Published in final edited form as:

Biomaterials. 2019 February ; 192: 235–244. doi:10.1016/j.biomaterials.2018.11.004.

pH-sensitive polymer micelles provide selective and potentiated lytic capacity to venom peptides for effective intracellular delivery

David J. Peeler¹, Salina N. Thai¹, Yilong Cheng¹, Philip J. Horner², Drew L. Sellers^{*,1}, and Suzie H. Pun^{*,1}

¹Department of Bioengineering and Molecular Engineering and Sciences Institute, University of Washington, Seattle, Washington, 98195, United States

²Center for Neuroregeneration and Department of Neurosurgery, Houston Methodist Research Institute, Houston, Texas, United States

Abstract

Endocytosed biomacromolecule delivery systems must escape the endosomal trafficking pathway in order for their cargo to exert effects in other cellular compartments. Although endosomal release is well-recognized as one of the greatest barriers to efficacy of biologic drugs with intracellular targets, most drug carriers have relied on cationic materials that passively induce endosomal swelling and membrane rupture with low efficiency. To address the endosome release challenge, our lab has developed a diblock copolymer system for nucleic acid delivery that selectively displays a potent membrane-lytic peptide (melittin) in response to the pH drop during the endosomal maturation. To further optimize this system, we evaluated a panel of peptides with reported lytic activity in comparison to melittin. Nineteen different lytic peptides were synthesized and their membrane-lytic properties at both neutral and acidic pH characterized using a red blood cell hemolysis assay. The top five performing peptides were then conjugated to our pH-sensitive diblock copolymer via disulfide linkers and used to deliver a variety of nucleic acids to cultured mammalian cells as well as *in vivo* to the mouse brain. We demonstrate that the sharp pH-transition of VIPER compensates for potential advantages from pH-sensitive peptides, such that polymer-peptide conjugates with poorly selective but highly lytic peptides achieve safe and effective transfection both *in vitro* and *in vivo*. In addition, peptides that require release from polymer backbones for lysis were less effective in the VIPER system, likely due to limited endosomal reducing power of target cells. Finally, we show that certain peptides are potentiated in

*Corresponding authors: Drew L. Sellers, phone: (206) 616-8023, drewfus@uw.edu, Suzie H. Pun, phone: (206) 685-3488, spun@uw.edu.

Conflict of Interest: A patent application for the VIPER polymer has been filed by the University of Washington. The authors declare no other competing financial interest.

Data availability: The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

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lytic ability by polymer conjugation and that these peptide-polymer constructs are most effective *in vivo*.

Graphical Abstract

Keywords

Endosomal escape; lytic peptide; polymer; nucleic acid delivery; central nervous system transfection; pH-sensitive

1. Introduction

Selective endosomal membrane disruption technology remains the “missing key” in most intracellular delivery vehicles for biologic therapeutics. While viral vectors possess efficient mechanisms for endosomal escape,[1,2] few, if any, synthetic systems offer matching levels of cytosolic delivery efficiency for macromolecular drugs such as nucleic acids.[3–5] Moreover, the commercial availability of polycationic materials like polyethylenimine (PEI) and poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) has facilitated their widespread use as all-in-one multifunctional gene delivery vectors.[6–8] A model polycation, pDMAEMA’s average pKa value of 7.5 enables partial protonation in the extracellular environment that promotes endocytosis and also “proton sponge” endosomal buffering that enables rapid osmotic swelling, membrane destabilization, and endosomal rupture in some cell types.[9,10] However, it is well documented that endosomal escape mediated by the proton sponge effect is not only very inefficient (1–2%) but also highly variable based on cell type specific properties (e.g. endosomal size and membrane leakiness).[10–12] The insufficiencies of passive, polycationic approaches in endosomal escape motivate the development of active escape strategies and materials that are more efficient, cell-type agnostic, and amenable to *in vivo* biomacromolecule delivery.

Advances in controlled polymerization technologies have enabled the facile synthesis of polymeric materials with more complex behavior.[13,14] For example, Jianjun Cheng’s group recently reported helical polypeptides with potent endosomal release properties that are effective in *in vivo* CRISPR/Cas9 delivery.[15,16] Inspired by adenovirus’ endosomal escape mechanism, which involves capsid rearrangement after cellular internalization to display membrane-lytic protein VI,[17,18] we recently reported a self-assembling block copolymer that responds to endosomal acidification by revealing a membrane-lytic peptide that is conjugated to a reversibly hydrophobic polymer block.[19] This Virus-Inspired Polymer for Endosomal Release (VIPER) combines highly sensitive and tunable state-switching polymeric materials[20–23] with reducible conjugation of the potent lytic peptide melittin to achieve safe and highly efficient plasmid and siRNA delivery *in vivo*.[19,24] In

comparison to the many strategies for active endosomal escape reviewed in depth elsewhere, [5,25] relatively few have employed the bioconjugation of membrane-lytic/pore-forming peptides to drug carriers, likely due to off-target lysis.[26–35] Moreover, past efforts have only utilized a select number of the many lytic peptides described in the literature and much remains unknown about the peptide conjugate properties that may be optimized to improve drug delivery.

