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Biocidal Activity of Polystyrenes That Are Cationic by Virtue of Protonation

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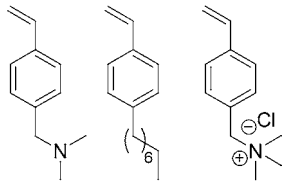
ABSTRACT



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Poly(1) kills bacteria (Gram-positive and -negative) and lyses human erythrocytes; this biocidal profile is similar to that of the peptide toxin mellitin. Poly(1) has antibacterial activity comparable to that of a potent derivative of the host defense peptide magainin II, but lacks magainin's selectivity for bacteria over erythrocytes. An analogous N-quaternized polymer, poly(3), is less biocidal than poly(1), suggesting that reversible N-protonation leads to greater biocidal activity than does irreversible N-quaternization.

Bacterial resistance to common therapeutic agents has prompted the search for new antimicrobial compounds.[1] Peptides, which play a central defensive role as innate antimicrobial agents,[2] have received increasing interest in this regard. Development of resistance to these "host-defense" peptides appears to be difficult, although not impossible. One large subset of host-defense peptides forms an amphiphilic α-helical structure.[3] These peptides appear to act by disrupting bacterial membranes. Their net positive charge attracts these peptides to the negatively charged bacterial membrane,[4] and the hydrophobic face of the helix allows the formation of aggregates that compromise membrane integrity.[2] Enantiomeric peptides retain full activity, suggesting that amphiphilic topology is the crucial feature of these molecules.[5]

Design principles suggested by host-defense peptides have been applied to other types of amphiphilic helical antimicrobial oligomers. β-Amino acid oligomers ("β-peptides") can adopt discrete helical conformations;[6] proper arrangement of cationic and lipophilic residues leads to formation of amphiphilic helices with antimicrobial activity.[7] Patch and Barron have reported that amphiphilic helix-forming peptoids (*N*-alkyl glycine oligomers) display antimicrobial activity.[8] DeGrado et al. extrapolated further from the host-defense

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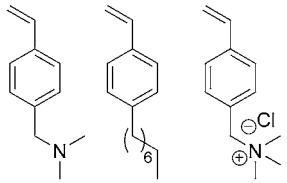
Table 1. Minimum Inhibitory Concentrations (μg/mL)

strain	*E. coli* JM109	*B. subtilis* BR151	*S. aureus* 5332	*E. faecium* A436
poly(**1**)	25	12.5	50	12.5
[Ala^{8,3,18}]-magainin 2 amide	12.5	6.3	12.5	3.2
poly(**3**)	>50	50	>50	>50
1 (monomer)	>50	>50	>50	>50

peptide model, creating oligomers with elongated conformations that can project lipophilic and cationic groups to opposite sides.[9]

The work reported here arose from our interest in determining whether conformational preorganization is required for antimicrobial activity in synthetic oligomers or polymers. The helical antimicrobial β-peptides developed by our group, composed of cyclically constrained β-amino acids, are quite rigid.[7b,c] As a result, antimicrobial activity is completely lost upon sequence scrambling to give a nonamphiphilic helix.[7b,c] In contrast, α-helical host defense peptides can be scrambled with only modest loss of activity.[10] This difference in the effect of residue scrambling can be explained by invoking the increased flexibility of the α-peptide backbone, which might allow scrambled sequences to populate nonhelical but nevertheless globally amphiphilic conformations. The highly preorganized β-peptide backbone cannot adopt such alternative, nonhelical conformations. Extending this speculation, we wondered whether any sufficiently flexible synthetic polymer backbone might be able to display a random sequence of cationic and lipophilic side chains in a manner that results in global amphiphilicity and, therefore, antimicrobial activity. Here we address this question by comparing polymers that contain 4-(dimethylaminomethyl)-styrene (**1**) to an α-peptide, Ala^{8,13,18}-magainin 2 amide, which is known to display potent antimicrobial activity.[11] Polymers cationic by virtue of quaternized nitrogen and structurally related to poly(**1**) have long been studied as antimicrobial agents;[12,13] polymers that contain **1** differ from the quaternized precedents in that, like host-defense peptides, polymers containing dimethylaminomethyl groups require protonation to develop positive charge.



1 **2** **3**

We prepared poly(**1**) and copolymers containing **1** and 4-octylstyrene (**2**) via AIBN-initiated radical polymerization.[14] Hydrophobic size exclusion chromatography (Sephadex LH-20) was used to purify the polymers and remove traces of unreacted monomer (as determined by ^{1}H NMR). Polymer molecular weight averages were determined by GPC.[15] Most samples had a number average molecular weight (M_n) near 3000, comparable to the molecular weight of the magainin derivative (MW = 2478) used as a standard in subsequent experiments. The PDI values of these samples were close to 3.0, indicative of the broad molecular weight distributions typically associated with AIBN-initiated radical polymerizations. For copolymerizations, a chromatographic assay demonstrated that these materials did not contain poly(**2**) and suggested that most or all of the material was copolymer formed from **1** and **2**.[15] Elemental and NMR analysis of the copolymers further indicated that the composition of the copolymers closely reflected that of the feed mixtures used.[15]

Minimum inhibitory concentrations (MICs) for poly(**1**) and a series of copolymers containing **1** and **2** (up to 40 mol % **2** as estimated by ^{1}H NMR)[16] were determined with *E. coli*,[17] *B. subtilis*,[18] methicillin-resistant *S. aureus*[19] (MRSA), and vancomycin-resistant *E. faecium*[20] (VRE) (Table 1). Turbidity-based assays for inhibition of bacterial growth were conducted at polymer concentrations up to 50 μg/mL, a limit determined by polymer solubility in the assay media. The data given are conservative estimates of MIC because some

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precipitation occurred, at concentrations above 12.5 μg/mL, over the 6 h incubation period, causing turbidity even in the absence of bacterial growth; in other words, the actual MIC values for the synthetic polymers may be lower than the reported values. Ala[8,3,18]-magainin 2 amide[11] was used as a positive control. To our knowledge, this study represents the first direct comparison between synthetic polymers and a peptide antibiotic.

