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Structure−activity Relationships among Random Nylon-3 Copolymers That Mimic Antibacterial Host-Defense Peptides

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Abstract: Host-defense peptides are natural antibiotics produced by multicellular organisms to ward off bacterial infection. Since the discovery of these molecules in the 1980s, a great deal of effort has been devoted to elucidating their mechanisms of action and to developing analogues with improved properties for possible therapeutic use. The vast majority of this effort has focused on materials composed of a single type of molecule, most commonly a peptide with a specific sequence of α-amino acid residues. We have recently shown that sequence-random nylon-3 copolymers can mimic favorable properties of host-defense peptides, and here we document structure−activity relationships in this polymer family. Although the polymers are heterogeneous in terms of subunit order and stereochemistry, these materials display structure−activity relationships comparable to those that have been documented among host-defense peptides and analogous synthetic peptides. Previously such relationships have been interpreted in terms of a specific and regular folding pattern (usually an α-helix), but our findings show that these correlations between covalent structure and biological activity do not require the adoption of a specific or regular conformation. In some cases our observations suggest alternative interpretations of results obtained with discrete peptides.

Introduction

Fending off infection by pathogenic bacteria is a constant challenge for eukaryotes. In the context of human medicine, this challenge becomes acute because of the rapidity with which bacteria can develop resistance to chemotherapeutic agents. Natural host-defense mechanisms are varied; in humans and other higher organisms these mechanisms include both adaptive and innate immune responses. An important component of the innate response is the release of peptides that display broad antibacterial activity.[1] These host-defense peptides have attracted considerable attention as prototypes for the design of new antibacterial agents. Many design efforts have been based on retention of the natural peptide skeleton (i.e., the resulting agents are oligomers of L-α-amino acids or "α-peptides"), but non-natural oligomeric structures have been explored as well for this purpose, particularly over the past decade. Early deviations from the natural peptide backbone included oligomers of D-α-amino acids[2] and oligomers containing both L- and D-α-amino acid residues.[3] Some recently explored agents have differed more substantially from the natural prototypes, including oligomers of β-amino acids ("β-peptides"),[4] both α- and β-amino acids ("α/β-peptides"),[5] N-alkyl glycines ("peptoids"),[6] N-acylated lysine,[7] and aromatic subunits.[8]

The structures and functions of natural host-defense peptides are diverse, but most exploration of host-defense peptide mimics has focused on one structural class and one type of function.

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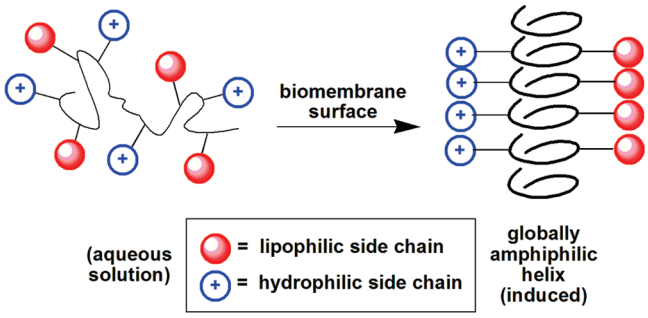

Figure 1. Hypothesis explaining the activity of many host-defense peptides, involving the adoption of a globally amphiphilic helical conformation upon approach to a biomembrane surface.

Specifically, these design efforts have centered on natural prototypes that form α-helices, such as magainins,[9] cecropins,[10] and the human peptide LL-37,[11] with the aim of reproducing the membrane-disrupting behavior displayed by many host-defense peptides. The α-helix-forming host-defense peptides do not fold in aqueous solution; helicity is induced at the target membrane surface.[12] These peptides are cationic under physiological conditions, and Coulombic forces apparently cause the initial attraction to the anionic surfaces of bacterial cells. The external surfaces of eukaryotic cells generally have a diminished negative charge density relative to prokaryotic cell surfaces, which is thought to underlie the selectivity for prokaryotic cells displayed by host-defense peptides.[13] The level of prokaryote vs eukaryote selectivity is generally assessed by comparing the ability of a given peptide to inhibit bacterial growth with the ability of that peptide to induce hemoglobin release from human red blood cells ("hemolysis").

