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‘Smart’ delivery systems for biomolecular therapeutics

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Structured Abstract

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Objective – There is a strong need for drug delivery systems that can deliver biological signals from biomaterials and tissue engineering scaffolds, and a particular need for new delivery systems that can efficiently deliver biomolecules to intracellular targets. Viruses and pathogens have evolved potent molecular machinery that sense the lowered pH gradient of the endosomal compartment and become activated to destabilize the endosomal membrane, thereby enhancing protein or DNA transport to the cytoplasmic compartment. A key feature of many of these biological delivery systems is that they are reversible, so that the delivery systems are not directly toxic. These delivery systems have the ability to change their structural and functional properties and thus display remarkable ‘smart’ material properties. The objective of this presentation is to review the initial development of smart polymeric carriers that mimic these biological delivery systems and combine similar pH-sensitive, membrane-destabilizing activity for the delivery of therapeutic biomolecules.

Design – We have developed new ‘smart’ polymeric carriers to more effectively deliver and broaden the available types of biomolecular therapeutics. The polymers are hydrophilic and stealth-like at physiological pH, but become membrane-destabilizing after uptake into the endosomal compartment where they enhance the release of therapeutic cargo into the cytoplasm. They can be designed to provide a range of pH profiles and membrane-destabilizing activities, allowing their molecular properties to be matched to specific drugs and loading ranges. A versatile set of linker chemistries is available to provide degradable conjugation sites for proteins, nucleic acids, and/or targeting moieties.

Results – The physical properties of several pH-responsive polymers were examined. The activity and pH profile can be manipulated by controlling the length of hydrophobic alkyl segments. The delivery of poly(propyl acrylic acid) (PPAA)-containing lipoplexes significantly enhanced wound

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healing through the interconnected effects of altered extracellular matrix organization and greater vascularization. PPAA has also been shown to enhance cytoplasmic delivery of a model protein therapeutic. Polymeric carriers displaying pH-sensitive, membrane-destabilizing activity were also examined. The pH profile is controlled by the choice of the alkylacrylic acid monomer and by the ratio of the carboxylate-containing alkylacrylic acid monomer to alkylacrylate monomer. The membrane destabilizing activity is controlled by the lengths of the alkyl segment on the alkylacrylic acid monomer and the alkylacrylate monomer, as well as by their ratio in the final polymer chains.

Conclusion – The molecular mechanisms that proteins use to sense and destabilize provide interesting paradigms for the development of new polymeric delivery systems that mimic biological strategies for promoting the intracellular delivery of biomolecular drugs. The key feature of these polymers is their ability to directly enhance the intracellular delivery of proteins and DNA, by destabilizing biological membranes in response to vesicular compartment pH changes. The ability to deliver a wide variety of protein and nucleic acid drugs to intracellular compartments from tissue engineering and regenerative scaffolds could greatly enhance control of important processes such as inflammation, angiogenesis, and biomineralization.

Key words: drug delivery; gene delivery; smart polymers; tissue regeneration; wound repair

Introduction

There are several classes of therapeutic macromolecular drugs, including peptides, proteins, plasmid DNA (pDNA), antisense oligodeoxynucleotides (ASODN), silencing RNA, and ribozymes. For many existing or potential macromolecular drugs, the targets of therapeutic action are intracellular. The internalization of macromolecules by passive or receptor-mediated endocytosis leads to subsequent accumulation in the endosomal-lysosomal compartments where degradation ultimately occurs (Fig. 1). The limited cytoplasmic delivery of enzyme-susceptible drugs thus remains a major barrier to their development as clinical therapeutics. Effective local delivery systems for these therapeutics may also open opportunities in the tissue engineering and regeneration field.

Several viruses and pathogenic organisms have evolved sophisticated intracellular delivery systems. These organisms utilize fusogenic proteins that display

membrane-destabilizing activity in response to acidic endosomal pH gradients, and have been shown to enhance DNA and protein transport from the endosomal compartment to the cytoplasm of targeted cells (1–5). The pH-sensing mechanism of hemagglutinin and diphtheria toxin is connected to the protonation of key carboxylate residues that triggers a shift in conformational equilibria towards the membrane-destabilizing state (2–5). Synthetic peptides with similar membrane-destabilizing mechanisms have been developed for use as endosomal-releasing agents in gene and protein delivery systems (6–10).

