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Bioinspired Polymers that Control Intracellular Drug Delivery

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Abstract One of the important characteristics of biological systems is their ability to change important properties in response to small environmental signals. The molecular mechanisms that biological molecules utilize to sense and respond provide interesting models for the development of "smart" polymeric biomaterials with biomimetic properties. An important example of this is the protein coat of viruses, which contains peptide units that facilitate the trafficking of the virus into the cell via endocytosis, then out of the endosome into the cytoplasm, and from there into the nucleus. We have designed a family of synthetic polymers whose compositions have been designed to mimic specific peptides on viral coats that facilitate endosomal escape. Our biomimetic polymers are responsive to the lowered pH within endosomes, leading to disruption of the endosomal membrane and release of important biomolecular drugs such as DNA, RNA, peptides and proteins to the cytoplasm before they are trafficked to lysosomes and degraded by lysosomal enzymes. In this article, we review our work on the design, synthesis and action of such smart, pH-sensitive polymers.

Keywords: biomimetic polymer, intracellular drug delivery, pH-sensitive polymer, polycarboxylate polymer

INTRODUCTION

The biotechnology and pharmaceutical industries have developed a wide variety of potential new drugs based on the key molecules of biology: DNA, RNA, peptides and proteins. While these therapeutics have tremendous potential, developing effective delivery formulations has been a great challenge. Among the important factors to the successful clinical application of such drugs are drug stability, administration, absorption, metabolism and bioavailability at the target site. While a number of creative delivery systems show significant potential for overcoming many of these problems with biomolecular drugs that act at cell membrane receptors, a major barrier for those drugs that function intracellularly is endosomal escape and cytoplasmic entry [1,2]. Passive or receptor-mediated endocytosis results in localization of biomolecules to the endosomal compartment, where the predominant trafficking fate is fusion with lysosomes and subsequent degradation [3].

One of the most significant barriers for the delivery of biomolecular therapeutics that function inside the cell is the endosomal barrier (Fig. 1). For therapeutics such as plasmid DNA, antisense oligonucleotides (ODNs), ribozymes, and immunotoxins, their ability to reach their intracellular target is dependent on their ability to escape from the endosome into the cytoplasm. Similarly, the delivery of plasmid-based or protein/peptide-based molecular vaccines is also dependent on getting the DNA plasmid into the nucleus, or getting the peptides or proteins into the cytoplasm for entry into the protein processing and display pathways. The magnitude of these challenges to drug delivery researchers can easily be seen in a comparison of viral versus non-viral gene delivery systems, where viral transfection efficiencies may be 4-6 orders of magnitude greater than those of non-viral delivery systems.

Why are viruses so efficient at intracellular delivery of DNA or proteins? Although there are different mechanisms that different viruses use for intracellular trafficking, viruses in general have evolved complex and specific protein coats that act to direct transport of their genetic contents from the endosome into the cytoplasm. The endosome develops one of the few large chemical gradients found in biology through the proton pumping activity of membrane-bound ATP-dependent proton pumps. As a result, the pH of these compartments drops during endosomal development to values of 5.5 or lower. This pH drop serves as a trigger for a conformational change of a peptide in the viral protein coat, leading to its conversion from a hydrophilic coil to a hydrophobic helix. The latter is membrane-active, and may fuse with and/or form pores in the endosomal lipid bilayer membrane, which destabilizes the membrane.

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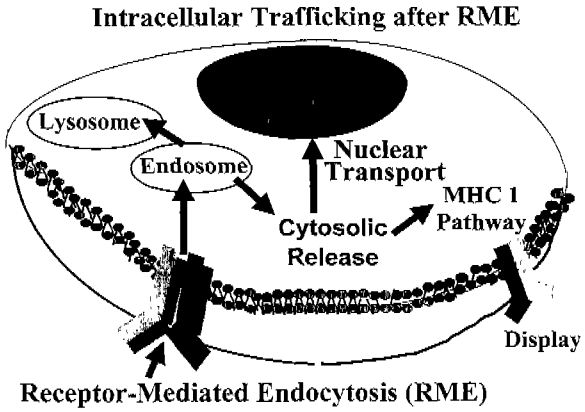


Fig. 1. Schematic of cell showing barriers and pathways involved in intracellular delivery of biomolecular therapeutics and vaccines.

