HPMA-Oligolysine polyplexes

2.6.1 Polyplex formulation—The pCMV-Luc plasmid was diluted in double distilled H₂O (ddH₂O) to a concentration of 0.1 mg/mL and mixed with an equal volume of polymer (in ddH₂O) at the desired lysine to phosphate (N/P) ratio. After mixing, the polyplexes were allowed to form for 5 minutes at room temperature.

2.6.2 Sizing of polyplexes by dynamic light scattering (DLS)—Polyplexes (2 µg DNA, 40 µL solution, N/P=3 and 5) were mixed with either 40 µL of ddH₂O or PBS and then used to determine the particle size of the polyplexes by dynamic light scattering (DLS, Brookhaven Instruments Corp ZetaPLUS). Particle sizing measurements were performed at a wavelength of 659.0 nm with a detection angle of 90° at RT. Experiments were conducted in triplicate.

2.6.3. Zeta Potential Measurements—Polyplexes (1 µg DNA, 20 µL solution, N/P=3 and 5) were mixed with 180 µL of double distilled H₂O and 800 uL of 10 mM NaCl. The zeta potential was determined using a Zetasizer Nano ZS (Malvern Instruments Inc., Southborough, MA). Experiments were conducted in triplicate.

2.6.4. Transmission electron microscopy (TEM)—Polyplexes (1 µg DNA, 20 µL solution, N/P=3 and 5) were deposited on glow discharged 400-mesh formvar/copper grids and incubated at room temperature for 30 minutes. Polyplex solutions were then wicked away and 1% uranyl acetate was then deposited on the grid and incubated for 10–20 sec, wicked away and grids were washed with double distilled water three times. The grids were allowed to air dry under a desiccator for overnight. TEM images were taken on a JEOL 1230 Transmission Electron Microscope equipped with the 2K × 2K ultrascan 100 bottom mount Gatan CCD camera for high resolution work and an 11 Megapixel SC1000 ORIUS side mount Gatan CCD Camera (Electron Microscopy Core Facility At Fred Hutchinson Cancer Research Center Seattle, WA) with 120 kV accelerating voltage at 30,000x.

2.6.5 Gel retardation assay—Polyplexes (1 µg DNA, 20 µL solution, N/P=3 and 5) with 10% (v/v) blue juice was loaded onto a 1% agarose gel containing TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 5 µg/mL Ethidium Bromide and electrophoresed at 100 V for 40 minutes. pDNA was then visualized using a UV transilluminator (laser-excited fluorescence gel scanner, Kodak, Rochester, NY).

2.7. Plasmid delivery of polymers

2.7.1. *In vitro* transfection efficiency—Human cervical epithelial adenocarcinoma cells (HeLa, ATCC # CCL-2) were seeded in D-MEM cell culture medium supplemented with 10% FBS and 1% antibiotic/antimicrobial at a density of 2.5×10^4 cells/well in a 24-well plate for overnight at 37 °C, 5% CO₂. Polyplexes were formed at desired N/P ratios using 1 µg of pCMV-Luc plasmid DNA in 20 µL total volume and then diluted to 200 µL with OptiMEM medium (Invitrogen). After washing with PBS, cells were incubated with transfection solution for 4 hrs at 37 °C, 5% CO₂, washed to remove transfection solution and then cultured with complete cell culture media for an additional 44 hours. Luciferase expression was quantified with a luciferase assay kit (Promega Corp.) according to the

manufacturer's instructions, except that a freeze-thaw cycle at -20°C was included after the addition of the lysis buffer to ensure complete cell lysis. Luminescence intensity was measured on the plate reader with integration for 1 s. The total protein content in each well was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions so that the luciferase activity could be normalized by the total protein content in each well. Each sample was tested with a sample size (n) = 4. PC-12 cell lines were transfected in a similar manner with few modifications. Before seeding, each well was coated with 200 μL of rat tail collagen IV (50 $\mu\text{g}/\text{mL}$ in 0.1 N acetic acid) and the solution was allowed to air dry for overnight. Plates were rinsed once with PBS and PC-12 cells were seeded at a density of 5×10^4 cells/well. In order to obtain a single cell suspension, cells were passed through a fire-polished glass pipette. Transfections were performed in triplicates for HeLa and quadruplicates for PC-12 cells.