The amino acid sequences of α-helical host-defense peptides lead to a global segregation of cationic and lipophilic side chains in the folded state,[12] with cationic groups arrayed along one side of the helix and lipophilic groups arrayed along the other (Figure 1). Inspired by this precedent, most designs of host-defense peptide mimics have been based on the adoption of a regular conformation that is globally amphiphilic. This approach requires control of subunit sequence so that folding to a specific conformation gives rise to distinct cationic and lipophilic regions of the molecular surface.[4d] Sequence control, in turn, requires stepwise synthesis, which is difficult to execute on a large scale. The expense of stepwise synthesis is recognized as a significant limitation with regard to potential applications of host-defense peptides, designed α-peptide analogues, and other discrete oligomers.[1c]

Unexpected results from our study of the antibacterial properties of helix-forming foldamers, β-peptides and α/β-peptides, led us to formulate a new strategy for generating host-defense peptide mimics that does not require control of subunit sequence.[5] The underlying hypothesis (Figure 2) is that an oligomer or polymer containing both cationic and lipophilic side chains could be induced by an interfacial environment to adopt a globally amphiphilic but *conformationally irregular* structure if the backbone were sufficiently flexible. This hypothesis arose

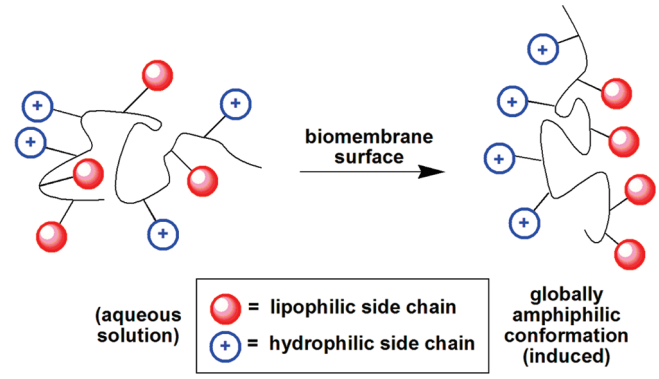

Figure 2. Alternative hypothesis (relative to Figure 1), involving the adoption of a globally amphiphilic *irregular* conformation, that explains the activity of our α/β-peptides[5] and the polymers reported herein.

from our observation that antibacterial activity among rigid β-peptides is very sensitive to β-residue sequence, with the ability to form a globally amphiphilic helix absolutely required,[4b] while antibacterial activity among more flexible α/β-peptides can be manifested whether or not the available helix conformations are globally amphiphilic.[5] Related trends have been observed among helix-forming α-peptides. Particularly noteworthy in this context are studies of Dathe et al.[3a] and Shai et al.[3b] showing that incorporation of a few D-α-amino acid residues into helix-forming α-peptides leads to a diminution of hemolytic activity without a corresponding decrease in antibacterial activity. Some of these heterochiral peptides may retain the ability to adopt an α-helical conformation,[3c] but more recent structural analysis has shown that such heterochiral peptides can be induced by a micellar environment to adopt specific but irregular conformations that result in global segregation of hydrophilic and lipophilic side chains.[3e−g]

If the hypothesis outlined in Figure 2 is valid, then it should be possible to develop polymers that disrupt bacterial membranes in preference to eukaryotic cell membranes without controlling subunit sequence. In other words, this hypothesis implies that random copolymers of cationic and lipophilic monomers might be able to mimic host-defense peptides. This prospect is appealing because random copolymers are often easy to prepare in quantity, and synthetic accessibility should minimize production cost and therefore facilitate application.