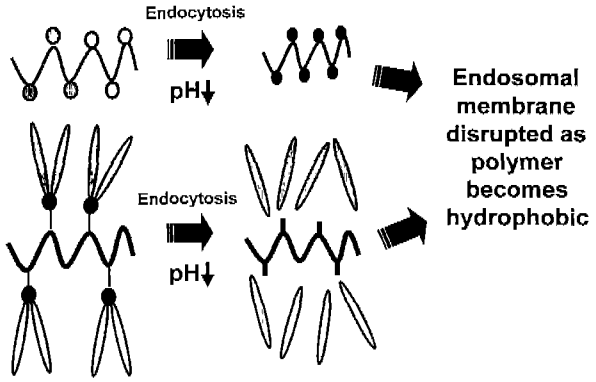


Fig. 2. Schematic of two types of pH-sensitive, membrane-disruptive polymers.

An example of such a peptide is hemagluttinin in the influenza virus protein coat; the mechanism of endosomal release is related to protonation of the carboxylate groups of acidic peptide residues, which, along with the hydrophobic residues also present in the peptide, lead to conversion of the peptide from an anionic, hydrated coil at pH 7.4 to a hydrophobic helical conformation at the lowered pH of the endosome [4-6] .

The use of viral proteins and synthetic biomimetic peptides (designed to exhibit similar activities) as endosomal releasing agents in gene and protein delivery systems has been investigated [7-9]. The GALA peptide family from Szoka and co-workers [10,11] was designed as a consensus sequence to display the key properties of naturally occurring endosomal releasing peptides (EDPs). The GALA repeating sequence contains the glutamic acid carboxylate group, whose average pKa is set by the local environment of alkyl side-chains. GALA peptides of the appropriate length show excellent activity as EDPs, with the desirable pH-dependent properties. While these peptides demonstrate that endosomal disrupting components can significantly increase the efficacy of some carrier systems, they potentially suffer from problems of stability, immunogenicity, and low activity.

Because of these potential problems with synthetic peptide mimics, and also inspired by the principle behind these biological strategies, we have been designing and investigating synthetic polymers that contain similar constituent pH-sensitive chemical functionalities, and testing them as new endosomal releasing components to enhance the intracellular endosomal delivery of biomolecules. These polymeric carrier systems are designed to be like viruses and pathogenic proteins, and to have modular components that possess different functional properties. Ringsdorf first proposed the potential of synthetic polymers to serve as multifunctional delivery vehicles [12].

We have designed two types of polymers that may be utilized to enhance endosomal release of biomolecular drugs. The first polymer backbone contains the combination of carboxylate groups and hydrophobic groups typified by viral peptides and also seen in the synthetic peptide, GALA. The other is based on a different approach, where the backbone is designed to be hydrophobic and membrane disruptive *de novo*, and it is masked by polyethylene glycol (PEG) chains to solubilize it. The PEG chains are conjugated to the backbone via acid labile, acetal linkages; these bonds are degraded at the lowered pH of the endosome, unmasking the backbone, which then disrupts the membrane. These two types of polymers are shown schematically in Fig. 2 Specific compositions will be described below. Both types of polymers may have targeting ligands conjugated to their backbones, and in the second type of polymer, the drug may also be directly conjugated to the backbone via acid degradable bonds, similar to the PEGs. We have also conjugated drugs such as oligodeoxynucleotides (ODNs) and peptides via disulfide bonds to the backbones, since these bonds would be reduced in the cytoplasm, releasing free drug. In summary, we are attempting to synthesize modular polymeric carriers that mimic the important elements of viruses and pathogenic organisms.

Membrane-Disrupting Properties of pH-Responsive Synthetic Polymers: A. Polycarboxylate Polymers and Copolymers

Our design and synthesis of various polycarboxylate polymers was inspired by the pioneering work of Tirrell and coworkers, who first described the pH-dependent disruption of liposomes using poly(ethylacrylic acid) (PEAA). [13,14] PEAA was found to destabilize liposomes at pHs within the range of endosomal acidification (pH 5.5-6.5). These properties suggested that related polymers could serve as endosomal releasing agents with biological membranes. Therefore, we synthesized and investigated similar polycarboxylates, including poly(propylacrylic acid) (PPAA) and poly(butyl-