2.7.2 GFP transfection—HeLa cells were seeded overnight in 24-well plates at a density of 2.5×10^4 cells per well (0.5 mL/well) at 37°C , 5% CO_2 . Polyplexes were formulated at as described previously and then diluted to 200 μL with OptiMEM medium (Invitrogen) and added to cells after washing twice with PBS. The cells were incubated at 37°C , 5% CO_2 for 4 h, washed and then incubated with complete cell culture media at 37°C , 5% CO_2 for another 44 hours. For analysis, cells were washed with PBS, trypsinized and pelleted at $1000 \times g$ for 5 min at 4°C . The pellet was resuspended in 0.3 mL propidium iodide (PI) solution (1 $\mu\text{g}/\text{mL}$ in 0.5% BSA in PBS), kept on ice and analyzed using flow cytometry, MACSQuant Analyzer (Miltenyi Biotec Inc., Auburn, CA). Intact cells were identified using the forward and side scatter data. The resulting cell population were gated into GFP+/PI+, GFP+/PI-, GFP-/PI+ and GFP-/PI- based on the green fluorescence and PI intensity from the control samples (cells transfected without the polymers but DNA only) and reported as the mean percentage of cell population that is GFP+/PI- including standard deviation (SD). All experiments were conducted in triplicate.

2.8. *In vivo* plasmid delivery of polymers

2.8.1. Intraventricular delivery—All animal procedures were done using protocols approved by the Institutional Animal Care and Use Committee at the University of Washington. Polyplexes were prepared as described above in 5% glucose using 2.5 μg of DNA in 10 μL at desired N/P. 7–9 week old female C57/Bl6 mice from Jackson Laboratories were housed for 1 week prior to experimentation. Mice were anesthetized by an intraperitoneal injection of Avertin. A 1 mm diameter craniotomy was made on the right-side of the skull using a dental drill and 10 μL polyplex or 5% glucose solution was stereotactically injected at 1 mm lateral, 0.5 mm caudal to bregma, and 1.75 mm deep from the dura using a 33 gauge 10 μL Hamilton syringe. The injection was made at 1–2 $\mu\text{L}/\text{min}$. and the syringe was kept in place for 2 min after injection to prevent backflow.

2.8.2. Lysate preparation—Brains were harvested from mice two days after injection and collected in lysis buffer supplemented by protease inhibitors (Roche, Nutley, NJ) and three freeze-thaw cycles were performed in liquid nitrogen. Tissues were mechanically homogenized and lysate was cleared by spinning at 21,000 g for 15 min at 4°C . 20 μL of lysate was assayed for luminescence with 100 μL of luciferase substrate. Luminescence was measured and were normalized by protein content in the three brain sections, determined using a BCA Protein Assay Kit (Pierce), and reported as relative light units (RLU) per mg brain.

3. Results and Discussion

3.1. Polymer design, synthesis and characterization

A diblock copolymer containing a DNA-condensing block and a membrane-lytic block was synthesized. The material was designed as a block copolymer because this architecture allows for plasmid binding and condensation via one block and display of endosomolytic agents on the second block (Figure 1).[36] HPMA was used as comonomers for both blocks and served as the hydrophilic segment that allows for steric stabilization.[41]

Scheme 1 shows the synthesis of the diblock copolymers via RAFT polymerization using a trithiocarbonate-based chain transfer agent. The first block was synthesized by copolymerizing HPMA and PDSMA in a 9:1 feed ratio to minimize steric hindrance for the thiol-reactive pyridyl disulfide groups. The block was then chain extended with HPMA and oligo-L-lysine (K10) monomer in 1M sodium acetate buffer at pH 5.2 to obtain the poly[(HPMA-co-PDSMA)-b-(HPMA-MaAhxK10)], **2**, diblock copolymer. The monomer feed ratio and the degree of polymerization for the second block was chosen based from our previous optimization studies: 4:1 HPMA to MaAhxK10 at degree of polymerization (DP) of 150.[15] Table 1 summarizes the molecular weights, polydispersities and comonomer compositions of **1** and **2**. As determined by SEC, copolymers **1** and **2** had number average molecular weight (M_n) of 13 kDa and 74 kDa respectively. Composition of the macroCTA **1** was determined by integration of three aromatic PDSMA resonances between 7.69 and 8.56 ppm to either the HPMA methyne or hydroxyl resonance (1H) between 3.59–3.76 ppm and 4.63–4.83 ppm and was found to have an actual relative monomer ratio of 89.5 % HPMA and 10.5 % PDSMA. Amino acid analysis was performed to determine the relative monomer ratio of the diblock copolymers and was found to have 86% HPMA and 14% MaAhxK10. The macroCTA **1** and diblock copolymer **2** had narrow polydispersity (1.13 and 1.16).

