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Intracellular Delivery of a Proapoptotic Peptide via Conjugation to a RAFT Synthesized Endosomolytic Polymer

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Abstract: Peptides derived from the third B-cell lymphoma 2 (Bcl-2) homology domain (BH3) can heterodimerize with antiapoptotic Bcl-2 family members to block their activity and trigger apoptosis. Use of these peptides presents a viable anticancer approach, but delivery barriers limit the broad application of intracellular-acting peptides as clinical therapeutics. Here, a novel diblock copolymer carrier is described that confers desirable pharmaceutical properties to intracellular-acting therapeutic peptides through site-specific molecular conjugation. This polymer was prepared using reversible addition−fragmentation chain transfer (RAFT) to form a pyridyl disulfide end-functionalized, modular diblock copolymer with precisely controlled molecular weight (M_n) and low polydispersity (PDI). The diblock polymer (M_n 19,000 g/mol, PDI 1.27) was composed of an *N*-(2-hydroxypropyl) methacrylamide (HPMA) first block (M_n 13,800 g/mol, PDI 1.13) intended to enhance water solubility and circulation time. The second polymer block was a pH-responsive composition designed to enhance endosomal escape and consisted of equimolar quantities of dimethylaminoethyl methacrylate (DMAEMA), propylacrylic acid (PAA), and butyl methacrylate (BMA). A hemolysis assay indicated that the diblock polymer undergoes a physiologically relevant pH-dependent switch from a membrane inert (1% hemolysis, pH 7.4) to a membrane disruptive (61% hemolysis, pH 5.8) conformation. Thiol−disulfide exchange reactions were found to efficiently produce reversible polymer conjugates (75 mol % peptide reactivity with polymer) with a cell-internalized proapoptotic peptide. Microscopy studies showed that peptide delivered via polymer conjugates effectively escaped endosomes and achieved diffusion into the cytosol. Peptide−polymer conjugates also produced significantly increased apoptotic activity over peptide alone in HeLa cervical carcinoma cells as found using flow cytometric measurements of mitochondrial membrane depolarization (2.5-fold increase) and cell viability tests that showed 50% cytotoxicity after 6 h of treatment with 10 μM peptide conjugate. These results indicate that this multifunctional carrier shows significant promise for proapoptotic peptide cancer therapeutics and also as a general platform for delivery of peptide drugs with intracellular targets.

Keywords: Therapeutic peptide; cancer; apoptosis; intracellular drug delivery; RAFT polymerization; bioconjugation; pH-responsive polymer; endosome escape

Introduction

Dysregulation of apoptosis was first linked to neoplasia by Vaux et al. upon the identification of the proapoptotic protein B-cell lymphoma 2 (Bcl-2) as a potential oncogene.[1] Subsequently, Bcl-2 overexpression was found to be a common hallmark of numerous malignancies, particularly lymphomas,[2] and to serve as a prognostic indicator for cancer chemoresistance and poor patient survival.[3,4] Improved understanding of this signaling pathway has enabled development of antagonists to antiapoptotic targets such as Bcl-2 with the pharmaceutical goal of eliciting cancer cell death and/or sensitization to chemotherapies. The most extensively tested proapoptotic drug, Bcl-2 antisense, has been used in clinical trials for multiple cancer types including melanomas, leukemias, and lymphomas.[5−8] Small molecule Bcl-2 inhibi-

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tors have also been explored. In this realm, one initial clinical trial has recently been reported[9] and human trials using other functionally similar compounds are currently in progress.[10,11]

These therapeutic approaches trigger apoptosis by indirectly activating the proapoptotic proteins Bax and Bak by decreasing the active pool of their natural inhibitors, the Bcl-2-like proteins.[12] Peptide fragments have also been derived from the third Bcl-2 homology (BH3) domain that are able to competitively inhibit Bcl-2-like proteins and indirectly trigger apoptotic signaling.[13−15] While delivery of BH3 peptides presents a logical approach for killing cancer cells, delivery hurdles have hindered the clinical use of peptide drugs. Peptides in general are rapidly cleared from the bloodstream and are susceptible to degradation by proteases *in vivo*, further limiting their effective half-life. Therapeutic peptides with cytosolic targets such as Bcl-2 can be especially challenging because they face the additional barrier of translocation across the cellular membrane. One strategy to achieve cell internalization is through fusion with peptide translocation domains (PTDs), short amino acid sequences that can facilitate intracellular transport of biomacromolecules. However, therapeutic cargo transported into cells via PTDs often suffers from compromised bioactivity due to internalization into and sequestration within intracellular vesicles that are trafficked for lysosomal degradation or exocytosis.[16,17]

Conjugation to polymeric carriers has been used to improve pharmaceutical characteristics such as circulation half-life of peptides.[18] More recently, the concept of diblock polymers that incorporate dual functions including endosomal escape have been described.[19] Here, the reversible addition−fragmentation chain transfer (RAFT) polymerization tech-