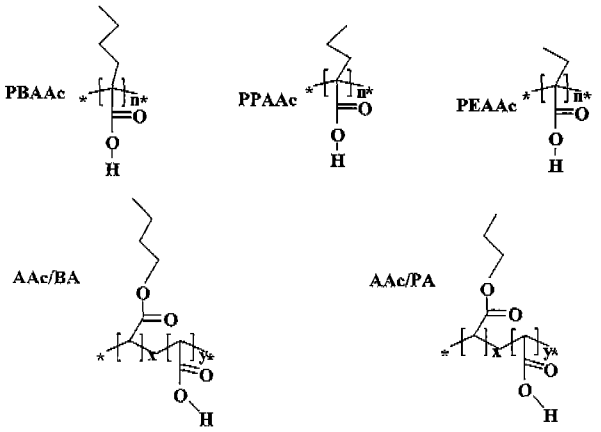


Fig. 3. Chemical compositions of carboxylate polymers.

acrylic acid) (PBAA). In addition, we also designed co-polymers that combined acrylic acid with acrylate ester monomers that contained different alkyl substituents (Fig. 3).

The polymers [15] and co-polymers [16] were initially screened in a red blood cell (RBC) hemolysis assay to determine the concentration dependence and pH profiles for RBC membrane disruption (Fig. 4). PEAA is inactive at physiological pH, and becomes strongly hemolytic as pH drops, with a sharp transition around pH 6.3. PEAA is approximately as active as the membrane disruptive peptide mellitin in this hemolysis assay. We next looked at PPAA, which differs from PEAA by the addition of a single $-CH_2-$ methylene unit. PPAA exhibited a surprising increase over PEAA in hemolytic efficiency at low pH, as well a sharp rise in hemolysis at a higher pH (~6.7-6.8) (Fig. 4). PPAA was approximately an order of magnitude more efficient at hemolysis than PEAA [15]. PEAA can also be activated by ultrasound at concentrations below where it is hemolytic [17]. The addition of another methylene unit with PBAA shifted the pH profile for membrane disruption even further toward physiological pH. The general shift in the pH profiles is consistent with the trend in carboxylate pKs expected with longer and more hydrophobic alkyl groups. Thus, PBAA is potentially cytolytic at physiologic pH (7.4), and could be used to lyse cells at those conditions, or if that is undesirable, BAA could be incorporated in copolymers with more hydrophilic co-monomers.

The concentration dependencies and pH profiles are also dependent on the polymer molecular weight, and it must be noted that the comparisons noted here are for similar molecular weights, usually about 40-60 kDa. In general, as molecular weight increases the profiles are shifted to higher pH transitions, and as molecular weight

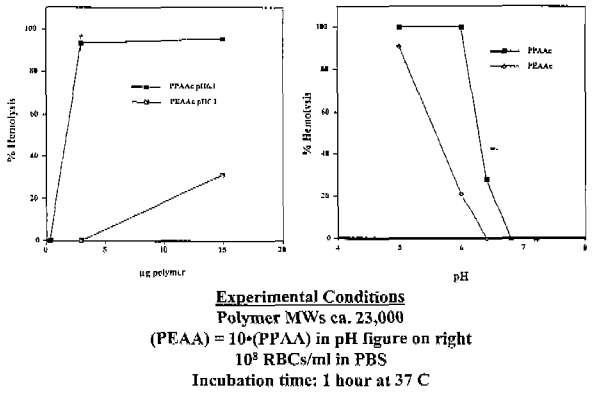


Fig. 4. Red blood cell disrupting (hemolysis) properties of PPAA and PEAA comparing concentration dependences (left) and pH profiles on right [15].