We review here the initial development of smart polymeric carriers that combine similar pH-sensitive, membrane-destabilizing activity for the delivery of therapeutic biomolecules. The smart polymers mimic key mechanistic aspects of the biological systems. The polymeric carriers 'sense' changes in environment pH and reversibly switch from a hydrophilic stealth-like conformation at physiologic pH to a hydrophobic and

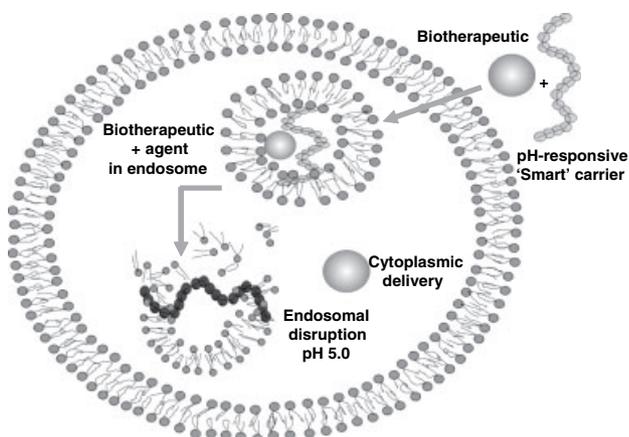


Fig. 1. A key barrier for many biomolecular therapeutics is cytoplasmic entry, where hydrophilic macromolecules need to transverse the endosomal membrane before trafficking to the lysosome exposes them to enzyme degradation.

membrane-destabilizing state in response to acidic stimuli. The general design of the smart polymeric carriers incorporates pH-sensing functionalities, hydrophobic membrane-destabilizing groups, versatile conjugation and/or complexation elements to allow the drug incorporation, and an optional cell targeting component.

General physical properties of pH-responsive polymers and delivery applications

The pH-dependent, membrane destabilizing activities of a family of poly(alkylacrylic acid) polymers have been characterized. This family includes poly(methylacrylic acid), poly(ethylacrylic acid) (PEAA), poly(propylacrylic acid) (PPAA) and poly(butylacrylic acid) (PBAA), where the alkyl group progressively increased by one methylene group (Fig. 2). Tirrell and coworkers previously described the pH-dependent disruption of lipid vesicles using PEAA (11,12). This series of polymer compositions was designed to examine the effect of increasing the length of the hydrophobic alkyl group on

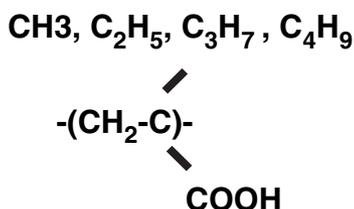


Fig. 2. The chemical structure of the poly(alkylacrylic acid) family.

the pH-dependent, membrane-destabilizing activity (13,14). PPAA was found to be approximately 15 times more active than PEAA (Fig. 3), and exhibited its maximum hemolytic activity at pH 6.0 and lower (Fig. 4) (13). The observed higher hemolytic activity of PPAA compared with PEAA and the change in pH profile are a result of the increased hydrophobic character

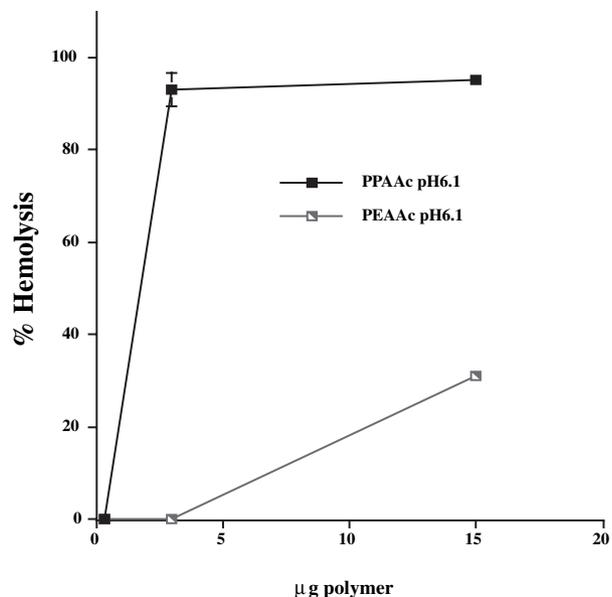


Fig. 3. The concentration-dependent hemolysis of RBC by PEAA (open square) and PPAA (filled square) at pH 6.1. Results shown are the average of triplicate measurements ± standard deviations of the mean.

