The Use of pH-sensitive Poly(propylacrylic acid) Polymers to Enhance the Delivery of Antigens for Therapeutic Vaccination

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Statement of Purpose: Therapeutic vaccination is an important new strategy in the treatment of diseases such as cancer and HIV. Antigenic proteins/peptides, or their corresponding mRNAs, are delivered to antigen-presenting cells such as macrophages and dendritic cells, in order to incite an immune response against any diseased cell presenting the antigen. Antigens expressed primarily by diseased cells have been identified, but their effective delivery into the potent class I immune presentation pathway requires escape from the endosomal compartment into the cell cytoplasm, which presents a significant challenge. The present research concentrates on the reversible conjugation of antigens to pH-sensitive, membrane-disruptive polymers able to enhance cytoplasmic delivery by disrupting the endosomal membrane at the lower pH (5-6) characteristic of the endosome. The polymer backbones consist mainly of the pH-sensitive component propylacrylic acid (PAAc), and conjugation is performed through a disulfide bond that is cleavable by glutathione once the antigen has been delivered to the cytoplasm. The membrane-disruptive abilities of several polymer and conjugate compositions have been determined, and the cytoplasmic delivery/class I presentation enhancement has been evaluated for both the model protein antigen ovalbumin and the cancer antigen epitope peptide, NYESO-1 157-165.

Methods: *Polymer Synthesis:* Several polymer compositions were produced by varying the amounts of pH-sensitive component PAAc and hydrophobic component butyl acrylate (BA). Pyridyldisulfide acrylate[1] (PDSA) was added at 3 mol% to provide the conjugation sites. Polymerization was carried out in bulk using both standard free radical and RAFT (reversible addition fragmentation chain transfer) techniques. The polymer was collected by precipitation in diethyl ether and characterized by NMR and GPC (gel permeation chromatography). *Polymer-antigen conjugation:* Conjugation was performed via disulfide exchange between PDSA on the polymer and free thiols on the antigen. Thiols were introduced onto ovalbumin using Traut's reagent, and the NYESO-1peptide contained a native cysteine residue. The polymer was reacted with the protein or peptide for several hours and the degree of conjugation determined by measuring A_{343} of the pyridine-2-thione group released from PDSA upon disulfide exchange. Conjugates were evaluated by GPC and gel electrophoresis. Ovalbumin conjugates were purified by Biocad anion exchange chromatography, and NYESO-1 conjugates were purified by gravity size exclusion chromatography. *Red blood cell hemolysis:* The pH-dependent membrane-disruptive abilities of the polymers and polymer-antigen conjugates were assessed using a red blood cell (RBC) hemolysis assay[2]. Briefly, RBCs were added to conjugate solutions of varying concentrations at pH values of 5.8, 6.6, and 7.4. The degree of membrane disruption (%hemolysis) was quantified by measuring A_{541} of hemoglobin released into the solution by lysed cells, in comparison to complete lysis by Triton X-100 detergent. *Class I antigen presentation assays:* The ability of the polymer to increase cytoplasmic delivery and subsequent class I presentation was evaluated using two assays. Ovalbumin conjugates were incubated with RAW 309.1Cr mouse macrophages, then B3Z T-cell hybridomas[3] were added. These cells express the *lacZ* gene upon recognition of the class I presentation molecule complexed with ovalbumin antigen fragment SIINFEKL. Thus, a lacZ assay[3] was used to determine T-cell activation and hence class I presentation. NYESO-1 peptide presentation was evaluated in primary dendritic cells. T-cell activation was measured via ELISA for the interferon γ produced by activated cytotoxic T-cells. Polymer-antigen conjugates, antigen only, polymer only, and physical polymer/antigen mixtures were compared.

Results and Discussion: *Polymer synthesis/conjugation:* Several polymers were obtained in the M_w range 15-40 kD, with M_w/M_n ranging from 2.0-3.0 for standard free radical synthesis and 1.3-1.7 for RAFT synthesis. NYESO-1 conjugates contained about 3 peptides per polymer chain, which represents saturation of the conjugation sites. Two main types of ovalbumin conjugates were explored: roughly 1:1 or 2:1 polymer:protein conjugates, and larger crosslinked conjugates. *RBC hemolysis:* The conjugates were considerably more hemolytic at low pH than physiological pH, as expected. As the pH decreases, carboxyl groups on the polymer backbone become protonated. This increased hydrophobicity allows the polymer to destabilize the cell membrane, releasing hemoglobin. A sample hemolysis result for an ovalbumin-30kD polymer conjugate at 5μg/ml was 10% at pH 7.4 and 80% at pH 5.8. Results were highly dependent on polymer concentration and polymer MW, with higher MW polymers being more hemolytic at all pH values. *Class I antigen presentaion assays:* The polymer-ovalbumin conjugates resulted in significantly greater T-cell activation/class I presentation than did delivery of free ovalbumin (*p* ranging from 0.0013 - 0.05 for various conjugate compositions; studen's t-test), or a physical mixture of ovalbumin and polymer (*p*: 6.1×10^{-5} - 0.0036). This result is in accordance with the increased endosomal escape and cytoplasmic delivery the conjugated polymer is expected to provide. Preliminary results from the NYESO-1 conjugate assay proved very promising, but a more detailed time-course study is needed before the results are conclusive.

Conclusions: It is possible to obtain and characterize protein/peptide-p(PAAc) conjugates that are able to disrupt membranes in a pH and concentration-dependent manner, as evidenced by RBC hemolysis. Furthermore, conjugation of the antigens to p(PAAc) can significantly increase class I antigen presentation and cytotoxic T-cell activation.

References: **1**.N.Murthy *Bioconjugate Chem* 14:412(2003) **2**.N.Murthy *J Controlled Release* 61:137(1999) **3**.S. Sanderson *Int Immunol* 6:369(1994)