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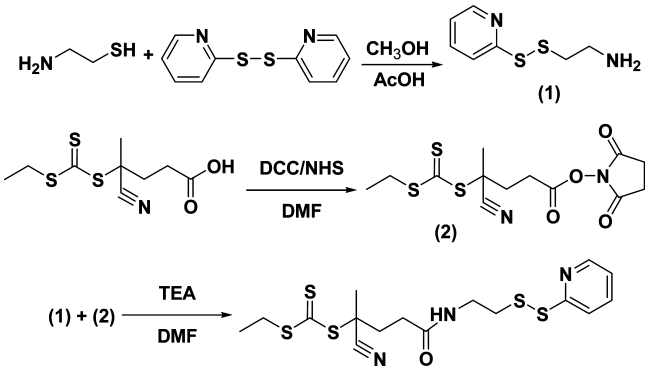
Scheme 1. Synthesis of Pyridyl Disulfide CTA



nique[20] was used to synthesize a carrier to be used to improve multiple facets of the pharmaceutical profile of peptide therapeutics through site specific peptide-polymer molecular conjugation. To do so, a diblock copolymer was synthesized that incorporates a neutral hydrophilic segment designed to enhance circulation characteristics, a pH-responsive block intended to promote endosomal escape, and a reversible peptide conjugation site that provides a means for peptide release from the carrier upon reaching the cytosol. Fusion of the therapeutic peptide sequence with the antennepedia (Antp) PTD sequence was used to trigger construct uptake into intracellular vesicles, and the ability of the polymer to enhance therapeutic peptide efficacy was assessed.

Materials and Methods

Preparation of Thiol Reactive Polymer. *Synthesis of Trithiocarbonic Acid 1-Cyano-1-methyl-3-[2-(pyridin-2-yldisulfanyl)ethylcarbamoyl]propyl Ester Ethyl Ester (PyrECT).* The 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanylpentanoic acid (ECT) precursor was synthesized as described previously,[19] and pyridyldithio-ethylamine was made as described by Zugates et al.[21] The pyridyl disulfide functionalized RAFT chain transfer agent (CTA) was synthesized by first converting ECT to the NHS ester followed by reaction with pyridyldithio-ethylamine using a procedure adapted from ref 22 (Scheme 1). Briefly, ECT (1.05 g, 4 mmol) and *N*-hydroxysuccinimide (0.460 g, 4 mmol) were dissolved in 100 mL of chloroform. The mixture was then cooled to 0 °C, at which time *N*,*N*′-dicyclohexylcarbodiimide (0.865 mg, 4.2 mmol) was added. The solution was maintained at 0 °C for 1 h and then allowed to react at room temperature for 22 h. The solution was then filtered to remove the dicyclohexyl urea and the solution concentrated via rotary evaporation. The resultant solid was then dried under vacuum and used without any further purification. NHS ECT (1.80 g, 5.0 mmol) and pyridyldithio-ethylamine (0.90 g, 5.0 mmol) were then separately dissolved in 200 and 300 mL of chloroform, respectively. The solution of pyridyldithio-ethylamine was added dropwise as three fractions 20 min apart. The mixture was then allowed to react at room temperature for 2 h. After solvent removal, two successive column chromatographies (silica gel 60, Merck) were performed (ethyl acetate:hexane 50:50; ethyl acetate:hexane 70:30 v/v) yielding a viscous orange solid. ^{1}H NMR 200 MHz ($CDCl_3$, RT, ppm): 1.29−1.41 [t, C*H*$_3$CH$_2$S: 3H], 1.85−1.93 [s, (C*H*$_3$)C(CN): 3H], 2.33−2.59 [m, C(CH$_3$)(CN)(C*H*$_2$C*H*$_2$): 4H], 2.86−2.97 [t, C*H*$_2$SS: 2H], 3.50−3.61 [t, NHC*H*$_2$: 2H], 7.11−7.22 [m, Ar para C*H*: 1H], 7.46−7.52 [m, Ar C*H* ortho: 1H], 7.53−7.62 [br, N*H*: 1H], 7.53−7.68 [m, Ar meta C*H*: 1H], 8.47−8.60 [m, meta C*H*N, 1H].

