pH-Responsive Copolymers for Intracellular Drug Delivery

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Statement of Purpose: Macromolecular therapeutics such as peptides, proteins, antisense oligodeoxynucleotides and short interfering RNA (siRNA) are active only in the cytoplasm of targeted cells. However, the primary route for cellular uptake of these molecules is endocytosis, which results in a loss of therapeutic activity due to their accumulation and degradation in the endosomal-lysosomal pathway. Despite ongoing research with viral fusion proteins, synthetic peptides, and cationic polymers, intracellular delivery of macromolecules through the endosomal membrane barrier remains a significant challenge. Here, we describe the synthesis of a novel "smart" polymer family that can enhance the cytoplasmic delivery of therapeutic macromolecules *via* pH-dependant membrane destabilizing activity. These polymers are propylamine, butylamine, and pentylamine derivatives of poly(styrene-*alt*-maleic anhydride) (PSMA) copolymers. At endosomal pH values, PSMA derivatives become membrane-disruptive, but remain inactive at physiological pH. Our results show that the pH-dependent membrane disruption caused by these polymers can be readily controlled by varying the length of the alkylamine group grafted onto the polymer backbone, the degree of alkylamine modification, and the molecular weight of the PSMA copolymer. We suggest that these copolymers could be used as platform intracellular delivery vehicles for a variety of macromolecular therapeutics.

Methods: *Polymer synthesis* PSMA was polymerized from mixtures of styrene and maleic anhydride in dimethylformamide (DMF) using azobisisobutyronitrile (AIBN) as a free radical initiator. Subsequent alkylamine modifications were performed by reacting a predetermined amount of propylamine, butylamine, or pentylamine with PSMA in DMF. PSMA copolymers and their derivatives were characterized using GPC and NMR.
Hemolysis Assay The pH-dependent membrane disruption of the PSMA derivatives was determined using a red blood cell (RBC) hemolysis assay[1]. Briefly, human RBCs were added to polymer solutions of various concentrations at pH values of 5.8, 6.6 and 7.4. At acidic conditions, membrane-disruptive polymers cause lysis of the red blood cells. The extent of membrane disruption (% hemolysis) was determined by measuring the A_{541} of hemoglobin released into solution relative to that released by a solution of Triton X-100 detergent.
Cytotoxicity Assay An assay for lactate dehydrogenase (LDH) was used to determine the cytotoxicity of PSMA copolymers and their derivatives. NIH3T3 fibroblasts were grown on tissue culture polystyrene 96 well plates. Adherent cells were treated with PSMA copolymer and propylamine, butylamine, and pentylamine derivatives of PSMA at various concentrations. At the end of the incubation period, the wells were aspirated and the adherent cells lysed. The LDH content of the wells was used to determine cell survival as a percentage of untreated controls.

Results / Discussion: *Hemolysis results* Reaction of PSMA with alkylamine groups introduces both a hydrophobic moiety (the alkyl chain) and a carboxylic acid group into the PSMA backbone. Protonation of these carboxylic acid groups causes the PSMA "smart" polymers to transition from a hydrophilic state to a hydrophobic one. We have found that by increasing the polymer hydrophobicity through alkylamine modification, membrane disruption is increased. Additionally, by increasing the molecular weight (Mw) of the PSMA backbone, it is possible to increase the hemolytic activity of a given alkylamine derivative. Specific results will be presented to illustrate how the type of alkylamine modifier and extent of modification can be altered to control the pH response of PSMA copolymers of various molecular weights. In general, our results show that propylamine derivatives of PSMA are not hemolytic, while the most hemolytic butylamine derivatives cause greater than 70% hemolysis at pH 5.8. These derivatives cause less than 10% hemolysis at pH 6.6 and less than 5% at pH 7.4, illustrating the "smart" behavior of the described polymers. Pentylamine derivatives were found to be more hemolytic than butylamine derivatives, causing greater than 80% hemolysis at pH 5.8, and greatly reduced levels of hemolysis at higher pH values. The hemolysis of both the butylamine and pentylamine derivatives was found to increase with the degree of alkylamine modification, polymer concentration, and polymer Mw.
Cytotoxicity results Alkylamine derivatives of PSMA are substantially less cytotoxic than the unmodified PSMA backbones. At a concentration of 40 μg/ml, PSMA derivatives caused less than 20% cell death, and cell death did not exceed 30% at any of the concentrations tested. No trends in cytotoxicity could be seen based on the type or extent of alkylamine modification.

Conclusions: The PSMA alkylamine derivatives we describe are "smart" pH-sensitive polymers capable of causing membrane disruption. By adjusting the degree and type of alkylamine modification, and the polymer molecular weight, we are able to engineer the pH range of the "smart" response. Anhydride groups which remain in the polymer backbone following alkylamine modification can be readily used for further functionalizations, such as the incorporation of cell-targeting ligands or the conjugation of biomolecular therapeutics[2]. We believe that these polymers are promising candidates for intracellular delivery applications, and can be incorporated in advanced drug delivery systems.

References: 1. Murthy, NM. J Cont. Rel. 1999;61:137-143. **2.** Maeda, H. J. Med. Chem. 1985;28:455-461.