pH-Sensitive Hemolysis by Random Copolymers of Alkyl Acrylates and Acrylic Acid

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SUMMARY: We have been designing and synthesizing synthetic polymers that mimic viral fusogenic peptides, which contain peptide residues having alkyl groups and carboxyl groups. We have synthesized two different types of such polymers, and their abilities to hemolyse red blood cells at pH 7.4 and 5.5 are compared here. The polymers are poly(2-alkylacrylic acid)s such as poly(2-propylacrylic acid), and random copolymers of poly(alkyl acrylate-*co*-acrylic acid) where the alkyl group is propyl or butyl. We have found that the poly(2-alkylacrylic acid)s such as poly(2-propylacrylic acid) are significantly more hemolytic at acidic pH than the random copolymers of equivalent propyl and carboxyl contents.

Introduction

At present there is considerable interest in the development of non-viral formulations that can enhance the intracellular delivery from the endosome to the cytoplasm of fragile biological molecular drugs such as DNA, oligonucleotides, peptides and proteins that would otherwise be trafficked to lysosomes and degraded. Endosomes and lysosomes exist at acidic pH between 5.0-6.0, in contrast to the cytoplasm of a cell, which is at pH 7.4. Some viruses, such as the influenza virus and the adenovirus, are very efficient at delivering their genetic material from the endosome into the cytoplasm by use of pH-sensitive membrane fusogenic peptides on their surfaces1). In order to avoid the potential toxicity of viral vectors in gene therapy, endosomal disruptive peptides have been designed and synthesized to mimic viral fusogenic peptides. One example is GALA or EALA, which has a repeat sequence of one carboxyl-containing amino acid followed by three hydrophobic amino acids2). Such peptides are converted from anionic, hydrated coils to hydrophobic helical forms at the lowered pH inside the endosome, and may then fuse with or otherwise disrupt the lipid bilayer membrane, leading to release of the endosomal contents to the cytosol. However, use of such peptide mimics is limited by their potential immunogenicity coupled with their high costs of synthesis and production. To avoid these disadvantages, we have been designing and synthesizing synthetic polymers that also mimic viral fusogenic peptides in that they also contain both alkyl groups and carboxyl groups. One such example is a family of polymers called poly(2-

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alkylacrylic acid)s, where the alkyl group may be ethyl (PEAA), propyl (PPAA) or butyl (PBAA). We have shown that the first two, in particular PPAA, can significantly enhance the disruption of red cell membranes at pHs characteristic of the endosome3). We have also recently found that PPAA can significantly enhance the transfection of cells in culture4), and in vivo in a mouse model5).

A common molecular characteristic of our endosomal disruptive polymers, such as PPAA and PEAA, is the presence of short hydrophobic groups and carboxyl groups, regularly spaced along the backbone of the polymer (Fig. 1).

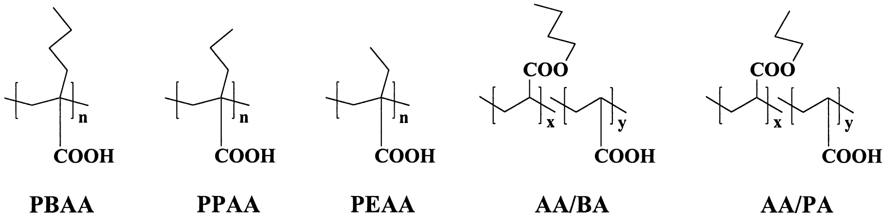

Figure 1: Endosomal disruptive polymers and copolymers.

This molecular combination leads to pH-induced membrane disruption because at pH 7.4 the polymer carboxyls are mostly ionized, causing the polymer to be too hydrophilic to partition into lipid bilayers. However, at acidic pH, the polymer carboxyls are significantly protonated and therefore sufficiently hydrophobic to partition into lipid bilayers and induce membrane disruption. (We do not believe that the synthetic polymer becomes helical at the lower pH of the endosome, as do the synthetic peptide mimics). The length of the hydrophobic moiety in the poly(alkylacrylic acid) is a critical parameter. The hydrophobic groups have to be of sufficient length to penetrate into the lipid bilayer. However, the interaction between the hydrophobic alkyl groups and the lipid bilayer has to be weak enough, such that when the polymer carboxyls are ionized at pH 7.4, it does not penetrate into lipid bilayers.