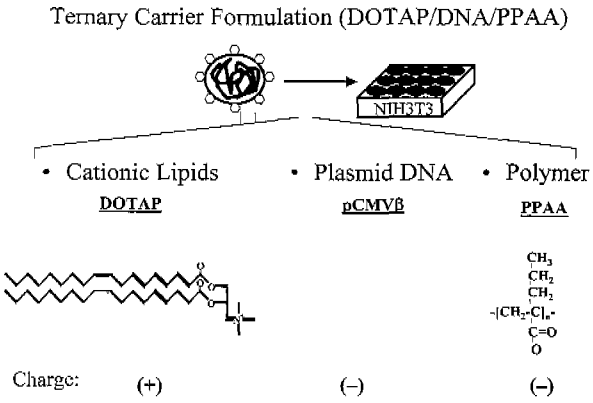


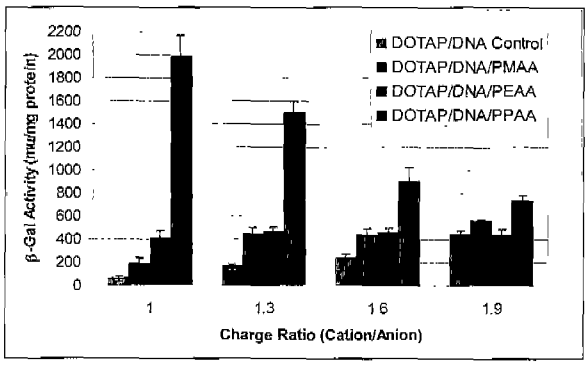
Fig. 5. Schematic of ternary DOTAP/DNA/PPAA gene delivery system for assessing activity of PPAA in NIH 3T3 fibroblast cell culture.

decreases, higher polymer concentrations are required to achieve the same degree of hemolysis.

These results suggested that random 1:1 copolymers of acrylic acid and alkyl acrylates which contain the same carboxylate and pendant alkyl group contents, but in different steric and spatial orientations, might also act as pH-responsive membrane disrupting agents. Random copolymers of acrylic acid with either propyl acrylate or butyl acrylate were synthesized and their membrane disruptive properties were assessed using the hemolysis assays. The extent of hemolysis as a function of concentration of the copolymer was dependent on both the alkyl acrylate monomer and on its relative proportion in the copolymer. Although all of the co-polymers displayed the ability to lyse RBC at lowered pHs, none of them was as effective as PPAA [16].

Enhancement of Gene Transfection with PPAA

These hemolysis studies suggested that PPAA was particularly membrane active at acidic pHs, and might



Polymer MW's: PMAA (52kD), PEAA (43kD), PPAA (61kD)

Fig. 6. β-galactosidase transfection levels for ternary complexes containing PMAA, PEAA, and PPAA versus the control binary DOTAP/Plasmid alone.

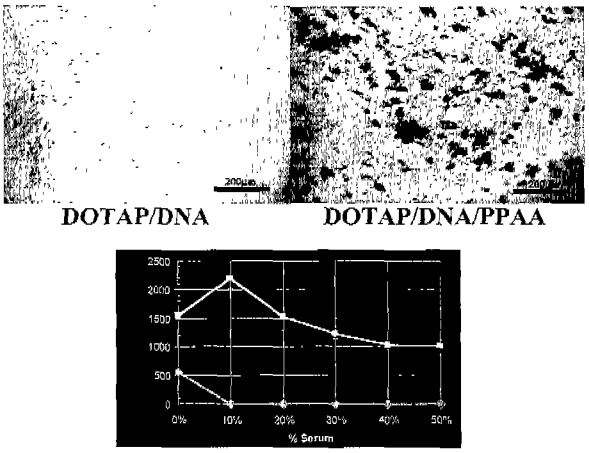


Fig. 7. Effect of serum on gene transfection by DOTAP/DNA with or without added PPAA. The upper panels represent the staining of adherent NIH 3T3 fibroblast cells for β-galactoside activity in 10% serum using the same formulation of Fig. 5. The bottom panel compares the transfection efficiencies of the DOTAP/DNA/PPAA formulations (top curve) to the control DOTAP/DNA (bottom curve) as a function of increasing serum levels (NOTE: The units of transfection on the y-axis of Fig. 7 are the same as those of transfection on the y-axis of Fig. 6).

serve as an efficient endosomal releasing agent. We first tested whether PPAA could enhance gene transfection in a model lipoplex delivery system using NIH 3T3 fibroblast cells in culture. (Fig. 5) Ternary particles of the cationic lipid dioleyl-trimethylammonium-propane (DOTAP), the pCMV (cytomegalovirus promoter) β-galactosidase reporter plasmid, and PPAA were formulated at a variety of solution charge ratios. In these lipoplexes, the PPAA was designed to serve in analogous fashion to the fusogenic peptides that have previously been physically incorporated into gene delivery systems. The resultant β-galactosidase activities were subsequently measured after transfection of the fibroblast cells with the DOTAP/DNA/PPAA and compared to a control of DOTAP/DNA alone. (Fig. 6) (C.C. Cheung, manuscript submitted) The PPAA-containing lipoplex formulations displayed a significant increase in transfection efficiencies. Transfections with equivalent amounts of added poly(methacrylic acid) (PMAA) or PEAA, both of comparable molecular weights to PPAA, showed much smaller or no increases in gene expression over the control, when compared to PPAA, implying that the greater hydrophobicity of PPAA is essential for transfection enhancement.

