Antimicrobial Polymers

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Antibacterial and Hemolytic Activities of Pyridinium Polymers as a Function of the Spatial Relationship between the Positive Charge and the Pendant Alkyl Tail**

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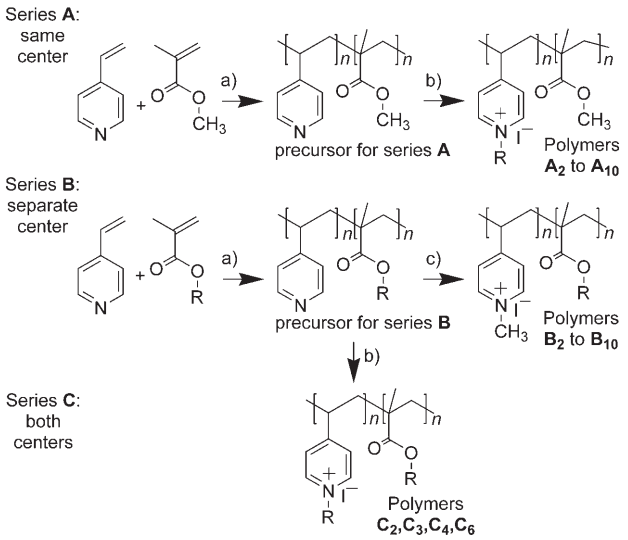
Synthetic amphiphilic polymers that mimic the membrane-disrupting properties of natural antimicrobial peptides[1] show potent biocidal activity towards bacteria,[2–4] fungi,[5] and viruses.[6] Their easy synthesis, stability towards enzymatic degradation, and facile chemical tailoring make them promising candidates as novel chemical disinfectants and non-leaching biocides for a variety of biomedical applications. However, there is a delicate balance between useful biocidal activity and detrimental toxicity towards mammalian cells. Herein, we report the interplay between the chemical structure and antibacterial versus hemolytic properties of amphiphilic pyridinium polymers.

The membrane-disrupting activity of amphiphilic polymers is mainly dependent on the charge and hydrophobicity, which have to be optimized to cause membrane disruption.[4,7,8] Structure–activity relationships have previously been reported on the effect of the length of the alkyl tail, an increase in the positive charge, and the overall hydrophobicity/hydrophilicity of the polymer on the membrane-disrupting activity.[7–14] All these variables also influence the balance between the antimicrobial and the hemolytic (toxicity) properties of the amphiphilic polymers. An important, yet unexplored, question is how the activity of an amphiphilic polycation varies as a function of the spatial positioning of the positive charge and the hydrophobic alkyl tail. For example, would the antibacterial and hemolytic activity of a polycation be any different if the positive charge and the alkyl tail were on the same center, as opposed to being spatially separated? We address this and related questions by comparing series of homologous amphiphilic pyridinium polymers that differ only in how the positive charge and the alkyl tail are spatially related. We observe that the spatial positioning of the charge and tail significantly influences the toxicity of these polymers, and this result may be used as a guiding principle in the design of polymeric antimicrobial compounds with reduced toxicity.

Two series (**A** and **B**) of amphiphilic pyridinium–methacrylate copolymers differing only in the spatial positioning of the positive charge and the alkyl tail were synthesized as shown in Scheme 1. Series **A** consisted of pyridinium–methacrylate copolymers in which the positive charge and the alkyl tail are on the same center. A copolymer consisting of approximately 50 mol% vinylpyridine (VP) units and about 50 mol% methyl methacrylate (MMA) units was synthesized by radical polymerization. All the pyridine units were then completely N alkylated by heating with an excess of the respective *n*-iodoalkane. This process yielded six cationic polymers in series **A**, that is, $\mathbf{A_2}$, $\mathbf{A_3}$, $\mathbf{A_4}$, $\mathbf{A_6}$, $\mathbf{A_8}$, and $\mathbf{A_{10}}$ with alkyl tails that were 2, 3, 4, 6, 8, and 10 carbon atoms long, respectively. Series **B** consisted of vinylpyridinium–alkyl methacrylate copolymers in which the positive charge and the alkyl tail are on separate centers. Copolymers of VP with different *n*-alkyl methacrylates were prepared by radical polymerization. The mole percentage of VP and each alkyl methacrylate in the copolymers was also tailored to be approximately 50% each by adjusting the feed ratios of the starting monomers. A positive charge was then introduced on