A key question regarding the design and synthesis of pH-sensitive membrane-disruptive polymers is how the spatial arrangement between the carboxyls and the hydrophobic alkyl moiety affects the pH sensitivity of membrane disruption. In the case of the poly(2-alkylacrylic acid)s, the carboxyl groups and alkyl groups are both linked to the same carbon atom, which repeats regularly along the backbone of the polymer chain. To determine if this special structural feature plays a critical role in the action of such polymers at lowered pH, we have synthesized copolymers in which the carboxyl groups and hydrophobic groups are located randomly along the backbone of the polymer and are not linked to the same carbon atom. We have done this by copolymerizing a hydrophobic alkyl acrylate monomer with a pH-sensitive carboxyl-containing monomer (acrylic acid). Acrylic acid (AA), butyl acrylate (BA) and propyl acrylate (PA) were chosen as the monomers because of their similarity to the composition of the endosomal disruptive polymer PPAA (carboxyl and propyl groups) and to the synthetic fusogenic peptide mimic, GALA, which has one carboxyl group along with two methyls and one isobutyl group. The random copolymers also have certain advantages over the poly(2-alkylacrylic acid)s such as PPAA in that they are based on commercially available monomers, and their compositions can be fine-tuned to be pH-responsive at specified pH.

Materials and Methods

Materials: The monomers, acrylic acid, butyl acrylate, and propyl acrylate used in this study were purchased from Aldrich Chemicals and distilled under vacuum prior to use. AIBN was purchased from Aldrich Chemicals and crystallized from methanol prior to use.

Monomer Synthesis: The monomers, 2-propylacrylic acid and 2-butylacrylic acid, were synthesized according to a previously reported procedure3).

Polymer Synthesis: The homopolymers and copolymers were synthesized through radical polymerization; the synthetic procedure is outlined briefly here. Appropriate amounts of the monomer solutions and 2 mole % AIBN with respect to the total monomer content were placed into a round-bottom flask, which was then fitted with a stopcock and stir bar. The polymerization mixture was then degassed by freezing it with liquid nitrogen and then applying vacuum; this was performed three times. The degassed solution was heated to 60 ^{o}C in an oil bath for 12 h, the resulting polymer was then precipitated in ether, isolated by suction filtration and then vacuum-dried overnight. ^{1}H NMR was performed on the purified polymers in deuterated DMSO, to ensure that the polymers were free of monomer contamination.

Hemolysis Assay: The hemolysis assay was performed according to the procedure previously described3). The copolymers were solubilized by, first, wetting them with an equal mass of triethylamine and then adding 1M NaOH. The alkaline polymer solutions were then added to red blood cells suspended in a buffered solution at either pH 5.5 or 7.4.

Results and Discussion

Two families of random copolymers and two homopolymers were synthesized, these are copolymers of butyl acrylate (BA) and acrylic acid (AA), copolymers of propyl acrylate (PA) and AA, PBAA and PPAA. The ability of these homopolymers and copolymers to act as endosomal disruptive agents was determined by measuring their ability to disrupt red blood cell membranes at pH 5.5 and 7.4. Red blood cells were used as the model system because of the excellent correlation observed between the ability of fusogenic peptides to disrupt red blood cells and also to disrupt endosomes in cell culture$^{1)}$.

Three different copolymers of BA and AA were synthesized using the AA/BA ratios 50/50, 75/25, 90/10. Figure 2a is a plot of the hemolytic activity of these copolymers. It demonstrates that random copolymers of BA and AA are effective membrane-disruptive agents. At pH 5.5, 10 μg/ml of the 50/50 AA/BA copolymer causes hemolysis of approximately 60 % of 10^8 RBCs. Under these conditions, this copolymer is comparable in hemolytic activity to the membrane-disruptive peptide mellitin at ca. 5 μg/ml, or the endosomal disruptive polymer PPAA at ca. 3 μg/ml. Importantly, at pH 7.4 and a concentration of ca. 10 μg/ml, the 50/50 copolymer only disrupts ca. 8 % of 10^8 red blood cells (see Fig. 2b). Thus the membrane-disruptive activity of this copolymer increases by a factor of 15 in going from pH 7.4 to 5.5.