RAFT Polymerization of Pyridyl Disulfide Functionalized Poly[HPMA]-b-[(PAA)(BMA)(DMAEMA)]. The RAFT polymerization of *N*-(2-hydroxypropyl) methacrylamide (HPMA) was conducted in methanol (50 wt % monomer: solvent) at 70 °C under a nitrogen atmosphere for 8 h using 2,2′-azo-bis-isobutyrylnitrile (AIBN) as the free radical initiator.[23] The molar ratio of CTA to AIBN was 10:1, and the monomer to CTA ratio was set so that a molecular weight of 25,000 g/mol would be achieved at 100% conversion. The poly(HPMA) macro-CTA was isolated by repeated precipitation into diethyl ether from methanol. The macro-CTA was dried under vacuum for 24 h and then used for block copolymerization of dimethylaminoethyl methacrylate (DMAEMA), propylacrylic acid (PAA), and butyl methacrylate (BMA). Equimolar quantities of DMAEMA, PAA, and BMA ($[M]_o/[CTA]_o = 250$) were added to the HPMA macro-CTA dissolved in *N*,*N*-dimethylformamide (25 wt % monomer and macro-CTA to solvent). The radical initiator V70 was added with a CTA to initiator ratio of 10:1. The polymerization was allowed to proceed under a nitrogen atmosphere for 18 h at 30 °C. Afterward, the resultant diblock polymer was isolated by precipitation 4 times into 50:50 diethyl ether:pentane, redissolving in ethanol between precipitations. The product was then washed 1 time with diethyl ether and dried overnight in vacuo. Gel permeation chromatography (GPC) was used to determine molecular weight and polydispersity (M_w/M_n, PDI) of both the poly(HPMA) macro-CTA and the diblock copolymer in DMF. Molecular weight calculations were based on column elution times relative to polymethyl methacrylate standards using HPLC-grade DMF containing 0.1 wt % LiBr at 60 °C as the mobile phase.

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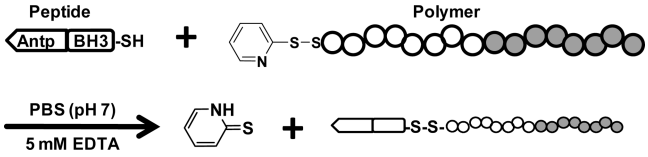
Scheme 2. Polymer−Peptide Conjugation via Thiol−Disulfide Exchange Reaction



pH-Dependent Membrane Disruption Hemolysis Assay. In order to assess the polymer's potential for aiding endosomal escape, a hemolysis assay was utilized as previously described[24,25] to measure the capacity of the polymer to trigger pH-dependent disruption of lipid bilayer membranes. Briefly, whole human blood was drawn and centrifuged for plasma removal. The remaining erythrocytes were washed three times with 150 mM NaCl and resuspended into phosphate buffers corresponding to extracellular (pH 7.4), early endosome (pH 6.6), and late endosome (pH 5.8) environments. The polymer (1−40 μg/mL) or 1% Triton X-100 was added to the erythrocyte suspensions and incubated for 1 h at 37 °C. Intact erythrocytes were pelleted via centrifugation, and the hemoglobin content within the supernatant was measured via absorbance at 541 nm. Percent hemolysis was determined relative to Triton X-100.

Polymer−Peptide Conjugation. Fusion with the Antp peptide sequence (RQIKIWFQNRRMKWKK) was utilized to synthesize a cell internalizing form of the Bak-BH3 peptide[14] (Antp-BH3) containing a carboxy-terminal cysteine residue (NH_2-RQIKIWFQNRRMKWKKMGQVGRQLAIIGDDINRRYDSC-COOH). To ensure free thiols for conjugation, the peptide was reconstituted in water and treated for 1 h with the disulfide reducing agent Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) immobilized within an agarose gel. The reduced peptide (400 μM) was then reacted for 24 h at room temperature with the pyridyl disulfide end-functionalized polymer in phosphate buffered saline (pH 7) containing 5 mM ethylenediaminetetraacetic acid (EDTA) as shown in Scheme 2. Conjugation reactions were done on an analytical scale at 1:1, 2:1, and 5:1 polymer to peptide molar ratios, and conjugations for in vitro experiments were all done with a 2:1 molar ratio. Reaction of the pyridyl disulfide polymer end group with the peptide cysteine creates 2-pyridinethione, which was spectrophotometrically measured to characterize conjugation efficiency based on its molar extinction coefficient in aqueous solvents at 343 nm (8080 M^{-1} cm^{-1}). To further validate disulfide exchange, the conjugates were run on an SDS−PAGE 16.5% tricine gel. In parallel, aliquots of the conjugation reactions were treated with immobilized TCEP prior to running SDS−PAGE to verify release of the peptide from the polymer in a reducing environment.

HeLa In Vitro Tests. HeLas, human cervical carcinoma cells (ATCC CCL-2), were maintained in minimum essential media (MEM) containing L-glutamine, 1% penicillin-streptomycin, and 10% FBS. Prior to experiments, HeLas were allowed to adhere overnight in 8-well chamber slides (20,000 cells/well) for microscopy or 96-well plates (10,000 cells/well) for other assays. For experimental treatments, polymer−peptide conjugates and controls were added in MEM containing L-glutamine, 1% penicillin−streptomycin, and 1% FBS, with the lower serum level intended to quiesce the cells and minimize potential confounding effects of rapid cell proliferation.

Microscopic Analysis of Conjugate Endosomal Escape. An amine reactive Alexa-488 succinimidyl ester was mixed at a 1:1 molar ratio with the Antp-BH3 peptide in anhydrous dimethylformamide (DMF). Unreacted fluorophore and organic solvent were removed using a PD10 desalting column, and the fluorescently labeled peptide was lyophilized. Alexa-488 labeled Antp-BH3 was conjugated to the polymer as described above. Free peptide or polymer−peptide conjugate was applied to HeLas grown on chambered microscope slides at a concentration of 25 μM Antp-BH3. Cells were treated for 15 min, washed twice with PBS, and incubated in fresh media for an additional 30 min. The samples were washed again and fixed with 4% paraformaldehyde for 10 min at 37 °C. Slides were mounted with ProLong Gold Antifade reagent containing DAPI and imaged using a fluorescent microscope.