Scheme 1. Synthesis of three series of pyridinium–methacrylate copolymers differing in the spatial positioning of the charge and tail as well as the length of the alkyl tail. Conditions: a) 1:50 molar ratio of azobisisobutyronitrile (AIBN), 65 °C in MeOH and/or $CHCl_3$, ca. 4–5 h; b) *n*-iodoalkane (RI: R = ethyl, propyl, butyl, hexyl, octyl, decyl), 65 °C in CH_3NO_2, 24 h; c) CH_3I, RT in CH_3NO_2, 24 h. Subscripts denote the number of carbon atoms in the alkyl tail R.

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all the pyridine units by methylating with excess iodomethane. This reaction yielded the six cationic polymers in series **B**, that is, $\mathbf{B_2}$, $\mathbf{B_3}$, $\mathbf{B_4}$, $\mathbf{B_6}$, $\mathbf{B_8}$, and $\mathbf{B_{10}}$ with alkyl tails of different lengths. In this series, the positive charge (on the pyridinium unit) is spatially separate from the alkyl tail (on the methacrylate unit). The molecular weights of the precursor polymers for series **A** and **B**, as determined by gel-permeation chromatography, were similar ($M_n = 27\,000$–$33\,000\ g\,mol^{-1}$). To better understand the spatial separation effect, a third polymer series **C** having the same pendant alkyl tail on both the positive center as well as on a separate center was prepared by N alkylating the precursor polymers for series **B** with an excess of the respective *n*-iodoalkane (Scheme 1).

The polymers in series **A** and **B** can be characterized by two quantities: backbone ratio (BR, the ratio between the number of moles of pyridinium/moles of methacrylate) and amphiphilicity ratio (AR, the ratio between the total number of moles of positive charge/moles of alkyl tails). Since the antibacterial activity of the polymers can be expected to intimately depend on both the structure (BR) and the positive charge (AR), the polymers being compared should have the similar values so as to obtain any meaningful comparison. Polymers in series **A** and **B** have similar BR and AR values of about 1. Thus, any difference between the antibacterial properties of these polymers ($\mathbf{A_2}$ versus $\mathbf{B_2}$; $\mathbf{A_3}$ versus $\mathbf{B_3}$) could be attributed purely to the spatial positioning of the charge and tail. Polymers from series **A**, **B**, and **C** were tested for their antibacterial activities towards Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis*, and their hemolytic activity towards human red blood cells (RBCs) by using the minimum inhibitory concentration method and HC_{50} method, respectively (see the Supporting Information). A lower MIC or HC_{50} value indicates a more potent membrane-disrupting polymer. The MIC and HC_{50} values of polymers from the three series are plotted in Figure 1. The MIC values for series **A** and **B** (Figure 1a, b) decreased and then increased as the tail length increased from C_2 to C_{10}, with the minima occurring at C_4 for the *E. coli* and C_6 for the *B. subtilis* experiments. This observation was consistent with findings by other researchers that suggested that an optimum tail length (generally medium-sized tails of C_3 to C_8) is required to cause membrane disruption.[7,8] It was observed that polymers from series **B** (separate centers) had lower MIC values (more potent in killing *E. coli*) than polymers from series **A** (same center). This trend was observed for all lengths of the alkyl tails, but was most pronounced for polymers with C_4 tails against *E. coli* and polymers with C_8 tails against *B. subtilis*. The hemolytic activity (HC_{50} values) increased with an increase in the tail length from C_2 to C_8 (Figure 1c), with polymer $\mathbf{B_8}$ being the most hemolytic (HC_{50} value of 0.11(0.08) $\mu g\,mL^{-1}$) in the two series (The HC_{50} values of $\mathbf{B_4}$, $\mathbf{B_6}$, and $\mathbf{B_8}$ are similar within the standard deviation of the measurements). This trend was consistent with reports that an increase in hydrophobicity increases the hemolytic activity because of stronger polymer–lipid core interactions.[10,11] Intriguingly, it was observed that separating the charge and the tail increases the hemolytic activity of these polymers much more than the antibacterial activity. Polymer $\mathbf{B_4}$ (separate center) was nearly 7500 times more potent than polymer $\mathbf{A_4}$ (same center) in causing RBC lysis, whereas it was only 3 times more potent in killing *E. coli*. The hemolytic activity results indicate that it may not be desirable to use the more antibacterial but far more hemolytic spatially separated polymers in settings such as biomedical devices and implants, where reducing mammalian cell toxicity is essential. The highest selectivity index (HC_{50}/MIC) of 34:1 was observed for polymer $\mathbf{A_4}$, thus indicating that it could act as a potent antimicrobial with low mammalian cell toxicity.