Melittin was then grafted onto the diblock copolymer via disulfide exchange between the sulfhydryl group of the cysteine-modified melittin and the pyridyl disulfide moiety in the polymer chain. Conjugation was achieved in 20 mM HEPES buffer at pH 7.0. The reaction was allowed to proceed for overnight followed by the measurement of pyridine 2-thione release by UV absorbance measurements. Approximately 5–6 melittin peptides per polymer were conjugated onto the polymer chain, which corresponded closely to the amino acid analysis. pHK10, a statistical copolymers of HPMA and oligolysine with a narrow PDI (1.14), actual molecular weight (65 kDa) and monomer composition (79.5% HPMA and 20.5% oligolysine), was used as a control polymer for all studies.[15]

3.2. Hemolysis assay

The membrane lytic activity of pHgMelbHK10 compared to pHK10, PEI and PLL at physiological pH was evaluated using hemolysis assay. This assay is utilized to measure the ability of lytic agents to disrupt the lipid bilayer of red blood cells and release hemoglobin. [40] About 40% cell lysis was observed at concentration as low as 0.8 $\mu\text{g/mL}$ and nearly complete cell lysis was observed across the range of concentrations (4–40 $\mu\text{g/mL}$) for pHgMelbHK10 while no lytic activity was observed for pHK10, PEI and PLL even at 50-fold higher concentrations (40 $\mu\text{g/mL}$) (Figure 2). From these results, melittin retained its endosomolytic activity upon covalent attachment to the polymer which is a critical aspect for efficient gene delivery.

3.3. Polyplex Characterization

The ability of the polymers to bind DNA was evaluated using gel retardation assay (Figure 3A). The electrophoretic mobility of plasmid DNA in different complexes showed that complete DNA retardation of pHgMelbHK10 was achieved at lower N/P ratio (N/P=1)

compared to pHK10 (N/P=3). These suggest that melittin incorporation into the polymer improved DNA binding efficiency due to increased charge density and α helicity attributed to the melittin peptide. The average hydrodynamic diameter of polyplexes were measured in both water and 150 mM PBS using dynamic light scattering (DLS) (Figure 3B). The complex formed by both copolymers displayed relatively stable particles in water and physiological salt concentrations with an average diameter of 200 nm at N/P 3 and 5. This is consistent with our previously reported result showing that HPMa-co-oligolysine polymers are salt stable when DP 100 and also confirmed that melittin conjugation does not reduce salt stability of the polyplexes.[15]

The surface charge of the complexes was also indicated through zeta potential measurements. A net positive charge is necessary for the efficient polyplex-mediated gene delivery. The excess positive charge interacts with the negatively charged cellular membrane thus allowing for an enhance uptake and delivery.[42] The polyplex formed by both copolymers at N/P 3 and 5 showed net positive charge (~ 25 kV) as shown in Figure 3B. The comparable zeta potentials for pHgMelbHK10 and pHK10 may be attributed to the similar DP and peptide length of the the DNA binding domain for both copolymers. TEM images for the pHK10 complexes revealed larger rod-like shapes while pHgMelbHK10 complexes showed more compact morphologies than pHK10 complexes (Figure 3C).

3.4. *In vitro* gene delivery

Gene transfection efficiency of pHgMelbHK10 was first screened in HeLa cells using pHK10 and PEI for comparison. A range of polymer to DNA charge ratios (N/P=2 through N/P=6) was tested. The transfection efficiency of pHgMelbHK10 polyplex at N/P=2 was at least 5-fold higher than pHK10 polyplexes at all of the tested charge ratios but transfection efficiency of pHgMelbHK10 decreased as charge ratios increased due to cell toxicity (data not shown). Boeckle et al. also previously reported that the cytotoxicity of melittin conjugation to polymers was significant, reducing the cell metabolic activity, a measure of cell viability, to as low as 2% of control values.[37] The cytotoxicity was attributed to the pronounced membrane destabilizing effect at neutral pH that results in membrane blebbing and cell lysis. It has been proposed that the mechanism of membrane destabilization by melittin depends upon its orientation to the lipid membrane and its membrane association can be improved upon conjugation to a polycationic molecule, such as PEI, due to enhanced electrostatic interaction with the negatively charged cellular membrane.[37] Since our base polymer, pHK10, is a highly dense cationic polymer, the observed toxicity during transfection was not surprising.