Measurement of Conjugate Proapoptotic Activity. The ability of the bioconjugate to trigger tumor cell death was determined using a lactate dehydrogenase (LDH) cytotoxicity assay. At the end of each time point, cells were washed two times with PBS and then lysed with cell lysis buffer (100 μL/well, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 1 h at 4 °C. Twenty microliters of lysate from each sample was diluted into 80 μL of PBS, and then 100 μL of the LDH substrate solution was added. Following a 10 min incubation, LDH was colorimetrically measured by absorbance at 490 nm. Percent viability was expressed relative to samples receiving no treatment.

Flow Cytometry Evaluation of Mitochondrial Membrane Potential. Loss of mitochondrial membrane potential, a known indicator for apoptosis, was assessed using the JC-1 dye. JC-1 forms red-fluorescent aggregates at the mitochondrial membrane in healthy cells while it exhibits dispersed green fluorescence in the cytosol of apoptotic cells due to loss of the electrochemical gradient across the mitochondrial membrane.[26] For this assay, HeLas were incubated for 2 h with 10 μM peptide or equivalent conjugate or polymer

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alone. JC-1 was added at a final concentration of 5 μg/mL and incubated for 15 min. Cells were washed 2 times with PBS, trypsinized, and resuspended in 0.5% BSA for flow cytometric analysis. Cells displaying mitochondrial depolarization were quantified based on the percent of the cell population staining positive for green and negative for red fluorescence.

Caspase 3/7 Activity Assay. Caspase 3/7 activation is a measurable marker of apoptotic signaling that occurs downstream of activation of Bak and Bax. Caspase 3/7 activity was measured using a commercially available assay kit that utilizes a profluorescent substrate that once enzymatically cleaved becomes fluorescent allowing for determination of relative caspase 3/7 activity using a fluorescence plate reader. Here, HeLas were incubated for 30 min with 25 μM peptide (alone or as polymer conjugate) in addition to polymer alone in a quantity equivalent to the conjugate samples. Afterward, a caspase 3/7 fluorigenic indicator was added directly to the culture media for each sample. Plates were shaken for 1 h and then assayed using a fluorescent plate reader. Data were expressed as percent caspase activity relative to samples receiving no treatment.

Results

Preparation of Thiol Reactive Polymer. The RAFT CTA (Pyr-ECT) synthesized for this study yields α-functionalized polymers with a terminal pyridyl disulfide moiety that can be used to form disulfide-linked conjugates to thiol-bearing molecules. Pyr-ECT was synthesized based on the concept initially introduced for atom transfer radical polymerization (ATRP)[27] and subsequently applied to RAFT for formation of protein−polymer and siRNA−polymer disulfide-linked conjugates.[28,29] Here, Pyr-ECT was synthesized and purified as verified by ^{1}H NMR as described in Materials and Methods and further validated based on molecular weight using electrospray ionization mass spectrometry (theoretical = 431.7 g/mol, experimental = 432.3 g/mol).

Pyr-ECT was employed to make a diblock polymer that contains a hydrophilic block designed to ensure conjugate solubility and potentially improve circulation properties in vivo and a pH-responsive endosomolytic block to enhance peptide intracellular delivery. For the first block, a poly(HPMA) macro-CTA with a molecular weight (M_n) of 13,800 g/mol with a narrow polydispersity (PDI = 1.13) was produced. HPMA was chosen for this formulation in order to enhance polymer solubility and potentially increase circulation time for eventual in vivo applications due to its well-characterized biocompatibility in drug delivery applications.[30] The second block was an ampholytic design intended to be a pH-responsive element that triggers endosomal release of the therapeutic peptide. This terpolymer block contained PAA, DMAEMA, and BMA in equimolar quantities. These monomers were chosen to produce an approximately charge neutral block at physiological pH that would generate a hydrophilic to hydrophobic transition (PAA), increase overall positive charge (PAA/DMAEMA), and promote hydrophobic interactions (BMA) within the lower pH environment of endosomes (Figure 1). The M_n of the second block was 5,200 g/mol for a diblock copolymer M_n of 19,000 g/mol and an overall diblock PDI of 1.27.