Cationic lipoplexes are often inactivated by interactions with serum proteins [18], and so the ability of PPAA to increase the serum stability of DOTAP/DNA lipoplexes was investigated. The DOTAP/DNA lipoplex was inactivated in media containing as little as 10% FBS, while the DOTAP/DNA/PPAA particles exhibited high levels of transfection throughout the entire range of serum levels tested. (Fig. 7) Nearly 70% of the transfection levels were preserved with DOTAP/DNA/PPAA particles at 50% FBS. Histological staining of the fibroblast cells for β-galactosidase activity confirmed the absence of transfection with DOTAP/DNA alone, while a significant number of stained cells were evident after transfection with DOTAP/DNA/PPAA (Fig. 7).

These results in cell culture suggested that the PPAA could provide significant transfection enhancement and serum stability for *in vivo* applications. Therefore, we carried out initial animal studies using local injection delivery in a mouse model of wound healing. This model is based on previous studies that have demonstrated that excisional wound healing is accelerated in thrombospondin 2 (TSP2)-null knockout animals [19]. Without TSP2, the excisional wounds exhibit an irregular deposition of extracellular matrix and enhanced vascularization that is associated with significantly accelerated wound healing. These results with the knockout mouse model suggested that delivery of a plasmid encoding an antisense oligonucleotide (cDNA) to inhibit TSP2 expression could enhance healing in the wild-type mouse [20]. In addition, delivery of a plasmid encoding TSP2 to the knockout mouse should reverse the TSP2-null phenotype and retard wound healing. This experiment is shown schematically in Fig. 8.

The deposition of TSP2 in WT mouse wounds was found to be absent during the early inflammatory phase, with peaking on day 10, coinciding with the period of maximal vascular regression [21]. In one protocol, wounds were injected with DOTAP/DNA/PPAA formulations on days 4, 8, and 12, with evaluation of the wounds at day 14 after sacrifice. In the TSP2-null mice, the DOTAP/DNA/PPAA formulations resulted in significantly higher TSP2 expression compared to DOTAP/DNA controls [20].

In the WT mice, the PPAA-containing lipoplexes with the antisense-encoding plasmid resulted in significantly enhanced disorganization of the wound extracellular matrix, resembling that seen with the TSP2-null wound

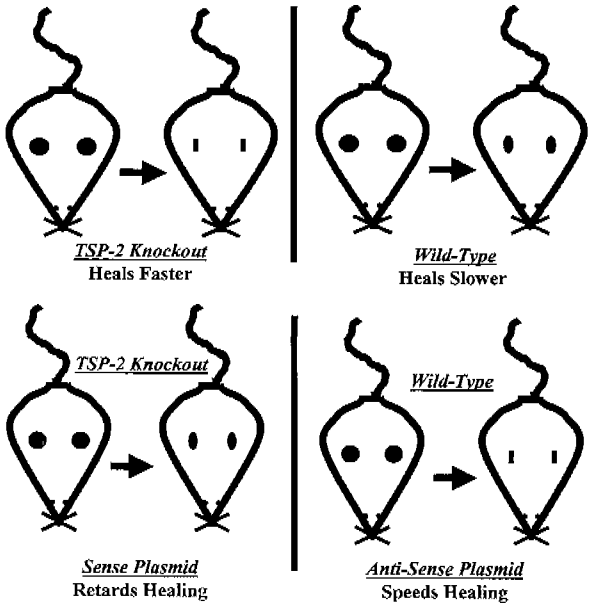


Fig. 8. Schematic of the mouse wound model used to test the *in vivo* transfection activity of DOTAP/DNA with added PPAA.

healing process. Immunohistochemical analysis of wound sections for the endothelial cell marker PECAM1 demonstrated that PPAA addition to the lipoplexes also resulted in significantly greater vascularization, again similar to that seen in the TSP2-null wounds. Taken together, the experiments with the knockout and WT mice demonstrate that inclusion of PPAA in the lipoplex plasmid formulations greatly enhanced *in vivo* transfection and resulted in the localized modulation of the wound healing response [20]. These remarkable results show that PPAA is effective *in vivo* in a simple physical mixture with the lipoplex DNA delivery system.