In this work, we present our expansive effort to (i) directly compare 19 reported membrane-lytic or cell penetrating peptides for their pH-dependent hemolytic activity, (ii) elucidate the impact of 5 select peptide sequences on resulting self-assembly properties of peptide-polymer conjugates, and (iii) evaluate the resulting materials for *in vitro* and *in vivo* gene transfer. We hypothesized that the backbone polymer of the VIPER system could serve as an effective scaffold for covalent conjugation and selective endosomal display of a wide variety of lytic peptides, thereby enabling a broad investigation into the peptide properties that enhance both polymer micellization and endosomolysis. In contrast to the vast majority of lytic peptide literature that has focused on the identification of peptides with dual antimicrobial potency and minimal lysis of mammalian cell membranes, we revisited this rich literature with a specific search for peptides with the ability to destabilize mammalian cell membranes. Because peptides in the VIPER system are concealed in the hydrophobic micelle core until environmental pH drops to < 6.4 in maturing endosomes, our pH-responsive polymer system should avoid non-specific extracellular lysis and enzyme-mediated peptide degradation before reaching its target. However, it is unknown how sequence-dependent variation in lytic peptide size, net charge, hydrophobicity, isoelectric point, and polymer conjugation would affect micelle stability, endosomolysis, and ultimately nucleic acid cargo delivery. We demonstrate that the sharp pH-transition of VIPER compensates for potential advantages from pH-sensitive peptides, such that polymer-peptide conjugates with poorly selective but highly lytic peptides achieve safe and effective transfection both *in vitro* and *in vivo*. Moreover, we show that certain peptides are poorly lytic in polymer-conjugated form such that the *in vivo* delivery efficiency of our disulfide conjugation system depends on the endosomal reducing power of target cells.

2. Experimental Section

2.1 Materials.

RAFT CTA 4-cyanopentanoic acid dithio-benzoate (CPADB), N,N'-Azobisisobutyronitrile (AIBN), anhydrous N,N'-dimethylacetamide (DMAc, HPLC, 99.9%), methanol (MeOH, HPLC, 99.9%), and methylene chloride (DCM, HPLC, 99.9%) were purchased from Sigma-Aldrich and used without further purification. Pyridyl disulfide ethyl methacrylate (PDSEMA) was synthesized as described previously.[23] 2-(Dimethylamino)ethyl methacrylate (DMAEMA) and oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA, Mn = 300 and pendent EO units DP 4~5), and 2-Diisopropylaminoethyl methacrylate (DIPAMA) were purchased from Sigma-Aldrich and were purified by passing through a column filled with basic alumina to remove the inhibitor prior to polymerization. Nile Red was purchased from ACROS Organics. Endotoxin-free plasmid pCMV-Luc™

(ProMega) and pmaxGFP™ (Lonza) were purified with the Qiagen Plasmid Giga kit (Qiagen) according to the manufacturer's protocol.

2.2 Peptide synthesis.

Lytic peptides (Table 1) were synthesized through solid phase peptide synthesis (SPPS) using a CEM Liberty Blue microwave peptide synthesizer (Matthews, NC) at 0.25 mmol scale using standard Fmoc amino acids and NovaPEG Rink Amide Resin (Millipore). Peptides were cleaved from the resin in a trifluoroacetic acid (TFA) cocktail with 5% dimethoxybenzene, 2.5% triisopropylsilane, and 2.5% ethanedithiol. Water was included at 2.5% for peptides containing arginine. Crude peptide was precipitated twice in cold diethyl ether and purified by reverse-phase HPLC using 0.1% TFA water and acetonitrile to >95% purity. Peptide molecular mass was determined by MALDI-TOF at the University of Washington Department of Medicinal Chemistry Mass Spectrometry Center. Peptide hydrophobicity was calculated as the average of each sequence's amino acid values using the Eisenberg scale.[36]

2.3 *In vitro* hemolysis of peptides.

A hemolysis assay was used to determine the lytic potency of synthesized peptides at pHs corresponding to extracellular (pH 7.4) and endosomal (pH 5.5) compartments using methods derived from others' work with endosomolytic polymers.[22] Briefly, plasma from human blood was removed by centrifugation, red blood cells (RBCs) were washed three times with 150 mM NaCl, and resuspended in 100 mM phosphate buffer (PB) at pH 7.4, or 5.5. Peptides were then incubated with RBC suspensions at various concentrations (0.012–100 μ M) in a sealed 96-well V-bottom plate for 1 h at 37 °C. After centrifugation, the released hemoglobin (supernatant) was transferred to a new plate and the absorbance at 541 nm was measured by with a plate reader. Percent hemolysis was calculated relative to the positive control (1% Triton X-100). Experiments were performed in triplicate and EC50 (50% hemolysis) values were determined using a four-parameter dose-response curve fit in Prism software (Graph Pad Software).

2.4 Cell culture.

HeLa cells (ATCC CCL-2) were maintained in Gibco Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. A549 cells (ATCC CCL-185) were cultured and transfected in F-12K medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S). PC-12 cells (ATCC CRL-1721) were cultured and seeded in growth medium (F-12K medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% P/S). For differentiation to a neuron-like phenotype and subsequent transfection, cells were cultured in differentiation medium (F-12K medium supplemented with 1% horse serum, 100 ng/mL nerve growth factor, and 1% P/S). Z310 cells[37] were cultured and transfected in high glucose 1:1 DMEM:F12K medium supplemented with 10% FBS, 1% P/S, 40 μ g/mL gentamicin, and 10 ng/mL nerve growth factor. Cells were passaged every three days or when 75% confluent and seeded to reach 75–90% confluency at time of transfection.