Based on these results, we reduced the melittin concentration in polyplexes by formulating with a mixture of pHgMelbHK10 and pHK10 polymers. Cationic polymer solutions containing 2%, 5%, 10% and 15% pHgMelbHK10 were prepared and used for polyplex formulation at N/P=3 and N/P=5. Polyplexes (N/P=3) containing 5–15% pHgMelbHK10 and delivering the luciferase reporter gene showed ~ 2 orders of magnitude higher luciferase activity compared to control pHK10 polyplexes without significant increases in cell toxicity (Figure 4).

The luciferase system, when analyzed for bulk photon generation, provides information about total reporter protein activity but not the percent of cells transfected. Therefore, we tested the high performing mixed formulation containing 15% pHgMelbHK10 for delivery of the green fluorescent protein (GFP) reporter gene to HeLa cells. The percentage of cells expressing GFP was determined by flow cytometry with propidium iodide (PI) as a cell viability marker. An increasing trend of GFP expression in live cells with increasing melittin content in polyplexes was observed: pHK10 (N/P=3, 2.1%) < pHK10 (N/P=5, 8.6%) < pHK10/pHgMelbHK10 15% (N/P=3, 14.7%) < PEI (N/P=5, 19.5%) < pHgMelbHK10 (N/

P=3, 36.4%) (Figure 5A). We included pHK10 (N/P=5) and PEI (N/P=5) in the transfection assay to show that the incorporation of melittin into the polymer significantly enhances the gene expression compared to both of the previously optimized formulations of pHK10 and PEI polyplexes. Cell viability was assessed by PI staining and an increase in PI positive cells, reflecting compromised plasma membranes, was observed as melittin content increased: pHK10 (N/P=3, 4.1%) < pHK10 (N/P=5, 10.3%) < PEI (N/P=5, 14.7%) < pHK10/pH_gMelbHK10 15% (N/P=3, 15.4%) < pH_gMelbHK10 (N/P=3, 25.9%) (Figure 5B).

Select high-performing formulations were then tested in neuron-like differentiated PC-12 cells. PC-12 cells are derived from a transplantable rat pheochromocytoma. These are suspension cells that have longer doubling time compared to HeLa cells, but respond to nerve growth factor (NGF) by differentiating to a characteristic neuron-like phenotype with sprouting neurites, accompanied by slow proliferation and changes in cellular composition. [43] Thus, 6-day differentiated PC-12 cells were used as an *in vitro* model for neuron delivery. Polyplexes formulations containing 3% and 5% pH_gMelbHK10 showed increase in luciferase expression compared to pHK10. The highest transfection efficiency was achieved by a mixed formulation containing 15% pH_gMelbHK10 (2 orders of magnitude higher than pHK10 and half an order magnitude higher than PEI) with no apparent cytotoxicity (Figure 6). pH_gMelbHK10 polyplexes showed a decreased luciferase expression that was attributed to the decreased cell viability. Thus, melittin incorporation increases transfection efficiency of HPMa-oligolysine polyplexes to differentiated neuron-like cells.

3.5 Gene delivery to the brain

In order to evaluate the effectiveness of incorporating an endosomolytic peptide *in vivo*, polyplexes composed pHK10, pHK10/pH_gMelbHK10 15% and pH_gMelbHK10 copolymers formulated at N/P=3 were injected into the right lateral ventricle of mice to deliver the luciferase plasmid. These formulations were well-tolerated and no significant gross morbidities or mortalities were observed due to polyplex injection. Brain tissue was harvested 2 days after injection and analyzed for luciferase activity (Figure 7). *In vivo* transfection efficiency was shown to correlate with melittin content in the formulations. There was a statistically significant increase ($p < 0.02$) in luciferase activity for both pHK10/pH_gMelbHK10 15% (5-fold higher) and pH_gMelbHK10 polyplexes (35-fold higher) compared to pHK10. The *in vivo* delivery efficiencies correlate well with the *in vitro* delivery efficiencies seen in differentiated PC-12 cells, except at high melittin concentrations less cytotoxic impact was observed *in vivo* likely due to polyplex dilution and therefore lower melittin concentrations. In our earlier studies we found poor correlation between *in vitro* and *in vivo* transfection efficiencies if polyplexes are administered systemically. However, *in vitro* delivery is more similar to cerebrospinal fluid (CSF) delivery in the CNS as opposed to systemic delivery into the circulation due to lower protein and cell composition of CSF. In addition, we have shown previously that transfected cells reside in the subventricular space.[44] In contrast, polyplexes delivered systemically are likely decomplexed prematurely in the liver before efficient transfection to other tissues can occur.[45] Thus, we demonstrate that incorporation of the membrane-lytic melittin peptide into polyplexes results in nearly two order magnitude increase in transgene activity in mouse brain.