Intracellular Delivery of Proteins with PPAA

There are many other biomolecular therapeutics in development that require intracellular delivery, especially protein therapeutics and vaccines. Therefore, we investigated whether PPAA could also enhance the delivery of proteins to the cytoplasm (C. A. Lackey *et al.*, manuscript submitted). The immunotoxins represent a good model therapeutic system where drug action requires escape from endosomes to the cytoplasm. In initial studies, we asked the question whether PPAA can enhance the cytoplasmic delivery of an immunoconjugate that utilizes a targeting antibody. MoAb 64.1 is an anti-CD3 antibody that has been extensively characterized as a T-cell lymphoma targeting agent [22]. MoAb 64.1 is rapidly localized to the lysosome after receptor-mediated endocytosis [23], and thus serves as an excellent model for endosomal release. (Fig. 9)

Rather than conjugate PPAA directly to the antibody, we biotinylated both the antibody, MoAb 64.1, and PPAA, and complexed them in sequence to streptavidin (SA), which is used as the linker. (Fig. 10) Because of the very high affinity of biotin to SA, the complexcan can be considered as a conjugate for the duration of the experiments. PPAA did not alter the uptake of the MoAb 64.1-SA complex into the Jurkat T-cell lymphoma cell, as demonstrated by fluorescence-activated cell sorting (FACS) analysis. The trafficking fates of the antibody-SA complexes were initially characterized by laser scanning confocal microscopy using fluorescein-labeled SA. Without the PPAA, the antibody-SA complex was localized in vesicular compartments (*e.g.*, endosome/lysosome) as seen by punctate fluorescence emission patterns. In contrast, a broadly diffuse fluorescence emission was observed in the cytoplasm of the Jurkat cells after a four hour incubation of the cells with the PPAA-containing complexes. This diffuse fluorescence indicated that the polymer aids escape of the SA and its complexed, biotinylated MoAb 64.1 and PPAA to the cytoplasm. Because the nucleus becomes visible as a darkened region after the appearance of the diffuse cytoplasmic fluorescence, it is likely the fluorophore is still attached to SA (which is too big to diffuse into the nucleus).

More direct confirmation of the endosomal releasing

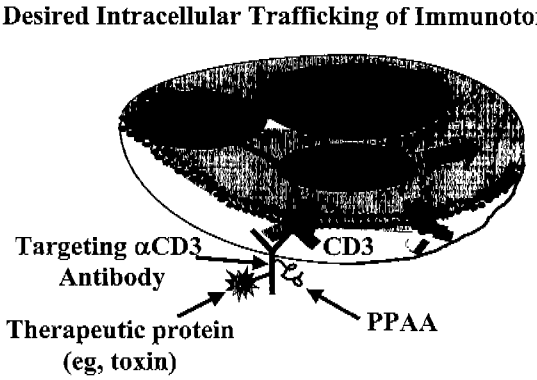


Fig. 9. Schematic illustration of the anti-CD3 delivery system for the Jurkat T-cell lympoma model that tests the ability of PPAA to enhance the cytoplasmic delivery of the antibody-targeted immuno-conjugate.

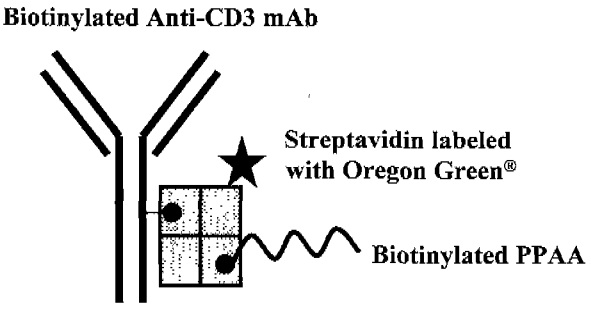


Fig. 10. Schematic model of the antibody-streptavidin-PPAA bioconjugate that was used to test the activity of PPAA in enhancing the endosomal release of therapeutic antibodies.