2.5 Polymer synthesis and characterization.

Block copolymer p(OEGMA_{8,60}-*co*-DMAEMA_{50,0})-*bl*-p(DIPAMA_{25,32}-*co*-PDSEMA_{1,00}) (“control polymer”, CP) was prepared with RAFT polymerization and purified by dialysis as in previous work.[19] ¹H NMR spectra were recorded on a Bruker AV 500 (Bruker Corporation) nuclear magnetic resonance (NMR) instrument in deuterated chloroform (CDCl₃). The molecular weight and molecular weight distribution (PDI) of the polymers were determined by size exclusion chromatography (SEC). To prepare materials for SEC analysis, the purified polymer was dissolved at 10 mg/mL in running buffer (0.15 M sodium acetate buffered to pH 4.4 with acetic acid) for analysis by SEC. Samples were then applied to 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). Absolute molecular weight averages (M_w and M_n) was calculated using ASTRA software (Wyatt).

2.6 Conjugation of lytic peptides to block copolymer.

Lytic peptides were conjugated to CP through disulfide exchange as described in our previous work with slightly altered reaction conditions.[19] CP (10 mg, 0.625 μ mol groups) was dissolved in 500 μ L MeOH for each conjugate. Then, 1 equivalent (relative to PDS groups) of each peptide was added to the polymer stocks and purged with argon at room temperature for 5 min. The reaction was sealed and monitored by UV at 343 nm for the release of 2-thiopyridine. Each reaction mixture was then diluted 10-fold in 0.15 M sodium acetate (pH 4.4) and then concentrated to the original volume on an Amicon 10 kDa centrifugal filter. Each polymer-peptide conjugate (PPC) was washed three times and then passed through a PD-10 column to ensure removal of unreacted peptide prior to lyophilization. Conjugation efficiency was confirmed by analysis of residual pyridyl disulfide (PDS) content as previously reported with slight adaptation.[29] Briefly, aqueous CP or PPC solutions (2 mg/mL) were reduced with DTT (10 mM, 37 °C, 30 min), releasing one pyridine-2-thiol molecule per PDSEMA group left unreacted. Small molecule pyridine-2-thiol was purified from the polymer with a 3 kDa cut-off Amicon spin filter (Millipore) and the UV-Vis absorbance of the flow-through was measured with a Nanodrop One (Thermo Fisher). Percent reacted PDSEMA was calculated according to the equation in Supplemental Table 1.

2.7 Micelle formation and characterization.

Polymer micelles were formed through rapid pH-transition as in previous work.[19] Briefly, up to 20 mg of PPC was dissolved in 190 μ L of 200 mM monobasic sodium phosphate (~pH 5.4), followed by addition of 810 μ L of 200 mM dibasic sodium phosphate to bring the pH to 7.4. Micelles were allowed to form overnight before buffer exchange to nanopure distilled water on a 10 kDa cutoff Amicon centrifugal filter (Millipore) and stored at 4 °C as a 20 mg/mL solution until use in future experiments.

Critical micelle concentrations were determined using Nile Red as previously reported.[38] Micelle solutions at concentrations varying from 0.5 to 1024 mg/L were added to dry Nile Red and incubated for 24 hr before fluorescence intensity (excitation/emission: 556/625 nm) was recorded using an Infinite 200 PRO plate reader (Tecan). As shown in a representative plot in Supplemental Figure 5, linear regression was separately performed on flat and

exponential regions of fluorescence intensity; the CMC was taken to be x-value at the intersection of these lines for each sample. A slightly altered version of the Nile Red assay described above was performed to determine the pH transition point where micelles disassemble due to cooperative protonation of DIPAMA: micelle concentration was fixed at 0.2 mg/mL and incubation phosphate buffer pH was varied from 5.2 to 7.4 in increments of 0.2 pH units.

Micelle size was determined by dynamic light scattering using a ZetaPLUS instrument (Brookhaven Instruments Corporation). Micelle stock solutions were diluted to 0.1 mg/mL in 800 μ L 150 mM phosphate buffered saline (PBS; pH 7.4) and measured in triplicate after 30 min incubation at room temperature.

2.8 Preparation and characterization of DNA polyplexes.

Polyplexes were formed by adding polymer to DNA solution followed by 30 min incubation at room temperature. For the gel retardation assay, polyplexes with various N/P ratios were loaded onto a 1% agarose gel containing TAE buffer (40 μ M tris-acetate, 1 mM EDTA) and 0.5 μ g/mL ethidium bromide, and were electrophoresed at 100 V for 40 min before imaging on a UV transilluminator. The size and surface charge of the polyplexes were also tested on a ZetaPLUS instrument; polyplexes (1 μ g DNA, 20 μ L solution, N/P = 5) were diluted in 800 μ L in 10 mM NaCl and measured in triplicate.

2.9 *In vitro* hemolysis of polymer-peptide conjugates.

Peptide-polymer conjugates (PPCs) were assayed as described above for peptides. PPCs were also tested in the presence of 10 mM glutathione (GSH) to determine whether PPCs recovered lytic potency upon peptide liberation from the polymer backbone. RBCs were first suspended in 20 mM GSH PB to give 10 mM GSH final when mixed with polymer dilutions. It should be noted that pH 6.0 was used in place of pH 5.5 in this assay because RBCs were damaged during incubation with GSH at pH 5.5 but not at pH 6.0. Concentration ranges and all other methods were otherwise performed identically to the assay described above for peptides.

2.10 *In vitro* plasmid transfection.