A recent report by Rathinakumar and Wimley[14] proposes that de novo design of host-defense peptide analogues based on a specific structure and sequence is not as effective as design based on the overall amino acid composition of the peptide. To test this hypothesis, these workers generated a combinatorial library of short peptides with fixed amino acid residues at regular intervals but varying hydrophobicity at the remaining sites. They

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identified a small number of peptides that share very little sequence similarity outside of the fixed residues but are nonetheless highly membrane-lytic. Our polymer-based efforts can be seen as complementary to that of Rathinakumar and Wimley, since we focus on chemical composition, without regard to a specific structure or sequence.

Cationic oligomers and polymers, mostly containing quaternized ammonium or guanidinium groups, have been explored as antibacterial agents for some time,[15] but this research avenue has until recently lacked a conceptual link to host-defense peptide mimicry. Early studies of polycations included polystyrene-based quaternary ammonium systems;[16] our initial foray into this field involved polystyrenes that contain tertiary amines and therefore require protonation to become cationic.[17] These new polystyrenes display antibacterial activity comparable to that of previously reported quaternary ammonium analogues, and we showed for the first time that such polymers are highly hemolytic. Thus, these polystyrenes lack the selectivity for prokaryotic vs eukaryotic cells that is characteristic of host-defense peptides. Trends observed among designed α-peptides suggest that bacterial selectivity is lost if overall hydrophobicity is too high.[12] We therefore hypothesized that host-defense peptide mimicry by random copolymers would require a backbone that is less hydrophobic than polystyrene.[17]

The search for antibacterial polymers with minimal hemolytic activity has recently broadened to include a variety of backbones. DeGrado et al. examined poly(arylamides)[18] and amphiphilic poly(methyl methacrylate) copolymers[19] but were unable to identify examples that display significant antibacterial activity in the absence of hemolytic activity. Tew et al. explored polydisperse *m*-phenylene-ethynylenes,[20] and Tew, Coughlin, et al. studied materials generated via ring-opening alkene metathesis polymerization.[21] Some recent examples of the latter type have shown good selectivity in terms of antibacterial activity vs hemolytic activity.[22] Our efforts[23] have focused on amphiphilic nylon-3 copolymers generated via ring-opening polymerization (ROP) of β-lactams.[24]

We were drawn to nylon-3 polymers because they share the backbone of the β-peptide oligomers previously shown to display promising biological activities.[4] The polyamide backbone of nylon-3 was attractive as potentially comparable in polarity to the backbone of natural peptides and proteins (which can be considered as nylon-2 derivatives). β-Lactams that lead to lipophilic subunits within poly-β-peptides are readily prepared via reaction of *N*-chlorosulfonyl isocyanate (CSI) with alkenes;[25] we expanded this methodology to enable large-scale synthesis of β-lactams that bear protected amino groups in a side chain.[23,26] Our preliminary studies of random copoly-β-peptides containing both cationic and lipophilic subunits identified examples that match the activity profile of host-defense peptides and established that membrane lysis is one mechanism by which they are likely to act.[23,27] In the present study we evaluate the impact of structural parameters such as lipophilic subunit identity, cationic subunit identity, lipophilic:cationic proportion, length, and end group on the antibacterial and hemolytic activities of nylon-3 copolymers. Each of our nylon-3 copolymer samples is generated from a racemic mixture of β-lactam monomer units and is therefore necessarily heterogeneous in terms of subunit sequence and stereochemistry. Despite this heterogeneity, we find that the nylon-3 family displays structure−activity relationships comparable to those previously documented among host-defense peptides and discrete oligomer analogues. Previously, such relationships have been interpreted in terms of an α-helical folding pattern, but our findings show that these correlations among covalent structure, antibacterial activity, and hemolytic activity do not require that the biologically active form adopt a regular conformation. In some cases, our observations raise questions about the interpretation of results from discrete peptides.

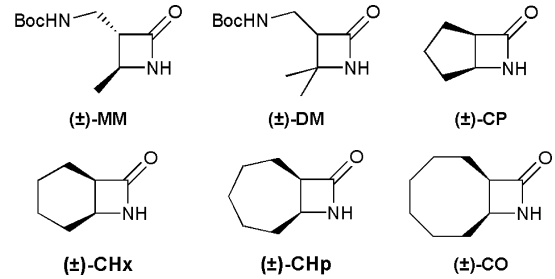

Figure 3. Monomers used for copolymerization. All β-lactams used are racemic mixtures.