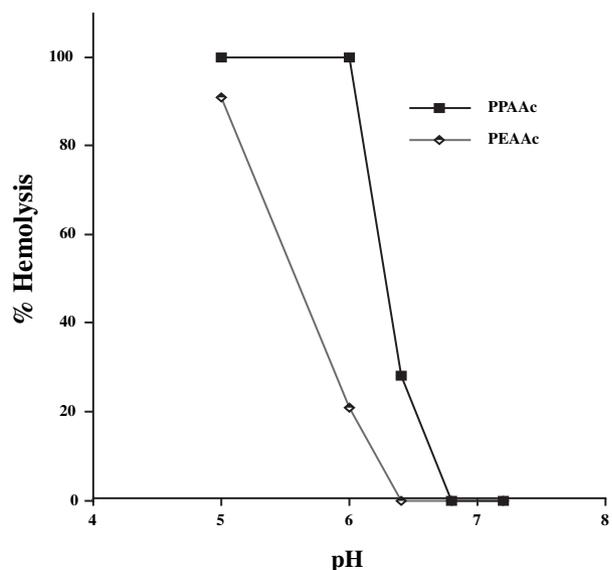


Fig. 4. The pH-dependent hemolysis of RBC by PEAA at 100 mg/ml (open square) and PPAA at 10 mg/ml (filled square) at pH 6.1. Results shown are the average of triplicate measurements ± standard deviations of the mean.

of the pendant alkyl chain by one methylene group. The pH profile of PBAA was shifted to higher pH values with hemolysis observed at physiological pH (14). These results demonstrate that the activity and pH profile can be manipulated by controlling the length of the hydrophobic alkyl segment.

The excellent pH-dependent hemolytic activity of PPAA motivated an investigation of its ability to enhance gene expression and serum stability when incorporated in cationic lipoplexes formulations (15). PPAA enhanced transfection significantly and increased the stability of ternary lipoplexes in serum. DNA condensation, cell uptake, and transfection studies collectively showed that incorporation of PPAA greatly improved the complex stability of these formulations at concentrations similar to those found in whole blood (16). The favorable transfection results motivated further *in vivo* evaluation. A mouse model of wound healing was utilized for the *in vivo* evaluation of cationic lipoplexes with/without PPAA (17–20). This model was based on previous studies that demonstrated excisional wound healing is accelerated in thrombospondin-2 (TSP2)-null knockout mice (19). The delivery of PPAA-containing lipoplexes significantly enhanced wound healing through the interconnected effects of altered extracellular matrix organization and greater vascularization (17).

PPAA has also been shown to enhance cytoplasmic delivery of a model protein therapeutic. Antibody-directed toxins enter the endosomal-lysosomal trafficking pathway, where cytoplasmic entry is a major barrier (21,22). The pH-dependent, hemolytic activity of PPAA was first shown to be retained when conjugated to streptavidin (23). PPAA was then studied with

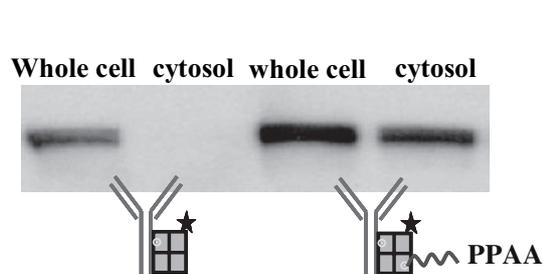


Fig. 5. Quantitative western blot analysis of whole cell and isolated cytoplasmic homogenates. Jurkat cells were incubated with $20 \mu\text{g}$ antibody/ 10^7 cells and equimolar biotinylated PPAA. The gel lanes are: (1) MoAb 64.1-biotin/streptavidin whole cell homogenate; (2) MoAb 64.1-biotin/streptavidin isolated cytoplasmic homogenate; (3) MoAb 64.1-biotin/streptavidin/PPAA-biotin whole cell homogenate; (4) MoAb 64.1-biotin/streptavidin/PPAA-biotin isolated cytoplasmic homogenate.

a model biotinylated anti-CD3 antibody/streptavidin complex (24). The monoclonal anti-CD3 antibody 64.1 (MoAb) is internalized with minimal translocation to the cytoplasm (21,25,26). The quantity of the protein complex present in the cytoplasmic fraction was compared with that present in the total cell homogenate using quantitative western blotting techniques (Fig. 5). Approximately 70% of the protein complexes with PPAA-biotin were detected in the cytoplasmic fraction vs. no detectable quantity when PPAA was absent. These results demonstrate that PPAA enhances the cytoplasmic delivery of antibody-targeted conjugates that are internalized through receptor-mediated endocytosis.