For pmaxGFPTM transfection, HeLa cells were seeded at a density of 25,000 cells/well in complete cell culture medium in 24-well plates. Cells were first incubated at 37 °C, 5% CO₂ for 16 h. Polyplexes were prepared at different N/P ratios using 1 μ g of pmaxGFPTM pDNA (0.1 mg/mL) and allowed to rest for at least 10 min. Each polyplex solution was then added dropwise directly to each well (in triplicate). After 4 h, cells were washed with PBS and given fresh complete cell culture medium. After 40 additional hours, cells were washed with PBS, trypsinized and pelleted at 300 $\times$ g for 5 min at 4 °C. Cells were washed once more with PBS, resuspended in Zombie VioletTM viability dye (BioLegend) diluted 500-fold in PBS, and incubated at room temperature for 10 min. Cells were washed twice with cold 1% BSA in PBS and analyzed using an Attune NxT flow cytometer (Thermo Fisher). Singlet cell events were serially gated to analyze viable (Violet-) and transfected (GFP+) cells as shown in Supplemental Figure 8. Transfection efficiency is reported as the mean percentage of single cells that were GFP+/Violet-. Luciferase transfection was performed at N/P 5 in the

same manner as above. HeLa, A549, and Z310 cells were seeded at 25,000 cells/well and transfected 16 h after seeding. PC-12 cells were seeded at 50,000 cells/well in growth media, exchanged to differentiation media after 16 h, and transfected in fresh differentiation media 3 d after initiation of differentiation. Polyplexes formed with commercially available branched polyethyleneimine (bPEI; MW = 20 kDa; Sigma) were also included as a control group in each transfection assay. At time of harvest (48 h post-transfection), cells were washed once in PBS and lysed directly in the plate using Reporter Lysis Buffer (ProMega). Lysates were subjected to one freeze-thaw cycle and clarified by centrifugation at 14,000 g for 10 min at 4 °C. Clarified lysate was assayed for luminescence with luciferase substrate (ProMega) using a plate reader and relative light units (RLU) were normalized by protein content as determined by BCA Protein Assay Kit (Pierce). All experiments were performed in triplicate and variance is reported as the standard deviation of the mean.

2.11 *In vivo* luciferase plasmid transfection in the brain.

All animal procedures were completed according to protocols approved by the Institutional Animal Care and Use Committee at the University of Washington. Intracerebroventricular (ICV) injection was performed as described before.[39] Polyplex formulations were prepared in 5% glucose solution containing 2.5 µg of luciferase plasmid DNA (N/P 10). Female C57/BL6 mice (8 wk old) were anesthetized by intraperitoneal injection of Avertin (500 mg/kg body weight). After craniotomy, a burr hole (1 mm diameter) was made on the right-side of the skull using a dental drill, and 10 µL of polyplex was stereotactically injected (Bregma, -0.5mm; Medial/Lateral, 1.0mm; Dorsal/Ventral, 1.8mm) using a 33 gauge Hamilton syringe. The injection was made at 2 µL/min and the syringe was kept in the injection site for 2 min to prevent backflow prior to needle removal. After two days, brain compartments were harvested as distinct tissues (left, right and hind brain) from mice and collected in lysis buffer supplemented with protease inhibitors (Roche, Nutley, NJ). Three freeze-thaw cycles were performed in liquid nitrogen, tissues were mechanically homogenized, and lysate was cleared by centrifugation at 21,000 g for 15 min at 4 °C. Clarified lysate was assayed in the same manner described for the *in vitro* assays. Thus, gene expression was reported as the average RLU/mg protein for each brain region with error reported as the standard deviation of the mean. Whole brain gene expression was calculated as the sum of RLU/mg values of all regions per brain. N=4 for Melittin, C6M3, MEP-2, CP; N=3 for CMA-2, FL-20, DNA.

2.12 Histology and epifluorescence microscopy

Polyplexes formulated with pmaxGFPTM and either CP or VIPER (melittin-PPC) were administered by intraventricular injection to mice as described above (N=2). Two days post-injection, mice were euthanized and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion and fixation, the brains were excised and equilibrated to 30% sucrose in phosphate buffer. Brains were embedded in OCT and sectioned into 30 µm-thick coronal slices. For immunofluorescent labeling, slides were rinsed with PBS and blocked in PBS, 0.3% TritonX-100, 2% bovine serum albumin (BSA) for 1 h. Primary antibodies (mouse anti-Tuj1 [1:500, Biolegend]; chicken anti-GFAP [1:500, Millipore]) were applied to the tissue sections in PBS, 0.3% TritonX-100, 2% BSA overnight at 4 °C. Sections were rinsed three times for 20min in TBS, 0.1% Tween 20 and

species appropriate secondary antibodies conjugated with fluorophore were incubated in PBS, 0.1% Tween 20, and 2% donkey serum for 2 h. Sections were rinsed three times for 20min in TBS–Tween, with the last rinse containing the nuclear marker, 4',6-diamidino-2-phenylindole (DAPI; 0.5 µg/ml). Sections were then mounted onto glass slides, sealed and cover-slipped with polyvinyl alcohol, and imaged using an epifluorescence microscope. Hematoxylin and eosin (H&E) staining and imaging was performed on unstained, dry-mounted tissue slices by the University of Washington Histology and Imaging Core.

2.13 Statistical analysis.

All statistical analyses were performed in Prism software (Graph Pad Software, La Jolla, CA) using a two-tailed Student's t- test with unequal variance and Welch's correction. All EC50 values were determined from curve fits with $R^2 > 0.95$.