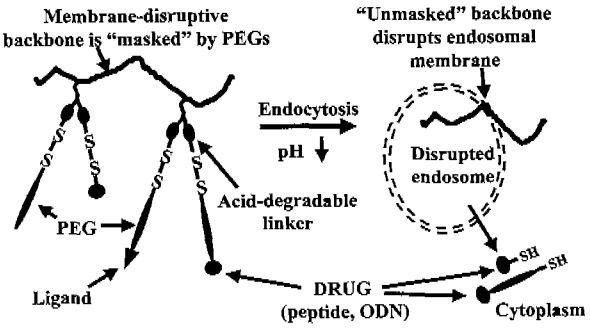


Fig. 11. Schematic illustration of the "encrypted" polymers that carry biomolecular therapeutics and cell targeting molecules via pH-degradable bonds. The backbone is membrane disruptive when unmasked by the hydrolysis of PEGylated grafts that releases the therapeutics to the cytosol.

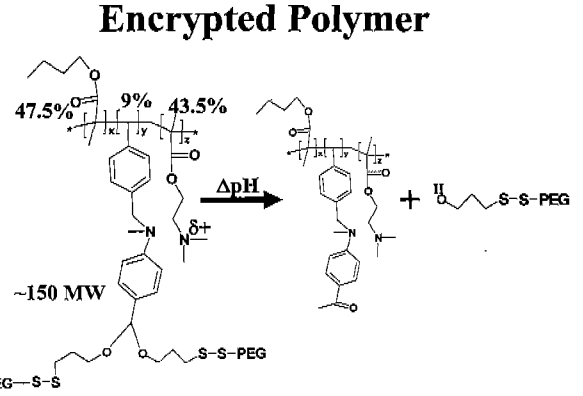


Fig. 12. Chemical composition of the first generation of encrypted polymers.

activity of PPAA was obtained by western blotting analysis after Jurkat cell fractionation, which showed that there was no detectable amount of the antibody released into the cytoplasm in the absence of the polymer, but that it was clearly taken up into the cells as evidenced by the intact antibody band in the total cell homogenate. When biotinylated PPAA was also complexed to the MoAb 64.1-SA complex, the cytoplasmic band displayed 73% of the staining intensity of the crude homogenate band. When free PPAA (non-biotinylated) was physically added to the MoAb 64.1-SA complex, it also displayed significant activity, with 29% of the total intracellular staining intensity observed in the cytoplasmic fraction. The western blotting analysis also verified that the protein detected in the cytoplasm had not been degraded, as the molecular weight of the cytoplasmic band was identical to that detected in the crude homogenate. These results suggest that endosomal-releasing polymers such as PPAA could be applicable to a wide range of protein/peptide intracellular delivery applications, including both direct protein drug delivery and vaccine development.

Membrane-Disrupting Properties of pH-Responsive Synthetic Polymers: B. "Encrypted" Polymers with Acid-degradable Side Chains

The interesting properties of the carboxylate polymers described above suggested that other designs could also incorporate pH-responsive elements that provide membrane disruptive capabilities at the low pHs of the endosome. With this in mind, we have developed a new class of polymers composed of a membrane disruptive backbone which is masked by poly(ethylene glycol) (PEG) chains that are grafted to the backbone by acid-degradable linkages, such as acetal bonds. Thus, in the low pH environment of the endosome, the backbone will be unmasked and then disrupt the endosomal membrane. In addition, a variety of drugs such as peptides and ODNs, as well as targeting ligands may also be linked to the backbone via similar bonds. They are similarly released in the acidic environment of the endosome. In some cases, they may also be released by disulfide bond reduction in the cytoplasm (Fig. 11).

These polymers are designed as self-contained carriers that incorporate the primary functionalities of viruses and toxins: targeting agents that direct receptor-mediated endocytosis, a pH-responsive element that selectively disrupts the endosomal membrane, and the biomolecular component which is delivered as a free and active agent into the cytoplasm. We have termed these polymers "encrypted" by analogy to encrypted domains in biological proteins. Active domains of several extracellular and matricellular proteins are initially masked but become exposed and activated by proteolytic processing at controlled timepoints [24]. Similarly, the encrypted polymers contain a masked membrane-disruptive element that is activated in the low pH environment of the endosome.