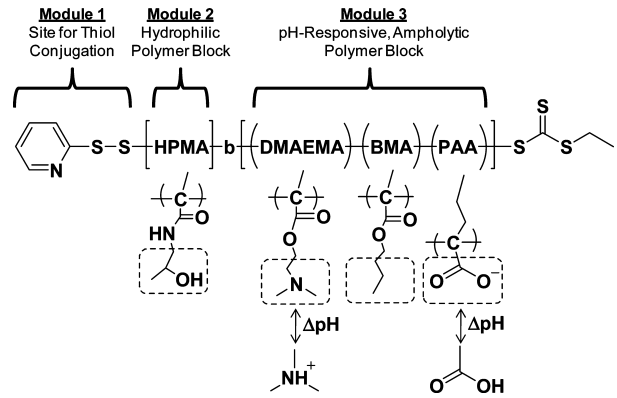

Figure 1. Poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)] polymer design. Multifaceted carrier properties were incorporated via RAFT polymerization using pyr-ECT to form a diblock architecture designed to possess aqueous solubility and pH-dependent membrane disruptive properties. The monomer chemical functionalities highlighted were chosen in order to produce the desired properties for each polymer block. Importantly, module 3 was designed to be near charge neutrality at physiologic pH (approximately 50% DMAEMA protonation and 50% PAA deprotonation predicted) but to undergo a transition to a more hydrophobic and positively charged state in lower pH environments.

pH-Dependent Membrane Disruption Hemolysis Assay. Red blood cell hemolysis was used to measure pH-dependent membrane disruption activity of the diblock copolymer at pH values mimicking physiologic (7.4), early endosome (6.6) and late endosome (5.8) environments (Figure 2). At physiologic pH, no significant red blood cell membrane disruption was observed even at polymer concentrations as high as 40 μg/mL. However, as the pH was lowered to endosomal values, a significant increase in hemolysis was observed, with greater membrane disruption

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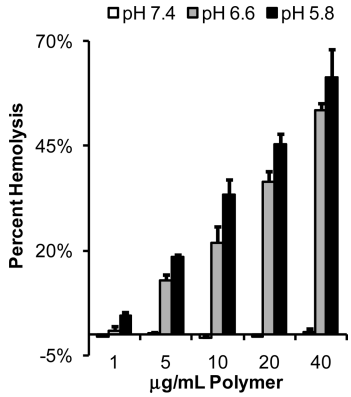

Figure 2. pH-Dependent membrane disruption by poly-[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)]. Polymer hemolysis was quantified at concentrations ranging from 1−40 μg/mL relative to 1% v/v Triton X-100. This experiment was completed 2 times in triplicate, yielding similar results. The data shown represent a single experiment conducted in triplicate ± standard deviation.

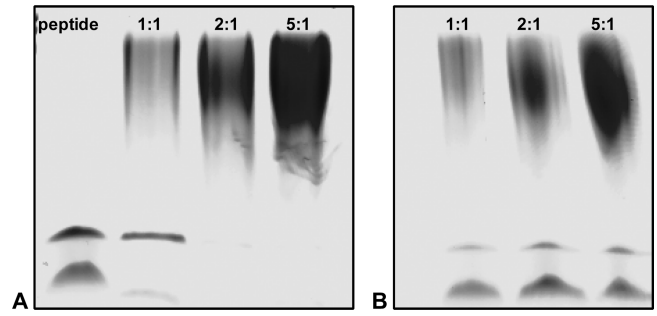

Figure 3. SDS−PAGE gel validating polymer−peptide conjugation via a reducible disulfide linkage. (A) Increasing the molar ratio of polymer:peptide to 2 or greater resulted in disappearance of the free peptide band as determined by staining with coomassie blue. (B) Treatment with the reducing agent TCEP disrupted the disulfide linkage, resulting in visualization of free peptide on the gel.

at pH 5.8 compared to 6.6. The hemolytic behavior of the polymer correlated to polymer concentration, with over 60% erythrocyte lysis occurring at 40 μg/mL polymer in pH 5.8 buffer. This sharp "switch" to a membrane disruptive conformation at endosomal pH combined with negligible membrane activity in the physiologic pH range demonstrates the desired functionality of the polymer design and further indicates its potential as a nontoxic, endosomolytic intracellular delivery vehicle.

Polymer−Peptide Conjugation. Conjugation reactions were conducted at polymer/peptide molar stoichiometries of 1, 2, and 5. UV spectrophotometric absorbance measurements at 343 nm for 2-pyridinethione indicated conjugation efficiencies of 40%, 75%, and 80%, respectively ([moles 2-pyridinethione released]/[moles of peptide reacted] X 100%). An SDS−PAGE gel was utilized to further characterize peptide−polymer conjugates. At a polymer/peptide molar ratio of 1, a detectable quantity of the peptide formed dimers via disulfide bridging through the terminal cysteine. However, the thiol reaction to the pyridyl disulfide was favored, and the free peptide band was no longer visible at polymer/peptide ratios equal to or greater than 2 (Figure 3A). By treating the conjugates with TCEP, it was possible to cleave the polymer−peptide disulfide linkages as indicated by the appearance of the peptide band in these samples (Figure 3B).