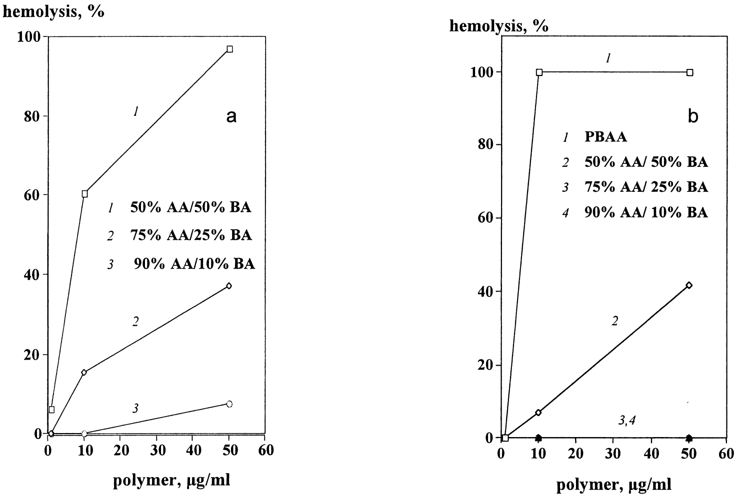

Figure 2: Effect of concentration of AA/BA random copolymers on red blood cell hemolysis at pH (a) 5.5, (b) 7.4. (10^8 cells/ml, incubation time 1 h, 37 °C, experiments in triplicate.)

The membrane-disruptive activity as well as the pH sensitivity of these copolymers are critically dependent on the AA/BA ratio. At pH 5.5 and ca. 50 μg/ml, the 75/25 copolymer is about one third as effective as the 50/50 copolymer, and the 90/10 copolymer is about one quarter as effective as the 75/25 copolymer (see Fig. 2a). However, Fig. 2b demonstrates that at pH 7.4, neither the 75/25 nor the 90/10 AA/BA copolymers show any membrane-disruptive activity at 50 μg/ml, whereas the 50/50 copolymer shows 45% hemolysis at those conditions. Thus, it appears that the membrane-disruptive activities of AA/BA copolymers are directly dependent on their hydrophobicity, since increasing the BA content (and decreasing the AA content) increases the membrane-disruptive activities both at pH 5.5 and 7.4. Thus by altering the AA/BA ratio, it is possible to make membrane-disruptive polymers which are active only at pH 5.5 but not at pH 7.4.

The homopolymer PBAA is significantly more effective in membrane disruption than any of the AA/BA random copolymers at pH 7.4 (see Fig. 2b). PBAA is insoluble at pH 5.5 and has no membrane-disruptive activity at this pH. The AA/BA random copolymers at pH 5.5 have comparable membrane-disruptive activity to PBAA at pH 7.4. The 50/50 AA/BA random copolymer has a similar hydrophilic/hydrophobic content to PBAA, yet the 50/50 random copolymer requires a significantly lower pH to achieve its maximum membrane-disruptive activity in comparison to PBAA. PBAA has its hydrophobic butyl groups regularly spaced, in contrast to the 50/50 copolymer which presumably has its butyl groups randomly interspersed with carboxyl groups along the polymer backbone, and thus has shorter sequences of regular hydrophobic butyl groups in comparison to PBAA. This difference in the length of the consecutive hydrophobic sequences could lead to the different membrane-disruptive behaviors of PBAA vs. the 50/50 AA/BA random copolymer.