3. Results and Discussion

3.1 Lytic peptide selection and synthesis.

We conducted an intensive review of literature describing venom-derived antimicrobial peptides and synthetic derivatives, focusing on peptides that are as small or smaller than melittin (~2.9 kDa and 27 a.a.) yet reported to display similarly potent hemolytic activity. We also sought to diversify our panel by selecting peptides that differed from melittin in regards to hydrophobicity (e.g. FL-20), isoelectric point (e.g. CMA-2), net charge (e.g. IsCT), and pH-dependent lytic behavior (e.g. C6M3). Peptides containing cysteine (e.g. defensins) were excluded so that a C-terminal cysteine could be added to facilitate polymer conjugation via pyridyl disulfide exchange as in previous work with cysteine-modified melittin.[19] A final panel of 19 lytic and cell-penetrating peptides (Table 1) were synthesized via solid phase peptide synthesis (SPPS) on Rink Amide resin, purified by HPLC, and characterized by MALDI-TOF mass spectrometry before further testing.

3.2 *In vitro* hemolysis of lytic peptides.

Lytic peptides functionalized with a C-terminal cysteine were first evaluated with a standard hemolysis assay for direct comparison of membrane-lytic ability. In this assay, red blood cell lysis is quantified by monitoring hemoglobin release through absorption measurements. Although conjugated peptides are expected to reside in the hydrophobic core of VIPER micelles until disassembly at pH 6.4, we hypothesized that peptides with minimal lytic activity at neutral pH and high lytic activity at acidic pH would confer additional safety and endosomolytic selectivity. Thus, peptide hemolysis was tested at both extracellular (7.4) and endosomal (5.5) pH in order to prioritize polymer conjugation according to hemolytic potency at acidic pH (Table 1). Indeed, many peptides displayed much less potent hemolysis (e.g. SPFK) or no pH-selective lysis (e.g. CMA-2) compared to reported values when tested in our hands, although the discrepancy in potency may be due to the addition of a C-terminal cysteine or assay differences such as the species of red blood cell used. Peptides with redundant characteristics were eliminated and four peptides with potent hemolytic activity at acidic pH were selected for conjugation to the VIPER polymer backbone (Figure 1): FL-20 (short melittin relative with greater hydrophobicity); CMA-2 (melittin analog with identical length but neutral isoelectric point); MEP-2 (bee venom-derived and similar in charge to

melittin but shorter in length); and C6M3 (shorter than melittin, selectively lytic at low pH, but greater positive charge).

3.3 Polymer synthesis and characterization.

VIPER block copolymers with the general block formula of p(OEGMA-*co*-DMAEMA)-*b*-p(DIPAMA-*co*-PDSEMA) were synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization according to protocols established in previous work.[19] Molecular weight analysis via GPC revealed a monodisperse polymerization product characteristic of living polymerization ($\bar{D} = 1.19$), while ^1H NMR analysis confirmed nearly identical monomer composition relative to the previously reported polymer. (Supplemental Fig. 1) All experiments, p(OEGMA_{8.60}-*co*-DMAEMA_{50.0})-*b*-p(DIPAMA_{25.32}-*co*-PDSEMA_{1.00}) is referred to hereafter as the control polymer (CP); various polymer-peptide conjugates (PPCs) are referred to by the name of the respective peptide conjugate.

Lytic peptides were separately conjugated to CP through disulfide exchange between cysteine and pyridyl disulfide (PDS) followed by centrifugal dialysis and size exclusion to remove any unreacted peptide (Supplemental Figure 3). As in previous work, UV/Vis measurement of released pyridine-2-thiol confirmed reaction saturation by 20 hr. A secondary analysis of residual, unreacted polymer PDS content through UV/Vis also demonstrated 75% conversion, indicating that the vast majority of polymer chains had been conjugated with one peptide for each PPC (Supplemental Table 1).

3.4 Micelle formation and characterization.

We first characterized the impact of varied peptide biochemical properties such as size, isoelectric point, and hydrophobicity on PPC micelle size and pH-dependent stability. Micelle diameter as determined by DLS was consistent across all PPCs (21.7–26.0 nm), indicating that VIPER micelles can encapsulate peptides with a variety of biochemical properties and molecular weights without affecting size (Table 2). The critical micelle concentration of PPCs was determined using a Nile Red encapsulation assay (Table 2 and Supplemental Figure 5); the CMCs of all 6 polymers (control and five peptide conjugates) were all in relatively narrow range of 46.2 mg/L (for CMA-2 PPC) to 59.4 mg/L (for FL-20 PPC).

We next monitored micelle disassembly as a function of pH between 5.4 and 7.2 in 0.2 pH unit increments using the Nile Red encapsulation assay. Again, micelle disassembly for all 6 polymers occurred within a narrow range of pH of 6.38 to 6.47, dominated by the pKa of the p(DIPAMA) block (Table 2, Supplemental Figure 6). Notably, the most hydrophobic PPC (FL-20) displayed a lower and more gradual pH transition than CP, while all other PPCs transitioned sharply at approximately the same pH as the melittin PPC (Supplemental Figure 5). Thus, increasing micelle core hydrophobicity effectively lowered monomer pKa as a result of decreased water and ion transport that limits “all-or-nothing” cooperative protonation characteristic of charge/state-switching polymeric micelles.[56–59]