Characterization of Intracellular Peptide Delivery in HeLa cells. The potential of poly[HPMA]-*b*-[(PAA)(BMA)-(DMAEMA)] as a peptide intracellular delivery vehicle was investigated following bioconjugation to the Bak-BH3 peptide fused with the Antp (also known as penetratin) PTD. BH3 peptides fused with Antp have been extensively studied as cell-internalized proapoptotic peptides, and they have previously been found to trigger apoptotic signaling.[31] However, it is believed that therapeutics delivered via peptide transduction domains may suffer from hindered potency due to sequestration within intracellular vesicles.[32] The following in vitro studies were completed to validate that Antp-BH3 peptide cytoplasmic delivery and proapoptotic functionality was enhanced by conjugation to the pH-responsive polymer.

Microscopic Analysis of Conjugate Endosomal Escape. To study the effects of polymer conjugation on peptide endosomal escape, the peptide was labeled with Alexa-488 for analysis with fluorescent microscopy. The fluorescently labeled peptide was then delivered alone or as the polymer bioconjugate. Microscopic analysis revealed distinct differences in peptide intracellular localization following polymer conjugation (Figure 4). The peptide alone displayed punctate staining, indicative of endosomal compartmentalization. HeLas delivered polymer−peptide conjugate exhibited a dispersed fluorescence pattern, consistent with peptide diffusion throughout the cytoplasm.

Measurement of Conjugate Proapoptotic Activity. To assess polymer−peptide conjugate bioactivity, a cytotoxicity study was conducted in HeLa cervical cancer cells. The peptide delivered as the Antp-BH3−polymer conjugate was found to potently trigger HeLa cell death in a dose dependent fashion. Less than 50% HeLa viability was detected after 6 h of treatment with 10 μM peptide conjugate, and samples receiving 25 μM peptide conjugate showed little if any viable cells following as little as 4 h of exposure. Control samples receiving peptide or polymer alone displayed no significant treatment effect, and there was no difference between these control treatment groups. Importantly, it was also shown in this experiment that peptide conjugation to poly(HPMA)

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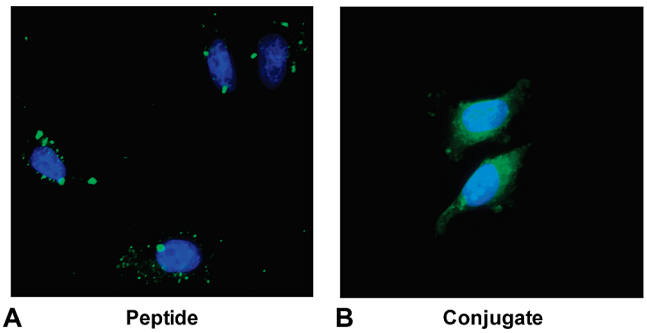

Figure 4. Polymer enhanced intracellular peptide delivery. Representative images illustrating (A) punctate peptide staining (green) in the samples treated with peptide alone and (B) dispersed peptide fluorescence within the cytosol following delivery of peptide−polymer conjugate. Samples were treated for 15 min with 25 μM peptide and prepared for microscopic examination following DAPI nuclear staining (blue).

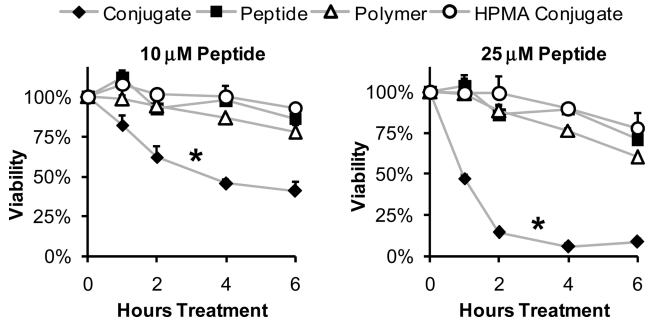

Figure 5. Poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)]−Antp-BH3 conjugate induction of HeLa cell death. HeLas were delivered (A) 10 μM or (B) 25 μM peptide conjugated to the Poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)], peptide alone, an equivalent amount of the polymer alone, or peptide conjugated to a non-pH-responsive poly-(HPMA). At 1, 2, 4, and 6 h, cell lysate was collected and assayed for LDH content relative to samples receiving no treatment. Representative data ± standard deviation are shown from 1 of 3 independent studies done in quadruplicate. * indicates conjugate treatment was significantly different ($p < 0.05$) from peptide, polymer, and HPMA conjugate groups.

produced effects similar to the control groups and did not result in significant toxicity, further validating functional enhancement of the peptide due to the pH-responsive, endosomolytic block (Figure 5).

Flow Cytometry Evaluation of Mitochondrial Membrane Potential. The loss of the electrochemical gradient across the mitochondrial membrane is a very early event in cellular apoptosis that can be detected using cytofluorimetric dyes.[33] Polymer controls were similar to cells receiving no treatment while Antp-BH3 alone showed an insignificant trend ($p =$ 0.13) toward increased mitochondrial depolarization. Polymer−peptide conjugate triggered a statistically significant approximately 10-fold increase in percent of cells exhibiting loss of mitochondrial polarity, indicating activation of proapoptotic programs by the peptide conjugate (Figure 6A).