The antibacterial and hemolytic activities of polymers from series **C** are shown in Figure 1d. These polymers have hydrophobic tails on both the positive center as well as on a separate center. One can consider series **C** as being derived from series **A** but with an additional spatially separated tail. Polymers from this series, especially $\mathbf{C_3}$ and $\mathbf{C_4}$, have higher hemolytic activities than $\mathbf{A_3}$ and $\mathbf{A_4}$, clearly indicating that introducing spatially separated alkyl tails dramatically increases the hemolytic activity. However, the antibacterial activities of series **C** were similar or slightly lower than those of series **A**. This result can be attributed to series **C** polymers being sparingly soluble in Lauria Bertani bacterial growth media. All three series were completely soluble in hemolytic assay media.

Higher molecular weight polymers from series **A** and **B** having butyl tails ($\mathbf{A_4}$ and $\mathbf{B_4}$), with AR and BR values of approximately 1, had the same MIC values (50 and 15 $\mu g\,mL^{-1}$, respectively) as the lower molecular weight polymers, thus indicating that the observed trend was not an artifact of varying the molecular weight. Also, corresponding polymers with alkyl tails of up to four carbon atoms long ($\mathbf{A_2}$ versus $\mathbf{B_2}$, etc) have the same solubility in water, thereby ruling out solubility as the reason for the observed trend. The *N*-alkylpyridinium units in series **A** are somewhat more hydrophobic than the *N*-methylpyridinium units in series **B**, and this may be a factor complicating the comparison of polymers from series **A** and **B**. To establish that the observed difference in activities between the two series was

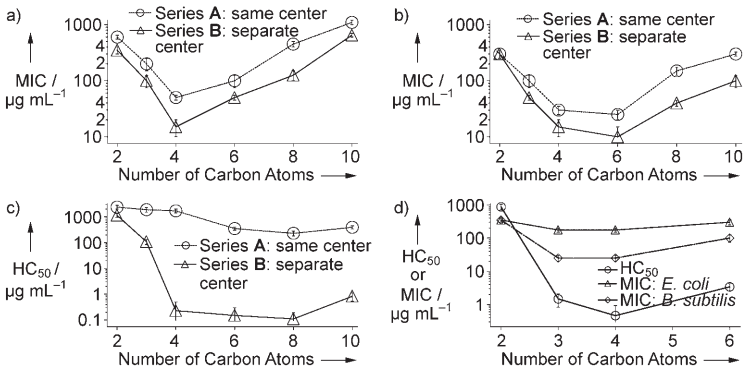
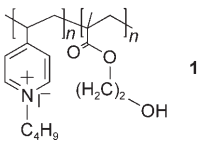


Figure 1. Average activities with error bars of pyridinium–methacrylate copolymers from series **A**, **B**, and **C** as a function of tail length: a) Antibacterial activity (MIC) of series **A** and **B** towards Gram-negative *E. coli*; b) antibacterial activity (MIC) of series **A** and **B** towards Gram-positive *B. subtilis*; c) hemolytic activity (HC_{50}) of series **A** and **B** towards human red blood cells; and d) antibacterial (MIC) and hemolytic (HC_{50}) activity of polymers from series **C**.

not an artifact of the difference in the hydrophobicity/polarity, we synthesized a modified derivative of polymer $\mathbf{A_4}$ with a polar and hydrophilic hydroxyethyl side chain. A 50–50 mol% copolymer of 4-vinylpyridine and hydroxyethyl methacrylate was prepared by radical polymerization, and was N butylated to yield the polymer **1**.