4. Summary and Conclusion

In this work, we have demonstrated the synthesis, characterization, and evaluation of HPMa-oligolysine-based block copolymers containing melittin, as an endosomolytic peptide that improves polyplex-mediated gene delivery. Incorporation of melittin into the

polymer resulted in polyplexes with more compact morphologies, increased DNA binding ability and enhanced delivery of reported genes, luciferase and GFP, to both HeLa and neuron-like PC-12 cell lines. To mitigate the cytotoxic impact of melittin, mixed formulations with the melittin-free pHK10 polymer was used. A statistically significant increase in luciferase expression in mice brain was achieved using melittin-based copolymers. Overall, incorporation of an endosomal escape peptide, melittin, enhanced the gene delivery efficiency of peptide-based polymeric carriers. In future work, we will investigate the use of these carriers for growth factor gene delivery to cells in the subventricular zone as an approach to enhance neurogenesis. We will also further improve these materials by incorporating different melittin analogs that were reported to mediate efficient transfection without compromised cell viability.[30, 32, 37, 46, 47]

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Figure 1.
Schematic representation of polyplexes formed by plasmid DNA and pHgMelbHK10 copolymers.

Figure 2.
Hemolysis activity of polycations at various concentrations at pH 7.4.

Figure 3. Characterization of polyplexes. A) Agarose gel electrophoresis of polymer/DNA complexes prepared at different N/P ratios using pHK10 and pHgMelbHK10. Lane 1 is free DNA; lanes 2–7 correspond to N/P ratios. B) Sizing and zeta potential measurements of pHK10 and pHgMelbHK10 polyplexes at N/P ratio 3 and 5. C) TEM images of A) pHK10 and B) pHgMelbHK10 polyplexes. TEM images were conducted on 400-mesh formvar/copper grids stained with uranyl acetate. Scale bar represents 100 nm.

Figure 4. Efficiency of pCMV-Luc plasmid delivery and cell viability using pHK10, pH_gMel_gbHK10 and mixed polymers to transfect HeLa cells at N/P 3 and 5. Relative luminescence was normalized against the total amount of protein.

Figure 5.

A) GFP expression in live cells (PI negative) and B) Cytotoxicity (PI positive) of cells treated with PEI, pHK10, pHK10/pHgMelbHK10 (15%) and pHgMelbHK10polyplexes analyzed by flow cytometry.

Figure 6. Efficiency of pCMV-Luc plasmid delivery and cell viability using PEI, pHK10, pHgMelbHK10 and mixed polymers to transfect differentiated PC-12 cells at N/P=3. Relative luminescence was normalized against the total amount of protein.

Figure 7.

Bulk luciferase activity in mouse brains two days after intraventricular injection using glucose control, pHK10, pHK10/pHgMelbHK10 (15%) and pHgMelbHK10 complexes to deliver the luciferase plasmid. Error bars represent standard deviation. * $p < 0.02$ relative to pHgMelbHK10, ** $p < 0.02$ relative to pHK10/pHgMelbHK10 (15%) where $n=6$ mice.

Scheme 1.

Synthesis of melittin-grafted diblock copolymers of HPMA and oligolysine.

Table 1

Characterization data for polymers.

Polymer	Polymer Name	Mn (kDa, actual)	PDI	mole % HEMA	mole % PDSMA	mole % K10	mole % Melittin
1	pHPDS	13	1.13	89,5	10,5*	-	-
2	pHPDS/MeIbHK10	74	1.16	86	-	14**	-
3	pH ₂ gMeIbHK10	84	1.09	83	-	14	3***

Relative % monomer ratio is *based on NMR, **based on AAA, ***based on pyridine-2-thione release and corresponded closely to AAA.