The first example of an encrypted polymer that we synthesized is the terpolymer of dimethylaminoethyl methacrylate (DMAEMA), butyl methacrylate (BMA) and styrene benzaldehyde (SBA). (Fig. 12) The DMAEMA/BMA backbone was chosen as a membrane-disruptive component on the basis of our previous work with copolymers discussed above, and the SBA was incorporated to provide sites for generation of acetal linkages. The PEG grafts were attached to the SBA unit of the backbone through acid-degradable acetal linkers. The acetal linkages degrade at rates that are proportional to the hydronium ion concentration, and therefore will hydrolyze 250 times faster at pH 5.0 than at pH 7.4. The rate constants can also be controlled over a wide range of time scales to optimize the degradation properties for specific delivery requirements [25]. The PEG grafts were chosen because of their well-characterized biocompatibility, and their ability to improve stability and biodistribution properties by maximizing circulation times.

The hydrolysis of PEG grafts from the terpolymer shown in Fig. 12 exhibited a half-life of 15 min at pH

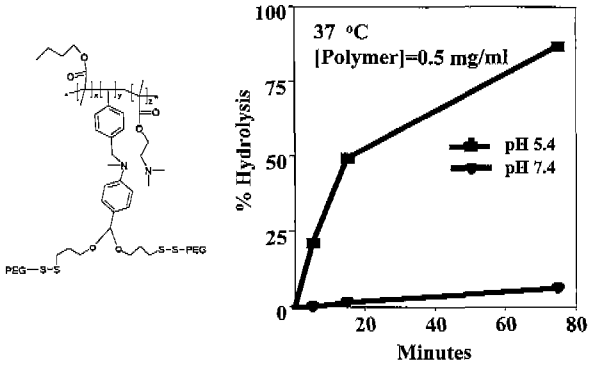


Fig. 13. Hydrolysis of the PEG grafts from the encrypted polymer as a function of pH.

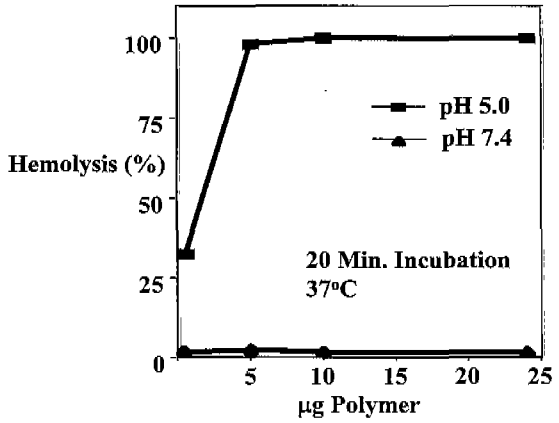


Fig. 14. Hemolytic activities of the encrypted polymers before and after hydrolysis of the PEG grafts.

5.4, with only 38% of the PEG grafts hydrolyzed after 12 h at pH 7.4 (Fig. 13). These kinetics match the general time frame for trafficking of internalized biomolecules to the lysosome. The hydrolysis properties also correlated with the hemolysis properties, with ca. 1 μg/mL of this polymer achieving 100% hemolysis of 10^8 red blood cells after a 20 min incubation at pH 5.0. (Fig. 14) No hemolysis was observed after incubation at pH 7.4. We then investigated whether the encrypted polymer could enhance the cytoplasmic delivery of oligonucleotides to hepatocytes in cell culture. We conjugated lactose to the terminal end of a small percent of the PEG grafts, for targeting of the asialoglycoprotein (ASGP) receptor. This receptor is known to rapidly direct lysosomal localization, and thus represents a challenging model system for determining if our polymer can induce escape from this trafficking fate.

Hexalysine was linked to approximately 20% of the PEG grafts, which then was used to ionically complex the rhodamine-labeled, negatively-charged oligonucleotides. After a three hour incubation of this carrier with hepatocytes, a striking localization of the rhodamine fluorescence was observed in the cell nuclei. In contrast, hepatocytes incubated with the oligonucleotides alone exhibited a punctate vesicular localization. These results demonstrate that the encrypted polymer enhances escape of lysosomal trafficking, and results in cytoplasmic delivery of the oligonucleotides (where they can subsequently diffuse rapidly to the nucleus). These results suggest that our new polymers could provide significant enhancement of antisense ODN therapeutic delivery. They are also well suited to carry peptides and proteins for vaccine delivery, through the versatile linkage chemistries available with this platform design.

CONCLUSION

We have designed, synthesized and tested a family of novel pH-sensitive polymers that show great promise for enhancing both the *in vitro* and *in vivo* intracellular delivery of important biomolecular drugs such as DNA plasmids, antisense oligonucleotides, peptides and proteins.

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