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The presence of hydrophilic hydroxyethyl side groups makes this derivative of $\mathbf{A_4}$ more hydrophilic than $\mathbf{B_4}$ while maintaining charge/tail relationship. The MIC and HC_{50} values for this derivative were similar to those of $\mathbf{A_4}$ (MIC: 50(5) and 30(5) $\mu g\,mL^{-1}$ towards *E. coli* and *B. subtilis*, respectively; HC_{50}: 1245-(397) $\mu g\,mL^{-1}$). This observation suggests that the observed differences between series **A** and **B** are not due to subtle differences in the hydrophilicity/polarity of the polymers, and are indeed due to the charge/tail positioning. Moreover, since polymers from series **C**, such as $\mathbf{C_3}$ and $\mathbf{C_4}$ (both of which are significantly less hydrophilic than $\mathbf{A_3}$ and $\mathbf{A_4}$) have high hemolytic activities, polymer hydrophilicity arising from the presence of polar groups does not seem to be a factor affecting hemolytic activity. In summary, both the antibacterial and hemolytic activity trends strongly suggest that separating the charge and tail increases the membrane-disrupting (hemolytic more than antibacterial) ability of an amphiphilic polymer.

We have also examined whether the observed trend in antibacterial and hemolytic activity with spatial positioning was valid over a range of AR values (the ratio between the positive charge and the alkyl tail on the polymer). Two series (**P** and **Q**) of amphiphilic pyridinium–methacrylate copolymers with C_4 tails having similar molecular weights were synthesized (Scheme 2). Each series consisted of five polymers, with the AR values increasing from 0.5 to 4.3. Note that an AR value of less than 1 is not possible for polymers in series **P** while maintaining true charge/tail union. For polymers in series **P**, the AR value is the ratio between the total number of moles of positive charge (all pyridinium groups) divided by the number of moles of C_4 tail (pyridinium groups having a C_4 tail). For polymers in series **Q**, the AR value is simply the ratio between the pyridinium groups and the butyl methacrylate units. Polymers were synthesized such that corresponding polymers had similar AR and BR values ($BR_P \approx BR_Q$ and $AR_P \approx AR_Q$). Corresponding polymers from the two series were compared for their antibacterial and hemolytic activity towards *E. coli*, *B. subtilis*, and RBCs.

Plots of the variation of the MIC value towards *E. coli* as well as of the HC_{50} values with increasing AR value are shown in Figure 2. The antibacterial potency first increased and then decreased as the AR valued varied from 0.5 to 4.3. The highest antibacterial potency (lowest MIC value) for polymers from both series was achieved with an AR value of 1. This trend was also followed for the activity towards *B. subtilis* (see the Supporting Information). Similarly, the hemolytic activity reached a maximum at an AR value of 1, and decreased as the AR values increased. When the amphiphilic polymer is too hydrophobic ($AR < 1$), interchain aggregation can occur,[7] which reduces the membrane-disrupting ability of the polymer. On the other hand, having too few hydrophobic groups ($AR > 1$) reduces the ability of the polymer to interact with the lipid core of the cell membrane, thereby making it less potent.[15–17] Thus, an optimum balance between charge and hydrophobicity is needed to disrupt the membrane (kill bacteria or lyse RBCs), which in this system is attained when equal numbers of cationic charges and tails are present. Interestingly, although series **Q** was always more potent (lower MIC and HC_{50} values) than series **P**, the effect of spatial positioning was more pronounced at $AR \approx 1$. The difference in the antibacterial activity as a result of spatial positioning was statistically insignificant at higher AR values.

