Effective Intracellular Delivery of an Anti-cancer Peptide via a RAFT-Synthesized Polymer Conjugate

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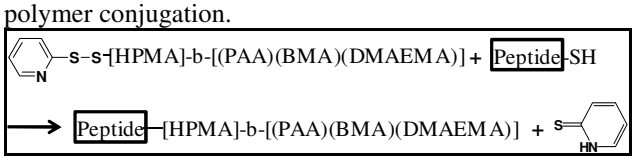
Statement of Purpose: Peptide drugs that act against intracellular targets hold great promise as therapeutics due to their high target specificity, small size, and manufacturability. However, substantial drug delivery hurdles must be overcome, including short circulation half-life, susceptibility to proteases, and poor cytosolic delivery. In this study, a multifunctional diblock copolymer was designed to address these challenges. This modular polymer was synthesized by the reversible addition–fragmentation chain transfer (RAFT) polymerization technique[1] from a functionalized chain transfer agent (CTA) that provided a streamlined and end-reactive peptide conjugation route. The diblock copolymer incorporated a hydrophilic, circulation-enhancing segment in addition to a pH responsive segment that strongly enhanced endosomal release and functional delivery of a pro-apoptotic peptide derived from the BH3 domain of the protein Bak (Bak-BH3). [2]

Methods: Thiol Reactive Polymer Preparation was completed using the RAFT technique. A pyridyl disulfide functionalized CTA[3] was synthesized and used to make a pyridyl disulfide α-functionalized diblock copolymer. The first block was polymerized with N-(2-hydroxypropyl) methacrylamide (HPMA), and the resultant HPMA macro-CTA was used for subsequent block copolymerization of dimethylaminoethyl methacrylate (DMAEMA), propylacrylic acid (PAA), and butyl methacrylate (BMA).

pH-dependent Hemolysis was used as a measure of the polymer's pH-dependent, membrane destabilizing activity. The percent red blood cell lysis was determined at physiologic (pH 7.4), early endosome (pH 6.6), and late endosome (pH 5.8) pH.

Reversible Polymer-Peptide Bioconjugation was done using a cell-internalizing Bak-BH3 peptide (ant-Bak-BH3) containing a carboxy-terminal cysteine. This thiol-containing amino acid was reacted with the pyridyl disulfide functionalized polymer (scheme shown below), and spectrophotometric measurement of the reaction byproduct 2-pyridinethione (ε=8080 $M^{-1}cm^{-1}$, 343 nm) and SDS-PAGE gels were utilized to confirm peptide-polymer conjugation.



HeLa In Vitro Tests were done in HeLas, human cervical carcinoma cells. Peptide was fluorescently labeled with Alexa-488 to track endosomal escape and cytosolic localization. Caspase 3/7 activation was measured using a commercially available assay kit, and bioconjugate efficacy for triggering cell death was determined using a lactate dehydrogenase (LDH) cytotoxicity detection kit.

Results: Thiol Reactive Polymer Preparation. The pyridyl disulfide CTA was synthesized as verified by NMR analysis. The poly(HPMA) macro-CTA had a molecular weight (MW) of 13,800 g/mol , and the poly[HPMA]-b-[(PAA)(BMA)(DMAEMA)] diblock copolymer MW was 19,000 g/mol with a narrow polydispersity of 1.27 as measured via gel permeation chromatography.

pH-dependent Hemolysis. At physiologic pH, the polymer exhibited negligible red blood cell disruption. At lower pH's, a significant increase in hemolysis was detected, and this effect was found to correlate to polymer concentration.

Reversible Polymer-Peptide Bioconjugation. Conjugation reactions conducted at polymer:peptide molar ratios of 1, 2, and 5 produced conjugation efficiencies of 40%, 75%, and 80%, respectively. In an SDS PAGE gel, the free peptide band was no longer visible at polymer:peptide ratios equal to or greater than 2.

HeLa *In Vitro* Tests. Peptide conjugation to poly[HPMA]-b-[(PAA)(BMA)(DMAEMA)] was found to trigger caspase activation and to enhance peptide endosomal escape and pro-apoptotic activity in HeLa cervical cancer cells (see Figure 1).

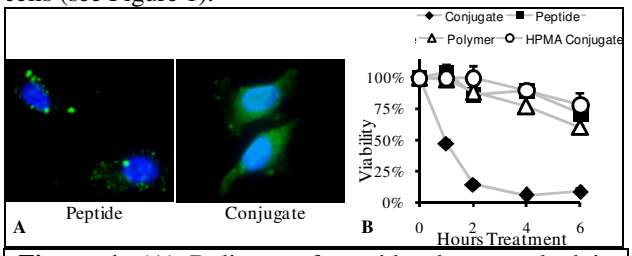


Figure 1. (A) Delivery of peptide alone resulted in punctate staining, indicative of endosomal entrapment while peptide delivered via conjugate displayed a dispersed appearance, suggesting endosomal escape. (B) HeLas exposed to the conjugate exhibited dramatic loss of viability while delivery of peptide alone, polymer alone, or peptide conjugated to non-endosomolytic poly(HPMA) had negligible effect.

Conclusions: In this work, a pyridyl disulfide end-functionalized diblock copolymer was made using RAFT and successfully utilized for peptide conjugation via disulfide exchange. Inclusion of a neutral hydrophilic segment made the polymer readily water soluble, and incorporation of a pH-responsive block successfully allowed the polymer to trigger pH-dependent membrane disruption. Furthermore, polymer conjugation was found to enhance peptide cytosolic delivery and bioactivity. Overall, the polymer described here shows great potential for use in applications requiring intravenous delivery of peptides (or other thiol containing biomacromolecules) targeted for intracellular delivery, and translation to *in vivo* testing will be the focus of future research.

References:

[1] Chiefari et al. Macromolecules.1998;31(16):5559-562.
[2] Holinger et al. J Biol Chem 1999;274(19):13298-304.
[3] Boyer et al. JACS 2007;129(22):7145-7154.