Polymeric carriers displaying pH-sensitive, membrane-destabilizing activity

The ability of PPAA to enhance protein and DNA intracellular delivery motivated recent work to construct more versatile carrier systems (Fig. 6). To provide a flexible conjugation route for therapeutic molecules, complexing agents, and/or targeting moieties, a new functionalized monomer was synthesized (27). The pyridyl disulfide acrylate (PDSA) monomer allows efficient conjugation reactions through disulfide linkages that can be reduced in the cytoplasm after endosomal translocation of the therapeutics. The general design of the PDSA family allows molecular tuning of the pH profile and membrane destabilizing activity. The pH profile is controlled by the choice of the alkylacrylic acid monomer and by ratio of the

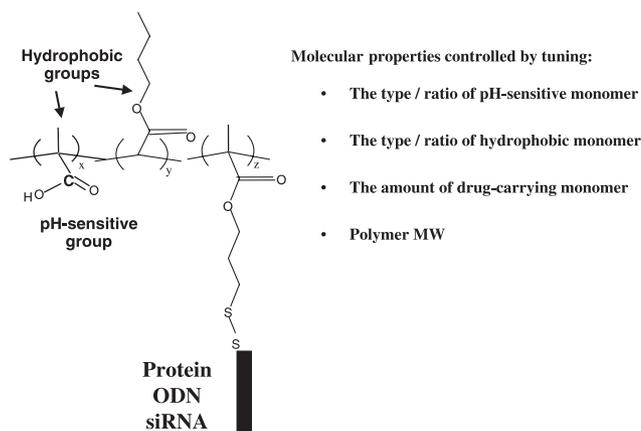


Fig. 6. The general design parameters of the PDSA family of polymeric carriers.

carboxylate-containing alkylacrylic acid monomer to alkylacrylate monomer. Similarly, the membrane destabilizing activity is controlled by the lengths of the alkyl segment on the alkylacrylic acid monomer and the alkylacrylate monomer, as well as by their ratio in the final polymer chains.

These factors have been characterized by studying the influence of increasing the length of the hydrophobic alkyl group substituted onto the pH-sensitive monomer, and the effect of incorporating butyl acrylate (BA) on the pH sensitivity and membrane destabilizing activity of new polymer compositions (28). For the pH-sensitive monomers, the α -substituted alkyl group was progressively increased in length by one methylene group from methylacrylic acid (MAA), to ethylacrylic acid (EAA) and propylacrylic acid (PAA). The second series of PDSA polymers was prepared by incorporating the hydrophobic BA monomer ratio in the polymer backbone. The monomer feed ratio, solvent/monomer ratio, and initiator concentration was controlled to allow the incorporation of approximately 5 mol % of PDSA units in the polymer backbone and to achieve a weight average molecular weight of 30–35 kDa. The hemolytic activity of these new polymer compositions was characterized as a function of pH and polymer concentration to identify the key structural features essential for pH sensitivity and membrane destabilizing activity of PDSA polymers.

In the first series of PDSA polymers, the poly(PAA-*co*-PDSA) exhibited a pH-dependent hemolytic activity, whereas poly(MAA-*co*-PDSA) and poly(EAA-*co*-PDSA) polymers were non-hemolytic at the examined concentrations and pH values (Fig. 7). In the second series of PDSA polymers, both poly(EAA-*co*-BA-*co*-PDSA) and poly(PAA-*co*-BA-*co*-PDSA) polymers exhibited pH- and concentration-dependent hemolytic activities (Fig. 8). These results demonstrated that the significant increase in polymer backbone hydrophobic character upon BA incorporation was directly tied to membrane destabilizing activity. Both the poly(EAA-*co*-BA-*co*-PDSA) and poly(PAA-*co*-BA-*co*-PDSA) polymers exhibited high hemolytic/membrane destabilizing activity at the low molecular weights of 9 and 12 kDa, respectively. This finding suggests that carriers below the renal excretion size limit can be designed to have potent pH-responsive, membrane destabilizing activity.

The ability to tune the pH profile and membrane destabilizing activity is particularly important because

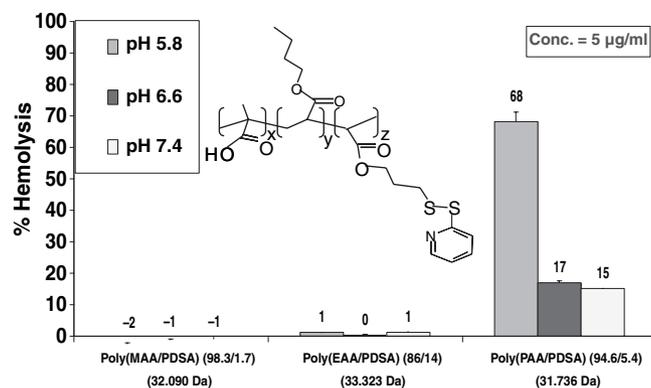


Fig. 7. The influence of increasing the length of the hydrophobic alkyl group substituted on the pH-sensitive monomer on the hemolytic activity of poly(MAA-*co*-PDSA), poly(EAA-*co*-PDSA), and poly(PAA-*co*-PDSA) polymers at pH 5.8 (■), 6.6 (■), and 7.4 (□). The hemolytic activity is examined at polymer concentrations of: (A) 5 µg/ml, (B) 10 µg/ml, and (C) 20 µg/ml.