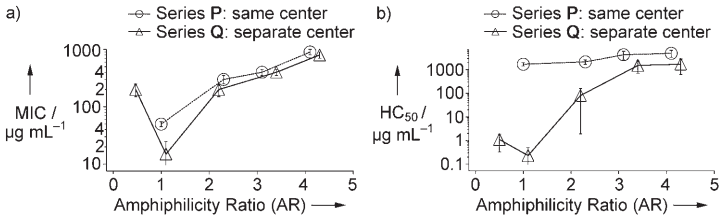
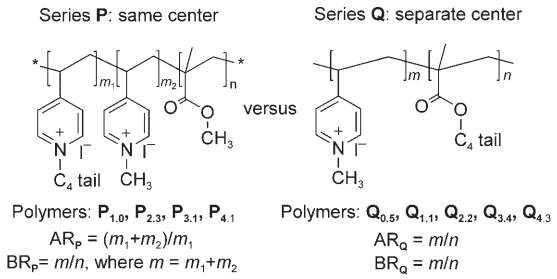


Figure 2. Average activities (with error bars) of pyridinium–methacrylate copolymers from series **P** and **Q** as a function of AR: a) antibacterial activity (MIC) towards *E. coli*, and b) hemolytic activity (HC_{50}) towards human red blood cells.



Scheme 2. Two series of pyridinium–methacrylate copolymers with increasing AR values and different spatial positioning. Subscripts refer to the AR value of the particular polymer, for example, polymer $\mathbf{P_{2.3}}$ from series **P** has an AR value (moles of charge/moles of C_4 tail) of 2.3.

Fluorescence confocal microscopy studies were also carried out to qualitatively investigate the underlying reason for the large observed difference in the hemolytic activities of polymers from series **A** , **B**, and **C**. Three representative polymers, $\mathbf{A_4}$ (HC_{50}, 1709 $\mu g\,mL^{-1}$), $\mathbf{B_4}$ (HC_{50}, 0.23 $\mu g\,mL^{-1}$), and $\mathbf{C_4}$ (HC_{50}, 0.46 $\mu g\,mL^{-1}$), were labeled with the fluorescent dye 5-(iodoacetamido)fluorescein (see the Supporting Information). Suspensions of human red blood cells (1.2% v/v) in TBS buffer (10 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM NaCl, pH 7.2) were incubated with the respective polymer (final polymer concentration: 5 $\mu g\,mL^{-1}$) for 5 minutes, and the RBC suspension was imaged under a confocal microscope. Note that the concentration of RBCs is six times that of those used in the hemolytic assays. The confocal and DIC micrographs are shown in Figure 3.