Results and Discussion

Polymer Synthesis and Characterization. Polymers were prepared from two types of β-lactam (Figure 3), some that lead ultimately to cationic subunits, **MM** (for "monomethyl") or **DM** ("dimethyl"), and others that provide lipophilic subunits, **CP** (for "cyclopentyl"), **CHx** ("cyclohexyl"), **CHp** ("cycloheptyl"), and **CO** ("cyclooctyl"). These β-lactams were prepared by reaction of CSI with the appropriate alkenes. The four bicyclic

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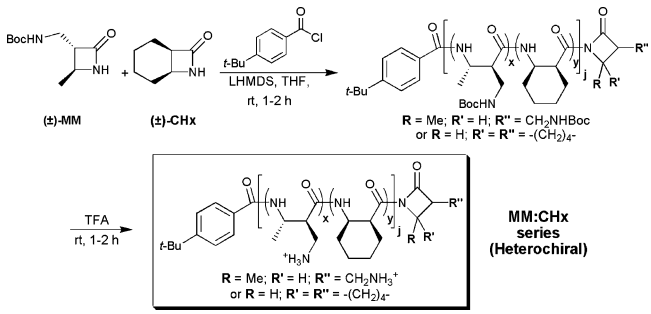
Scheme 1. Synthesis of **MM-CHx** Copolymers[a]



[a] All polymers presented in this report were synthesized using analogous chemistry.

monomers have been described previously,[28] and we reported the synthesis of **MM** and **DM**.[23,26a] Since each β-lactam is generated in racemic form, the resulting polymers are heterochiral. We used a recently reported modification of the standard base-initiated method for β-lactam ROP (Scheme 1). The standard method employs an imide as a co-initiator to ensure rapid and coordinated initiation of polymer chain growth,[24a] while the modified method employs an acid chloride or anhydride, which rapidly generates the corresponding imide co-initiator in situ.[26a] The latter approach enables facile variation of the fragment at the N-terminus of the nylon-3 molecules, which is derived from the co-initiator, because many acid chlorides and anhydrides are available.

The ROP products contain side chains bearing Boc-protected amino groups, derived from β-lactam **MM** or **DM**. These protected polymers were readily analyzed via GPC, with number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), and polydispersity index (PDI $= M_w/M_n$) determined based on data from a refractive index (RI) detector coupled to a multiangle light-scattering (MALS) detector. Analysis of the deprotected polymers proved more difficult, and the characterization data provided below are therefore based on the protected polymers. We make the chemically reasonable assumption that the Boc deprotection does not alter polymer length or composition (^{1}H NMR data were used to determine that deprotection had proceeded to completion). In our preliminary studies of poly-β-peptides derived from **MM** and **CHx**, we determined M_w, M_n, and PDI for protected polymers based on comparison to poly(methyl methacrylate) standards.[23] For a given sample, this poly(methyl methacrylate)-based method consistently leads to lower apparent molecular weights than does the RI/MALS method. Thus, for example, the **MM-CHx** copolymers studied previously appeared to have length distributions centered around 15−20 subunits based on the poly(methyl methacrylate) standards, while the RI/MALS analysis of these materials suggests length distributions centered around 28−37 subunits.

For nylon-3 derivatives containing 15−58 monomer units, the lengths were reasonably well controlled, with PDI values generally in the range 1.04−1.18; shorter polymers were more polydisperse. In a few cases longer polymers showed PDI values in the range 1.3−1.4. This greater polydispersity was most pronounced for the most hydrophobic **MM-CHp** polymers. In these cases GPC analysis revealed peaks with shoulders on the high-M_n side, which may arise from polymer self-association (discussed further below); these shoulders could interfere with accurate PDI determination. The small number of polymer samples with higher apparent PDI does not compromise the general conclusions drawn below.

