Mechanistic Study of Biologic Intracellular Delivery with pH-Responsive Polymers

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Statement of Purpose: Current treatments for cancer employ cytotoxic drugs such as small hydrophobic molecules that can pass thru cell membranes of non-targeted cells and cause many side effects. The use of cancer targeting biologics that reach the cytosol for action have enhanced specificity and can effect targets that are "undruggable" by small molecules. Antibodies can be chosen to internalize when bound to cell surface receptors and deliver small molecules after entry into cells. Cargo must then escape the endosomal/lysosomal pathway for effective cytosolic delivery. A pH-responsive, poly(propylacrylic acid) (PPAA) polymer has demonstrated enhanced intracellular delivery of biologics to the cytoplasm due to its membrane disruptive behavior at endosomal pH [1, 2]. In the present study, well-defined, biotinylated polymers were synthesized via controlled reversible addition-fragmentation chain transfer (RAFT) then combined with anti-CD22/streptavidin (HD39) conjugates to target and internalize into B-cell lymphoma via CD22 receptors [3]. Mechanistic studies were performed to quantify biologic intracellular delivery and characterize the intracellular trafficking pathways with PPAA. There are no current therapeutics that work against intracellular targets, but if a delivery system to get past this barrier were developed, a new class of drugs could be enabled.

Methods: PPAA and poly(methacrylic acid) (PMAA) were polymerized by RAFT using a biotinylated chain transfer agent, with PMAA serving as a similarly structured negative control polymer with a lower pKa that makes it less endosomolytic than PPAA. The polymers were characterized by gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy. Stoichiometries were determined using a 4-hydroxyazobenzene-2-carboxylic acid (HABA) assay to produce 4 PPAA or PMAA polymers per HD39 for cell studies. Uptake studies in RAMOS cells were performed to measure kinetics of real-time endosomal trafficking of HD39, HD39-PPAA and HD39-PMAA. Conjugates were dual-labeled with pH-sensitive (pHrodo, Molecular Probes) and pH-insensitive (AlexaFluor 488, Molecular Probes) fluorophores and the ratiometric fluorescence was measured to quantify the pH of conjugate-containing compartments [4]. Flow cytometry measured the average pH of the conjugates' intracellular environment, while real-time fluorescence microscopy identified the pH of individual conjugate-containing compartments. Single-labeled conjugates were also used for preliminary live-cell imaging and fixed-cell colocalization to characterize the trafficking pathway of the conjugates. After delivery, cells were fixed and labeled with early endosome, late endosome and lysosome markers (Santa Cruz Biotechnology) then imaged to quantify colocalization of HD39 conjugates within each compartment.

Results: SEC analysis of PPAA and PMAA showed that both polymers had a molecular weight of approximately 11 kDa. HABA analysis determined the molar excess of biotinylated polymer needed to achieve saturation of the 4 streptavidin binding sites. Results suggest that a polymer to HD39 ratio of approximately 12:1 and 4:1 was needed to achieve complete binding for PPAA and PMAA respectively. Preliminary live-cell imaging studies demonstrated cytosolic release of HD39 in the presence of PPAA as indicated by diffuse intracellular staining as seen in Figure 1, and quantitative analysis showed significantly increased intracellular accumulation of HD39-PPAA conjugates. Ratiometric fluorescence measured by flow cytometry demonstrated increasing fluorescence of pHrodo with HD39, to a measured pH of 4-5, associated with lysosomal trafficking. For HD39-PPAA conjugates, pHrodo fluorescence remains constant at a measured pH of 6-7. The pH of individual HD39-containing compartments measured by real-time microscopy has demonstrated a similar trend on the single cell level. Colocalization studies with the various markers have shown preliminary differences with HD39 in the presence and absence of PPAA and PMAA.

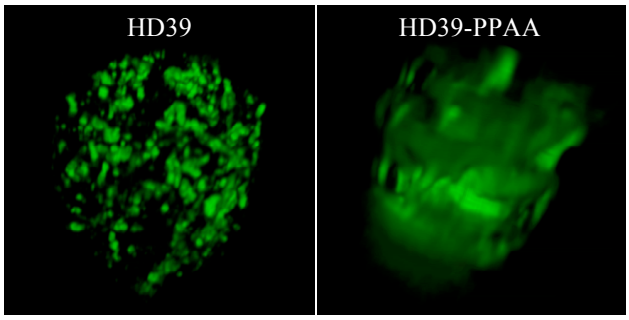

Figure 1. Comparison of fluorescently labeled HD39 and HD39-PPAA delivered to RAMOS cells for 40 minutes

Conclusions: RAFT-based polymeric carriers were utilized in mechanistic studies of biologic intracellular delivery. Ratiometric fluorescence measurements found that HD39–PPAA conjugates can manipulate endosomal pH and alter the intracellular trafficking pathway compared to HD39 alone. Further quantitative methods are being developed to better characterize the effects of endosomolytic polymers on intracellular trafficking. The authors would like to express their gratitude to the NIH grant EB002991 for funding. GYB is supported by a National Science Foundation Graduate Fellowship.

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