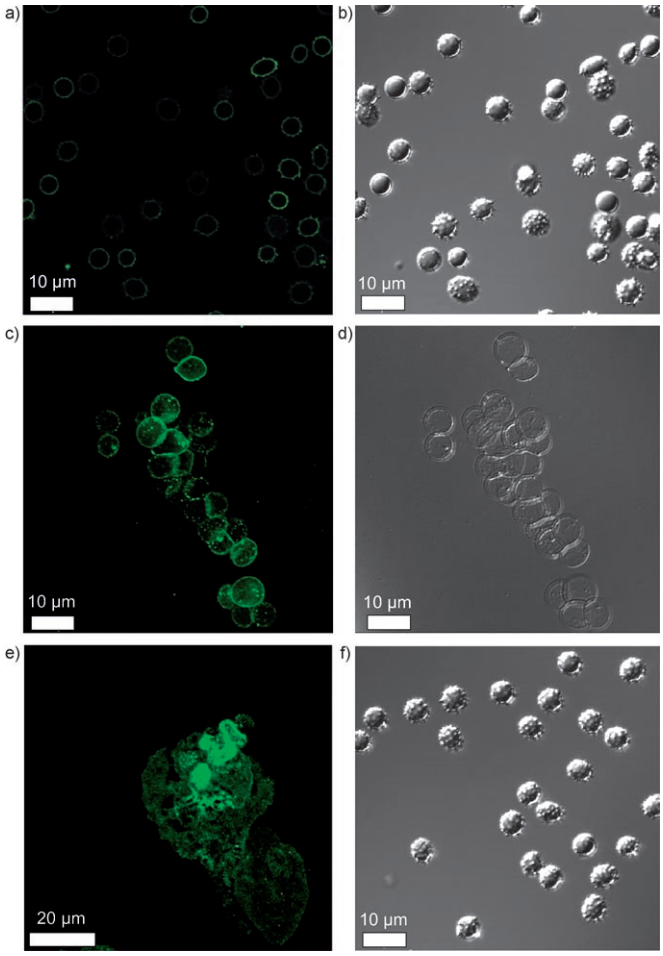


Figure 3. Confocal laser scanning microscopy images of human erythrocytes treated with fluorescein-labeled polymers $\mathbf{A_4}$ and $\mathbf{B_4}$: a, b) Fluorescence and corresponding differential interface contrast (DIC) image of RBCs treated with 5 $\mu g\,mL^{-1}$ of polymer $\mathbf{A_4}$; c, d) fluorescent and corresponding DIC image of RBCs treated with 5 $\mu g\,mL^{-1}$ of polymer $\mathbf{B_4}$; e) image of areas of nonstructured fluorescence indicating cellular debris observed with polymer $\mathbf{B_4}$; f) control DIC image of untreated RBCs in buffer.

Untreated human RBCs in TBS buffer have the morphology shown in Figure 3 f. The spiky appearance of erythrocytes is attributed to a dilution of the blood serum and has been reported by other researchers.[18] It should be mentioned that fractionated and purified RBCs were purchased from a biopharmaceutical company, and thus the samples are not expected to be contaminated by other types of blood cells. Distinct differences were observed between RBCs treated with polymers $\mathbf{A_4}$ and $\mathbf{B_4}$, as shown in Figure 3. Red blood cells treated with the more hemolytic polymer $\mathbf{B_4}$ agglutinated into large clusters of cells (Figure 3 c, d). Most of the cells present on the slide being imaged were agglutinated into clusters, and only a few cells remained as free singles or doubles. The cells in these clusters had a deformed morphology (slightly increased size and a flatter profile) compared to those of untreated RBCs. The fluorescently labeled polymer was also clearly internalized into the cell as shown by the presence of fluorescence inside the cell. Furthermore, regions of nonstructured fluorescence were observed throughout the sample (Figure 3 e). We attribute these regions to polymer–membrane debris resulting from cell clusters which had been completely lysed. Red blood cells incubated with labeled polymer $\mathbf{C_4}$ (both centers) also showed similar cellular agglutination and regions of nonstructured debris, consistent with its high hemolytic activity in HC_{50} assays (see the Supporting Information). In contrast, RBCs treated with polymer $\mathbf{A_4}$ exhibited no cellular agglutination, and the cells had a morphology similar to those of untreated cells (Figure 3 a b). The intensity of the fluorescence from the cell membrane resulting from the binding of $\mathbf{A_4}$ was much lower than that observed with $\mathbf{B_4}$ and $\mathbf{C_4}$, which qualitatively indicates that polymer $\mathbf{A_4}$ binds more weakly to the RBC membrane. Moreover polymer $\mathbf{A_4}$ remained localized on the cell surface and no polymer internalization into the RBCs was observed (sequential images traversed in the z direction showed no fluorescence inside the cell). Thus, confocal microscopy studies also suggests that $\mathbf{B_4}$ is much more hemolytic than $\mathbf{A_4}$. We attribute this to the increased membrane-binding ability and membrane permeability of $\mathbf{B_4}$. Furthermore, the ability of $\mathbf{B_4}$ to cause cell agglutination seems to be an important factor in causing RBC lyses and is being investigated further.

In summary, it was found for homologous polymers having similar backbone composition and charge/tail ratios that placing the charge and tail on spatially separated centers results in a higher membrane-disrupting ability, as evident from the increased antibacterial and hemolytic activities. Spatial separation was found to especially amplify mammalian cell toxicity. When the charge and the tail were on the same center, the highest selectivity (HC_{50}/MIC) obtained was 34:1. Clearly, it is not desirable to use the more antimicrobial, but far more hemolytic, spatially separated polymers in settings such as biomedical devices and implants, where reducing mammalian cell toxicity is essential. Confocal microscopy studies clearly indicated that polymers having spatially separated tails interacted with RBC membranes more strongly. Interestingly, cellular agglutination caused by spatially separated polymers seems to be an important factor leading to hemolysis. Since the physical and chemical properties of a polymer (solubility, surface properties, modulus, elasticity, etc.) are intimately dependent on its structure, these results will help guide the design of new antimicrobial polymers with improved biocompatibility for applications in which a specific property is desired.

Experimental Section

Polymer synthesis and characterization, antibacterial, hemolytic and confocal microscopy assays are described in the Supporting Information.

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