Poly(**1**) displayed significant antimicrobial activity against all four bacteria, in contrast to monomer **1**, which was completely inactive. Copolymers containing up to 20 mol % **2** displayed similar or slightly improved activity, and higher proportions of **2** led to a drop in activity (data not shown). Poly(**1**) is somewhat less active than is the magainin derivative, particularly toward the pathogenic *S. aureus* and *E. faecium* strains; however, this variation seems relatively modest in light of the profound difference in molecular structure between the peptide and poly(**1**) and the difference in effort required for chemical synthesis of the peptide vs the homopolymer. We compared poly(**1**) with poly(**3**) (synthesized from monomer **3** via reported methods[13,21]) in order to evaluate the difference between N-protonation and N-quaternization as a source of positive charge. Poly(**3**) was reported to display antimicrobial activity in an agar-plate assay,[13,21] but in our liquid-medium assay, this material showed little or no activity.

Host-defense peptides, such as the magainins, are much more effective at disrupting bacterial cell membranes than at disrupting eukaryotic cell membranes.[10] Cell membrane selectivity is conventionally evaluated in vitro by comparing antibacterial activity to human red blood cell lysis ("hemolysis") activity; for host-defense peptides, there is a substantial concentration range in which only antibacterial activity is manifested.[22] However, other natural peptides such as melittin display comparable lytic effects toward both bacterial and eukaryotic cells. Poly(**1**) was highly hemolytic, somewhat more so than melittin itself (on a weight basis); thus, this polymer is more a mimic of melittin than of a host-defense peptide.

Both nonpolar and electrostatic forces are believed to be important in the interactions of host-defense peptides such as magainins and general toxin peptides such as melittin with cell membranes.[23] It therefore seemed curious that introduction of hydrophobic units, derived from **2**, did not cause a significant increase in lytic activity relative to poly(**1**). Titration data provided one potential explanation for the functional similarity between poly(**1**) and copolymers formed from both **1** and **2**. For the conjugate acid of monomer **1**, the pK_a is 8.5 in water and 7.5 in aqueous methanol. The conjugate acid of poly(**1**) is highly soluble in water, but poly(**1**) itself is not; thus, titration was possible only in aqueous methanol. In this solvent, the pK_a was indistinguishable from that of the conjugate acid of monomer **1**. Thus, under the neutral pH conditions of the antimicrobial assays, a significant fraction of the dimethylaminomethylbenzene groups is probably not protonated. These unprotonated groups should be relatively hydrophobic, allowing poly(**1**), formally a homopolymer, to function as an amphiphilic copolymer.

Monomeric quaternized ammonium compounds containing sufficiently hydrophobic organic groups are toxic to bacteria, presumably via a detergent mode of membrane disruption.[24] We wondered whether poly(**1**) might form micellar subdomains that disrupt cell membranes in a similar way, i.e., whether this material is simply a polymeric detergent. To evaluate this possibility, we tried to solubilize orange OT in aqueous solutions of poly(**1**). Orange OT is a highly hydrophobic dye that does not dissolve in water. This substance can be used to determine critical micelle concentrations of surfactants because orange OT dissolves in the nonpolar core of surfactant aggregates.[25] However, orange OT was not solubilized by aqueous solutions of poly(**1**) or by copolymers formed from **1** and **2**; thus, the biocidal properties of these polymers do not arise from a detergent-like membrane disruption mechanism.

The data reported here show that poly(**1**) is broadly biocidal, killing bacteria and lysing human red blood cells at relatively low concentrations. Poly(**1**) bears some similarity to a number of polystyrene-derived antibiotics that are cationic by virtue of N-quaternization; to our knowledge, the toxicity of these quaternized polymers toward eukaryotic cells has not been reported. Poly(**1**) displays comparable or slightly reduced activity toward both Gram-positive and Gram-negative bacteria, including human pathogens that are resistant to clinical antibiotics, relative to a potent derivative of the host-defense peptide magainin 2. This result supports the hypothesis that backbone preorganization is not essential for potent antimicrobial activity in oligomeric or polymeric materials.[26] Poly(**1**) is much less expensive to prepare than a peptide such as magainin 2 or unnatural oligomers[7−9] that have been shown to display magainin-like activity. The lack of discrimination between prokaryotic and eukaryotic cells by poly(**1**) diminishes its potential for biomedical utility relative to host-defense peptides or their oligomeric mimics, but structure−activity relationships established among peptides suggest strategies for improving the cell selectivity of synthetic polymers.[3] In particular, decreasing the hydrophobicity of the polymeric backbone seems to be a promising approach. We are currently exploring copolymers with amphiphilic backbones such as polyacrylates, polyacrylamides, and poly(ethylene glycol)s to test this hypothesis.

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Supporting Information Available: Synthetic protocol and characterization data for polymers, assay procedures, and hemolysis curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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