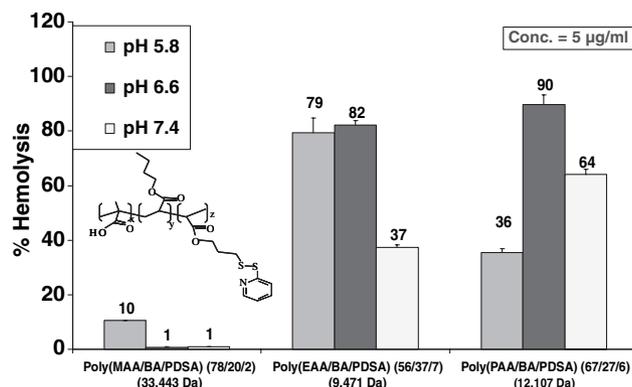


Fig. 8. The influence of incorporating the hydrophobic BA monomer in the polymer backbone on the hemolytic activity of poly(MAA-*co*-BA-*co*-PDSA), poly(EAA-*co*-BA-*co*-PDSA), and poly(PAA-*co*-BA-*co*-PDSA) polymers at pH 5.8 (■), 6.6 (■), and 7.4 (□).

the biomolecular therapeutics are hydrophilic macromolecules that can significantly shift the pH profile and membrane destabilizing activity. The polymeric carrier can be engineered to have high membrane destabilizing activity, including some activity at physiological pH, because when the drug is added it alters the overall hydrophilic/hydrophobic balance back toward activation at lower pH values. This principle is illustrated in Fig. 9 where the hemolytic activity of poly(MAA-*co*-BA-*co*-PDSA) both free and after conjugation to a cationic [(Cys)-(Gly)₃] peptide is shown. The addition of the hydrophilic peptide shifts the pH profile significantly, with the pH of activation occurring at pH values approximately 0.7 units more acidic than the polymer alone. The precise pH profile and activity will depend

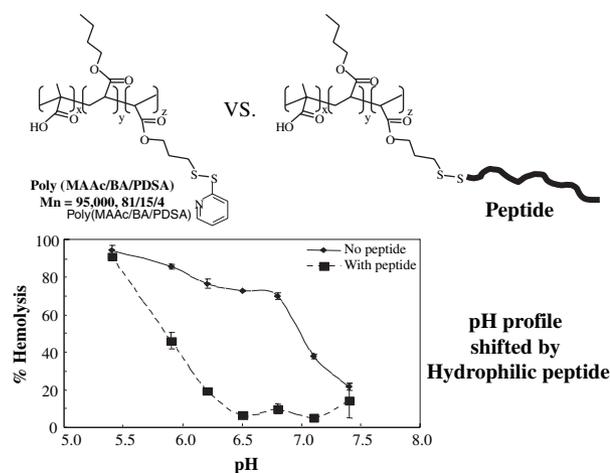


Fig. 9. Comparison of the intrinsic pH-dependent, membrane-destabilizing activity of poly(MAA-co-BA-co-PDSA) vs. the peptide Cys-Gly₃ conjugate. The Mn of the poly(MAA-co-BA-co-PDSA) was 95 000 with a MAA:BA:PDSA ratio of 85:15:4.

on the nature of the macromolecule (e.g. DNA vs. protein) and the quantity of drug loading, so that the ability to tune the intrinsic pH profile and membrane destabilizing activity is critical.

Clinical utility and implications

The ability to control the tissue remodeling processes of angiogenesis, inflammation, morphogenesis, etc. is critical to the tissue engineering and regeneration field. The delivery of biological signals from scaffolds and matrices to control these processes is thus an active research area. Our group has developed new 'smart' polymeric carriers to more effectively deliver and broaden the available types of biomolecular therapeutics used in this setting. The polymers are hydrophilic and stealth-like at physiological pH, but become membrane-destabilizing after uptake into the endosomal compartment where they enhance the release of therapeutic cargo into the cytoplasm. They can be designed to provide a range of pH profiles and membrane-destabilizing activities, allowing their molecular properties to be matched to specific drugs and loading ranges. Finally, a versatile set of linker chemistries is available to provide degradable conjugation sites for proteins, nucleic acids, and/or targeting moieties.

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