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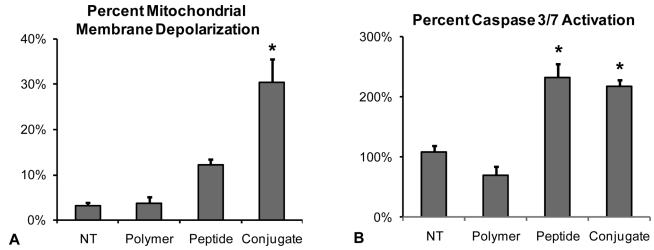

Figure 6. Proapoptotic indicators of peptide-polymer conjugate bioactivity. In each test, HeLas were given fresh media (NT), media containing 10 μM Antp-BH3 (peptide), 10 μM Antp-BH3−polymer conjugate (conjugate), or an equivalent amount of the polymer alone (polymer). (A) The JC-1 dye was added following 2 h of treatment, and flow cytometry was used to assess the percent of cells exhibiting loss of mitochondrial membrane integrity. Representative data ± standard error mean are shown from combined data from 2 independent studies done in triplicate. * indicates $p < 0.05$ vs NT, polymer, and peptide treatments using Tukey's method for pairwise comparisons. (B) To measure caspase activation, after 30 min of incubation, a fluorescent caspase 3/7 substrate was added to the media, and fluorescent readings were taken after 1 h. Caspase activity was expressed relative to samples receiving no treatment (NT). Representative data ± standard error mean are shown from combined data from 3 independent studies done in triplicate. * indicates $p < 0.05$ vs NT and polymer treatments using Tukey's method for pairwise comparisons.

Caspase 3/7 Activity Assay. Activation of caspases 3 and 7, which is indicative of proapoptotic signaling, can be measured using a profluorescent substrate specific to these proteases. In the current study, controls containing the polymer alone displayed equivalent caspase activity relative to negative controls receiving no treatment. However, rapid caspase activation (approximately 2.5-fold) was detected following treatment with the Antp-BH3 peptide by itself or in the polymer conjugate form (Figure 6B). The similar effects of Antp-BH3 alone or as a polymer conjugate could indicate that caspase signaling is saturated by treatment with the peptide alone or that other positive feedback mechanisms exist for amplification of perturbations in the baseline caspase activation state. These results also suggest that there was no steric hindrance or other reductions in peptide-induced caspase activity as a result of conjugation to the polymer.

Discussion

Proapoptotic peptides have significant anticancer potential if they are able to overcome in vivo delivery barriers to reach their desired intracellular targets. Here, previously introduced

methods for synthesis of pyridyl disulfide functionalized polymers[27−29] were modified to enable direct conjugation to a proapoptotic peptide to provide desirable pharmaceutical properties. The current report is one of the first to use this end-conjugation route for peptide drug delivery, to extend this concept beyond model biomacromolecules into the formation of a therapeutic conjugate, and to utilize this technique to formulate a complex diblock copolymer with multiple, tunable functionalities. Specifically, this new polymer design incorporated mechanisms for enhancing aqueous solubility and endosomal escape, in addition to providing a reversible conjugation site. This polymer was found to display a desirable pH-dependent membrane disruption profile and efficient conjugation to a peptide terminal cysteine. In vitro tests proved polymer conjugation to Antp-BH3 resulted in enhanced peptide cytosolic delivery and proapoptotic activity, indicating the polymer's potential for delivery of peptide drugs with cytosolic targets.

A modular polymer design was used to incorporate multiple peptide carrier properties. R group functionalization of the RAFT CTA with a pyridyl disulfide group was chosen due to the reversible nature of disulfide conjugation, which releases the drug cargo upon exposure to the cytoplasmic reducing agent glutathione.[34] Using this CTA, HPMA was chosen for polymerization of the first block due to its ability to enhance circulation time and its overall record for safety and efficacy in clinical drug delivery.[30,35,36] As in previous reports,[23,37] poly(HPMA) was made with relative synthetic ease using RAFT to achieve fine-tuned molecular weight and narrow polydispersities. The poly(HPMA) was then used as a macro-CTA to block the endosomolytic polymer "module" modified from a cationic siRNA-condensing polymer recently described.[19] This ampholytic terpolymer was designed to exist at near charge neutrality at physiological pH. Upon exposure to a lower pH environment such as that found in endosomes, the polymer sharply transitions to a state with net positive charge and increased hydrophobicity (Figure 1), promoting endosomal membrane disruption and diffusion to cytosolic targets.