3.5 Characterization of DNA polyplexes.

PPC and CP polyplexes formed by electrostatic complexation of cationic micelles with plasmid DNA were characterized by gel retardation (DNA condensation), DLS (diameter), and electrophoretic mobility (zeta potential/surface charge). Imaging polyplex gel retardation showed that all PPCs fully condensed plasmid DNA (pDNA) at N/P 2, while CP condensed pDNA at N/P 1 (Supplemental Figure 7). Since only the number of polymeric tertiary amines was used to determine the N/P ratio (same mass concentration used for each polymer), the difference in N/P required for full DNA condensation is attributed to the mass of peptide present in PPC samples as in previous work.[19]

Statistical analysis of polyplex DLS Z-avg diameters revealed that MEP-2 and melittin PPC polyplexes had significantly larger diameters compared to CP. (Table 2) However, all diameters were between 105–145 nm and therefore typically representative of polyplexes described by our lab and others.[4,19,24] Because peptides are conjugated to the polymer chains that occupy the micelle core at neutral pH, the lack of statistically significant differences in zeta potential (surface charge) between CP and PPC polyplexes is also unsurprising. However, the most hydrophobic PPC (FL-20) registered the lowest surface charge of polyplexes tested (12.8 mV), which was significantly lower than that of the PPC with the highest net peptide charge (C6M3, 19.4 mV; $p = 0.0028$).

3.6 *In vitro* hemolysis of polymer-peptide conjugates.

PPC-mediated hemolysis of human RBCs was evaluated in buffer conditions mimicking various cellular compartments to determine which PPCs possessed specificity for lytic behavior in acidic endosomes. The majority of literature support the claims that the cytoplasm is a reducing environment with high concentrations of glutathione (1–10 mM GSH) and relatively neutral pH (7.4),[60,61] while endosomes are essentially as oxidative as the extracellular space (1–100 μ M GSH) but establish pH as low as pH 5.4 through ATP-dependent proton pumps.[62–64] Thus, we tested PPC hemolysis without GSH at pH 7.4 (extracellular mimic) and pH 5.5 (endosomal mimic), as well as with 10 mM GSH at pH 7.4 (cytoplasm mimic) and pH 6 (non-physiologic control; see note in Methods 2.9). A broad range of polymer concentrations spanning four orders of magnitude (0.01 to 100 μ M polymer) was tested (Figure 2a; Supplemental Figure 4).

No hemolysis was observed at pH 7.4 without GSH for all samples, confirming that lytic peptides remain hidden inside the DIPAMA micelle core in extracellular environments (Figure 2a). Hemolysis was observed only at high concentrations of melittin and MEP-2 PPCs ($>10 \mu$ M) at pH 7.4 in the presence of GSH, likely due to GSH penetration into the micelle core and subsequent release of small amounts of lytic peptide (Supplemental Figure 4). Most interestingly, in endosomal mimicking conditions (acidic pH without GSH), the EC_{50} s of the three melittin-like PPCs tested (melittin, CMA-2, FL-20) were lower than the corresponding peptide-only EC_{50} s (2.0, 2.3, 1.9 μ M, respectively), whereas the EC_{50} s of MEP-2 and C6M3 PPCs increased relative to peptide-only values (23.5, 22.9 μ M, respectively) (Figure 2b). We hypothesized that certain peptide sequences must be released from the polymer backbone to retain lytic activity and tested this hypothesis by conducting the PPC hemolysis assay at acidic pH with 10 mM GSH. As expected, all EC_{50} values of

GSH-reduced PPCs at acidic pH are similar that of the free peptide at acidic pH (Figure 2b; Supplemental Figure 4). This fact indicates that melittin-like PPCs may be better suited to endosomal lysis than MEP-2 or C6M3 as a result of the former's ability to retain and/or enhance peptide activity following conjugation to a polymer backbone through disulfide bond. Importantly, no hemolysis was observed for the control polymer (CP) at any concentration tested in any condition, demonstrating that the polymer backbone itself is non-lytic and therefore all lysis observed in PPC samples was peptide mediated.

3.7 *In vitro* plasmid transfection.

Next the gene transfer efficiency and cell compatibility of PPCs were tested in cultured mammalian cells. HeLa cells were transfected in serum-containing media with PPC polyplexes formulated at various N/P ratios with pmaxGFPTM plasmid (Figure 3A; Supplemental Figure 8). Cell viability decreased with increasing N/P as expected, but was acceptable (>75%) even at the highest N/P (N/P 8) tested. Moreover, no significant differences in viability were observed between any PPC and CP at each N/P. Therefore, the previously observed cytotoxicity was not due to the presence of a particular lytic peptide as we had hypothesized, but rather due to charge-dependent toxicity associated with increased p(DMAEMA) content.[65,66] All PPCs transfected exceptionally well even in the presence of serum proteins (>60% GFP⁺) at N/P 5 and above and were significantly more effective than CP (<20% GFP⁺) at all N/P tested ($p < 0.002$), confirming results from our previous work that pH-dependent display of the lytic peptide is required for efficient transfection. [19,24] Of the PPCs, CMA-2, FL-20, MEP-2, and melittin transfected with >96% efficiency, while C6M3 transfected with 76% efficiency at N/P 8. The reduced transfection efficiency of C6M3 is likely due to compromised lytic activity when conjugated to the polymer backbone (Figure 2b). Since peptides are susceptible to lysosomal protease degradation that increases in activity as pH decreases to pH 5.5,[67–69] C6M3 activity may be compromised by degradation at the pH required for membrane lysis.