The ability of copolymers of AA and PA to disrupt red blood cell membranes was also investigated at pH 7.4 and pH 5.5. Figure 3a demonstrates that copolymers of different AA/PA monomer feed ratios can act as effective membrane-disruptive agents; at pH 5.5, the 50/50, 40/60 and 60/40 copolymers are all capable of causing hemolysis. However, these copolymers are in general less effective than the BA/AA copolymers at comparable AA contents and the PPAA homopolymer. At 10 μg/ml concentration, the 50/50 AA/PA copolymer causes only ca. 25% hemolysis of 10^8 red blood cells, whereas the 50/50 AA/BA copolymer causes ca. 60% hemolysis, and PPAA causes 100% hemolysis at this pH and concentration. PPAA is significantly more effective in membrane disruption than the 50/50 copolymer of AA/PA even though both polymers have a very similar hydrophilic/hydrophobic balance. Similar to the AA/BA copolymer vs. the PBAA homopolymer, this difference in membrane-disruptive activity could be due to regularly arranged hydrophobic propyl groups in PPAA vs. the 50/50 AA/PA copolymer, which presumably has its hydrophobic propyl groups randomly interspersed with carboxyl groups along the polymer backbone.

The AA/PA copolymers also show pH-sensitive membrane disruption. At 50 μg/ml concentration and pH 7.4, the 50/50 copolymer causes ca. 25% hemolysis (see Fig. 3b) whereas at pH 5.5, it causes ca. 52% hemolysis. The AA/PA copolymers, like the AA/BA copolymers, exhibit pH-sensitive membrane disruption, but are not as effective and are also not as pH-responsive as the AA/BA copolymers.

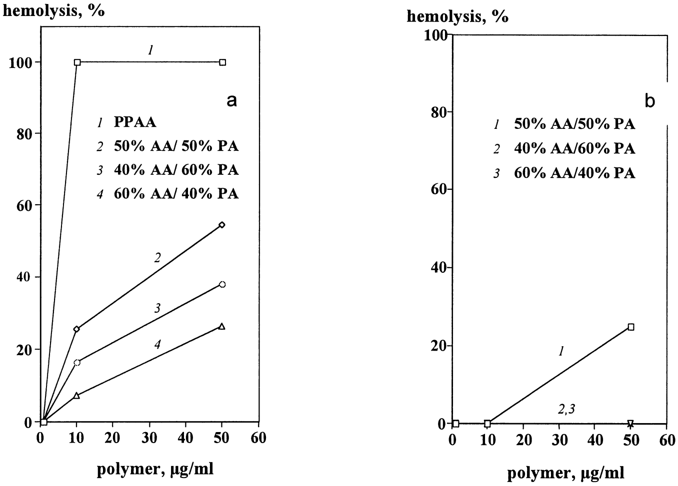

Figure 3: Effect of concentration of AA/PA random copolymers on red blood cell hemolysis at pH (a) 5.5, (b) 7.4. (10^8 cells/ml, incubation time 1 h, 37 °C, experiments in triplicate; for other conditions, see Fig. 2.)

Conclusions

In this report we demonstrate that random copolymers of alkyl acrylates and acrylic acid can act as pH-sensitive membrane-disruptive agents, possessing hemolytic activities at acidic pH in a similar way to homopolymers such as PPAA and peptides such as mellitin. Although the random copolymers are not as effective as the homopolymers of 2-alkylacrylic acids, the monomers used in the synthesis of the random copolymers are commercially available, while the 2-alkylacrylic monomers must be synthesized *de novo*. Thus, the random copolymerization of commercially available monomers has several advantages: (1) their synthesis is convenient and inexpensive, (2) they can be easily functionalized with end groups other than COOH through copolymerization with functionalized chain transfer agents, (3) in contrast to the 2-alkylacrylic acid monomers, acrylate monomers may be polymerized by group transfer and atom transfer polymerization and, therefore, these random copolymers can be prepared with precise control of molecular weight and molecular architecture, (4) the hydrophilic/hydrophobic balance and, therefore, the pH sensitivity to membrane disruption can be easily manipulated by simply changing the AA/alkyl acrylate ratio or the nature of the alkyl acrylate comonomer in these copolymers. Future work will focus on formulating these copolymers in drug delivery systems for DNA and oligonucleotide delivery.

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