To validate the peptide intracellular delivery potential of poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)], we developed a bioconjugate with the Bak-BH3 peptide fused with the Antp cell penetrating peptide. Antp-BH3 has been shown to antagonize the Bcl2-like family member Bcl-xL and to trigger apoptosis in HeLas via a cytochrome *c* independent mechanism.[14] Subsequently, the Antp-BH3 peptide was reported to lead to apoptotic cell death in head and neck squamous cell carcinomas, but, contrary to the initial report, the authors indicated that the peptide caused robust cytochrome *c* release from isolated mitochondria.[31] Finally, in prostate carcinoma cells, the minimal Bak-BH3 sequence delivered via electroporation was found to induce apoptosis but did not result in loss of mitochondrial polarity or cytochrome *c* release.[38] While there have been slight discrepancies on the mechanism of action, which could be dependent on cell type, delivery method, or model used, the peptide was found to trigger apoptosis in all of the reported circumstances due to Bcl-xL antagonism. It should be mentioned that numerous related peptide sequences derived from the BH3 domains of other Bcl-2 family members have proapoptotic properties worthy of further testing.[39] Also, significant breakthroughs in synthesis of chemically modified peptides have recently led to production of BH3 peptides with increased protease resistance and conformational stability,[40] further increasing their viability as pharmaceuticals. Related work in the field has discovered promising new peptide sequences that can directly activate proapoptotic members[41] or convert Bcl-2 into a prodeath factor.[42] The therapeutic potential for these and other intracellular-acting peptides may be further expanded by delivery via the carrier presented here.

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As in previous reports,[14] the current studies found that the Antp-BH3 peptide was internalized and triggered caspase activation in HeLas. The precise mechanism of uptake promoted by the Antp and other translocation peptide sequences is controversial, but the literature indicates that they are trafficked into vesicular pathways via either endocytosis or macropinocytosis, which can lead to entrapment in endosomes.[32] It is believed that the success of PTDs has been limited by relatively low efficiency of escape from intracellular vesicles, and it has been shown that endosome-releasing polymers and peptides can increase cytosolic delivery of PTD conjugates and fusion proteins.[17,43] Similarly, in this work, polymer-assisted vesicle escape improved cytosolic diffusion and bioactivity of the Antp-BH3 peptide. Significant cell death was seen with as little as 10 μM peptide delivered as a polymer bioconjugate, while previous reports indicated that longer time scales and higher concentrations (50 μM) were necessary to detect a robust apoptotic response in HeLas delivered the Antp-BH3 peptide alone.[14] Also, Antp-BH3 conjugated to a poly(HPMA) homopolymer showed similar activity to peptide alone (Figure 5), further emphasizing the importance of the pH-responsive element of poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)] in mediating peptide bioactivity.

Translation of the peptide−polymer conjugate efficacy observed in vitro to the in vivo setting may require further design optimization. We hypothesize that the HPMA polymer block will help to extend circulation half-life and lead to peptide−polymer conjugate tumor accumulation via the enhanced permeation and retention effect (EPR).[44] Upon extravasation into the tumor, it is posited that Antp would mediate conjugate uptake into tumor cells leading their death. However, further testing will be necessary to characterize conjugate pharmacokinetics and biodistribution in vivo, and dual tracking of the peptide and polymer will be required to validate that the conjugate disulfide linkage is not labile in blood. This may necessitate use of protected disulfides, which have been shown to be more stable in vivo.[45] Finally, fusion of the BH3 peptide with Antp was found to be critical to the current design in preliminary studies that indicated that poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)] conjugated to the minimal BH3 peptide was not internalized and did not trigger peptide activity (data not shown). However, it is possible that alternate cancer cell specific targeting approaches such as folate, transferrin, antibodies, or targeting peptides will be necessary to achieve a maximum tumor dose and minimal off-target effects in vivo. One useful feature of RAFT is that it provides a facile route to prepare telechelic polymers with distinct α and ω functionalities. A strong precedent exists for modification of the stable R-group of the CTA as done in this report for synthesis of α-functionalized polymers.[22,23,29] Modification of the CTA Z-group has been previously possible although more synthetically difficult,[46,47] and a new RAFT technique was recently developed that provides a simpler, more flexible method to prepare ω-functionalized polymers.[48] This feature could be adapted for future generations of the current carrier to achieve heterotelechelic end groups to synthesize well-defined polymeric conjugates containing both targeting and therapeutic moieties.

There is considerable unrealized potential for peptide therapeutics, particularly as anticancer agents. Here, a poly[HPMA]-*b*-[(PAA)(BMA)(DMAEMA)] pyridyl disulfide end-functionalized carrier was designed to form well-defined polymer−peptide molecular conjugates that enhance the pharmacological potential of intracellular-acting peptide drugs. The polymer's neutral hydrophilic segment made it readily water-soluble, and the pH-responsive polymer block enabled the polymer to produce pH-dependent membrane disruption, which enhanced peptide cytosolic delivery and bioactivity. Bioconjugation reactions and peptide−polymer conjugate bioactivity were validated in tests using the Antp-BH3 peptide, but the modularity of the polymer design makes it generally applicable for intracellular delivery of other peptides and other classes of biomacromolecules. Future directions will include preclinical assessments of the polymer's pharmacokinetics, testing cancer-specific targeting modules, and measuring the bioactivity of polymer−peptide conjugates in vivo. Based on the promising in vitro results presented here, there is optimism that translational testing will prove this carrier to be a significant breakthrough in the area of peptide drug delivery.

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