PPC transfection efficiency was also screened in several other cells lines using a luciferase plasmid in order to confirm the cell type-agnostic nature of our active endosomal escape mechanism. We conducted this screen at N/P 5 because transfection at this N/P yielded the largest total number of GFP⁺ cells in the previous studies. We chose to compare HeLa cells, A549 cells (which have larger endosomes than HeLa cells[12]), ventricular zone choroid plexus epithelial Z310 cells[37], and neuron-like 3-day differentiated PC-12 cells.[70] The resulting trends in transfection mirror those of the HeLa GFP transfection in that melittin, CMA-2, FL-20, and MEP-2 PPCs transfect more efficiently than C6M3 PPC (Figure 3B) and transfect with 1–3 orders of magnitude greater efficiency than “proton sponge” control polymers (CP, 20 kDa bPEI) in all cell types tested. These results demonstrate the broad applicability of our lytic peptides and highlight the large impact that “active” endosomal escape strategies and can have on non-viral gene delivery.

3.8 *In vivo* plasmid transfection in the brain.

As all PPCs displayed excellent transfection efficiencies *in vitro*, we evaluated *in vivo* delivery efficiency by delivering polyplexes formed with the reporter luciferase plasmid to the mouse brain by intracerebroventricular (ICV) injection (Figure 4). This route of

administration enables access to neural progenitor cells (NPCs) which reside in the subventricular zone (SVZ) and extend foot processes through the choroid plexus epithelium. [71] We are interested in transfection to this region with our non-viral vector because expression of transcription and growth factors in SVZ NPCs has shown therapeutic promise as a means for the regeneration of cortical neurons following ischemic injury.[72–76] We have previously shown that dividing NPCs can be transfected by N/P 10 polyplexes delivered through this administration route, [77] and have achieved higher transfection in the right brain over the left after injection into the right ventricle.[39,78,79] In this study we observed no significant differences between transfection in the left and right brains for any polymer tested (Supplemental Figure 9). Importantly, overall transfection efficiency was highest for PPCs that achieved potent lysis at low pH without GSH (i.e. melittin and its homologs CMA-2 and FL-20). Thus, cells transfected in the brain from intraventricular polyplex delivery likely do not have sufficient endosomal GSH levels to release disulfide conjugated peptides from PPCs.

Epifluorescence microscopy was also used to analyze the distribution of transfection in a separate *in vivo* study where mice received polyplexes formulated with pmaxGFP™ and either CP or melittin PPC. We observed greater transfection for brains receiving PPC polyplexes but saw similar localization of transfection throughout the SVZ for both treatments (Supplemental Figure 10). It is not surprising that both polyplexes (CP and melittin PPC) undergo non-specific uptake along the SVZ epithelial barrier because association with cellular membranes and endocytosis of polyplexes is driven by their positive surface charge.[80] Although co-localization was not observed between transfected cells (GFP⁺) and either Tuj1 (neuronal marker) or GFAP (glial marker), transfection was observed predominantly in the periventricular space throughout the brain as in our previous studies with other polycations.[79] Transfection of cells in the subventricular zone was also observed for melittin PPC polyplex-treated animals. Finally, injections were well-tolerated by the animals and H&E staining revealed neither gross toxicity nor major tissue disruption along the needle track (Supplemental Figure 11). These results confirm that lytic peptides are well-protected in polyplexes *in vivo* and that transfection efficiency *in vivo* correlates with the lytic activity of non-reduced PPCs.

4. Conclusion

Herein we report the synthesis and evaluation of a panel of synthesized lytic peptides and the transfection capabilities they convey to acid-sensitive block copolymer micelles. Peptide polymer conjugates (PPCs) created from selected lytic peptides all demonstrated pH-dependent lysis and significantly enhanced *in vitro* transfection relative to unconjugated polymer, indicating that various peptide sequences may be encapsulated in VIPER micelles and are sufficient for endosomal escape. Surprisingly, only melittin-like PPCs showed greater hemolysis at pH 5.5 than their corresponding free peptides, implying that polymer-conjugated peptides may have enhanced lytic properties as a function of peptide sequence. As these most potently hemolytic PPCs also achieved the highest transfection *in vivo*, we conclude that only certain lytic peptides are able to promote endosomal escape when covalently conjugated to a polymer backbone. In summary, these data show that our pH-responsive micelle system exerts highly sensitive control over peptide-mediated membrane

lysis regardless of the pH-responsiveness of the peptide conjugate, further establishing this technology as an active, universal mechanism for the endosomal escape of nucleic acid cargo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by NIH R01NS064404 and NIH R01CA177272. We are grateful to Shaoyi Jiang and Kim Woodrow for the use their ZetaSizer and plate reader instruments, respectively. We are grateful to Prof. Wei Zheng (Purdue University) for kindly providing the Z310 cells.

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Figure 1. Lytic peptide hemolysis at various pH.

Peptides were incubated with human red blood cells at pH 7.4, 6.4, or 5.5 and hemolysis measured by UV absorbance at 541 nm relative to the positive control, 0.1% Triton X-100.

Figure 2. Peptide-polymer conjugate (PPC) hemolysis.

(A) PPC hemolysis at various pH. (B) Compiled low pH hemolysis curves of free peptide, non-reduced peptide-polymer conjugates, and reduced peptide polymer conjugates.

Polymers were incubated with red blood cells at various effective peptide concentrations at pH 7.4 and pH 5.4 for 1 h. For CP, an equivalent concentration of polymer was used in place of PPC concentration. Hemolysis was monitored by UV absorbance for released hemoglobin at 541 nm.

Figure 3. *In vitro* plasmid transfection.

(A) HeLa transfection viability and GFP expression efficiency. Transfection efficiency of CP and PPCs to HeLa cells in serum-containing media as determined by flow cytometry analysis of GFP expression 48 hours post transfection. Cell viability was determined by flow cytometry using Zombie Violet staining. All values are reported as mean $\pm$ SD. (N = 3; * = $p < 0.002$) (B) Relative luciferase activity in cell lysates 48 h after transfection with N/P 5 polyplexes. All values are reported as mean $\pm$ SD with significance in comparison to CP, bPEI, DNA, and cells only. (N = 3; * = $p < 0.002$)

Figure 4. Relative luciferase activity in whole brain lysates 48 h after ICV injection of N/P 10 polyplexes.

N=3–4; Data are plotted as the mean + SD. Statistical significance derived from Student's t-test comparing expression to one of two controls. (* $p < 0.05$ vs CP; † $p < 0.05$ vs DNA)

Key properties of the 19 peptides evaluated in this work. Peptides are listed in order of decreasing membrane lytic activity (hemolysis) at endosomal pH (5.5).

Table 1.

Peptide	Biological origin	Amino acid sequence	# AA	Molecular weight	EC ₅₀ (μM) pH 5.5	pH 7.4	pH(I)	Net charge pH 7	pH 5	Hydrophobicity	Ref
Melittin	Bee venom	GIGAVLKVLTTGLPALISWIKRKRQQC	27	2949.76	5	6	12.2	6	6	0.09	[19]
C6M3	Synthetic	RLWHLLWRLWRRLLHRLRC	19	2681.00	6	>100	12.8	7	9	-0.23	[40,41]
MEP-2	Bee venom	GFLSILKKVLKVMAMKMC	18	2043.80	12	16	11.2	5	6	0.22	[42]
CaLL	Synthetic hybrid	KWKLFKKIKFRIVQRKIDFLRC	22	2892.52	13	9	12.0	9	9	-0.25	[43]
CMA-2	Melittin	GIGAVLKVLTTGLPALISWIIHHHEEC	27	2929.46	14	9	7.4	0	4	0.35	[29]
FL-20	Flora/Melittin	GIGAILKVLATGLPTLISWIC	21	2136.60	16	30	10.1	2	2	0.61	[44]
EBI	Penetratin	LIRLWSHLIHWFQNRRLKWKKKC	24	3205.11	18	9	12.5	8	10	-0.22	[45]
Meucin-18	Scorpion venom	FFGHLFKLATKIIPSLFQC	19	2207.25	20	12	10.7	3	4	0.39	[46]
LAH	KALA/synthetic	KKLAHALHLLALLWLHLAHALKKAC	25	2811.39	20	>100	11.2	5	9	0.34	[47]
V681-S11K	Synthetic	KWKSFLKTFKKAVKTVLHTALKAISSC	27	3064.24	22	10	11.5	8	9	0.04	[48]
Endopoter	Synthetic	LHKLLHLLHLLHKLHLLHLLHKLK	27	3374.48	31	>100	11.0	5	16	0.12	[49]
Crabrolin	Hornet venom	FLALIRKIVTALC	14	1571.86	38	16	11.4	3	3	0.42	[50]
LPE3-1	GALA/melittin	GWWLALAEAEAEALALASWIKRKRQQC	27	3099.13	75	>100	9.9	2	2	0.02	[51]
Aurein1.2	Southern bell frog	GLFDIHKIAESFC	14	1582.70	95	98	8.8	1	1	0.24	[30]
IsCT	Scorpion venom	ILGKIWEGIKSLFC	14	1604.71	>100	57.5	10.0	2	2	0.31	[52]
Lyc0-L17E	M-lycotoxin	IWLTKFLGKHAAKHEAKQQLSKLC	26	2962.34	>100	>100	10.9	5	7	-0.03	[28]
HE	Magainin 2	GIHHWLHSAHEFGEHFVHHMNSC	24	2860.38	>100	>100	6.8	0	5.5	0.19	[53]
SPFK	Bovine semen	PKLLKTFLSKWIGC	14	1631.82	>100	>100	11.1	4	4	0.23	[54]
TAT	HIV	YGRKKRRQRRRC	12	1661.20	>100	>100	12.4	9	9	-1.77	[55]

Table 2:
Micelle and polyplex characterization.

Critical micelle concentration (CMC) and pH transition point as determined by a Nile Red encapsulation assay. Micelle and polyplex diameter as determined by DLS. Polyplex Zeta potential as determined by electrophoretic mobility.

Polymer or Conjugate	CMC (mg/L)	pH transition point	Micelle diameter (nm)	Polyplex diameter (nm)	Polyplex Zeta potential (mV)
CP	46.9	6.38	21.7 ± 1.9	112.4 ± 4.2	16.2 ± 3.3
Melittin	49.9	6.41	22.7 ± 1.9	133.0 ± 3.4 *	17.8 ± 1.7
CMA-2	46.2	6.47	26.0 ± 1.2	108.3 ± 3.2	16.3 ± 1.4
FL-20	59.4	6.34	24.1 ± 1.5	103.3 ± 9.4	12.8 ± 1.0
MEP-2	54.1	6.40	22.7 ± 2.0	141.5 ± 1.5 *	15.4 ± 4.3
C6M3	52.6	6.42	23.8 ± 2.1	127.8 ± 17.9	19.4 ± 1.3

*
= p < 0.05