The final, deprotected copolymer trifluoroacetate salts were characterized using NMR spectroscopy and MALDI-TOF mass spectrometry. The highest intensity mass peak for each final polymer and the degree of polymerization and monomer ratio to which it corresponds are reported in the Supporting Information. The Boc-protected precursors did not produce MALDI-TOF mass spectra. We did not use MALDI-TOF-MS to determine M_n or PDI values because the sensitivity of this analytical method may vary as a function of molecular weight, with larger polymer chains underrepresented relative to smaller polymer chains.

Biological Characterization. A standard serial dilution method was used to assess the abilities of nylon-3 derivatives to inhibit bacterial growth in liquid culture.[4b] We used a panel of four bacteria, including one Gram-negative species, *Escherichia coli,*[29] and three Gram-positive species, *Bacillus subtilis,*[30] *Staphylococcus aureus*,[31] and *Enterococcus faecium*.[32] Laboratory strains of *E. coli* and *B. subtilis* were employed, but clinical isolates were used in the other two cases. Our strain of *S. aureus* is resistant to methicillin, and our strain of *E. faecium* is resistant to vancomycin. Antibacterial activity was evaluated in terms of the minimum inhibitory concentration (MIC), i.e., the lowest concentration of polymer in a series of 2-fold dilutions that completely prevents bacterial growth.

Assessment of the antibacterial activities and selectivities of host-defense peptides and their unnatural analogues typically involves MIC measurements and evaluation of the ability to cause hemoglobin release from human red blood cells (RBC). Hemolytic activity is interpreted to reflect the propensity to disrupt eukaryotic cell membranes. MIC measurement techniques are generally comparable from one laboratory to the next, but methods of quantifying hemolytic data vary considerably. Many researchers use the concentration of an agent necessary for 50% hemoglobin release (HC_{50}) as the index of hemolytic activity,[18−22,33] while others use the lowest concentration necessary for detectable hemoglobin release, i.e., the minimum hemolytic concentration (MHC).[34] Alternative metrics have been employed as well.[3b,4a,b,35] HC_{50} can be determined with greater precision than MHC, as illustrated below, but we have come to prefer MHC because this measurement is more analogous to MIC than is HC_{50}. Selectivity can be quantitatively captured in a ratio of hemolytic activity to antibacterial activity; obviously, however, meaningful comparison of selectivities reported by different laboratories will be impossible if the hemolytic activities have not been represented in a comparable manner.

Effects of Polymer Length on Biological Activity. Our previously reported preliminary studies focused on random **MM-**

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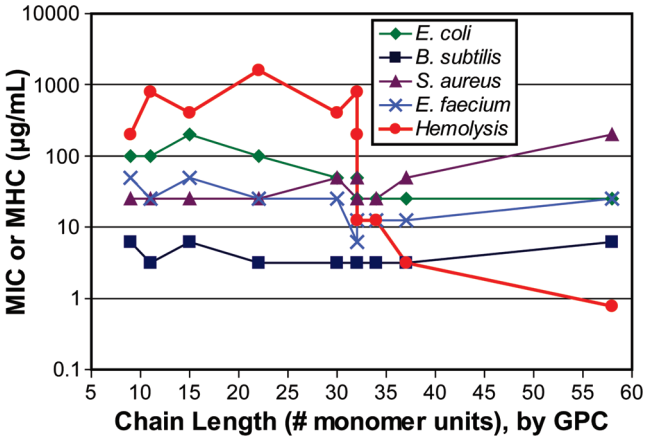

Figure 4. Dependence on average 63:37 **MM:CHx** copolymer chain length, determined as described in the text, of the biological activity profile. All polymers have an N-terminal *p*-(*tert*-butyl)benzoyl group. The lines simply connect the data points. Data points represent the median value of at least four independent antibacterial assays or three independent hemolytic assays.

CHx copolymers bearing a *p*-(*tert*-butyl)benzoyl unit at the N-terminus.[23] Exploration of the impact of the **MM:CHx** ratio on biological activities revealed optimal behavior for materials prepared from a β-lactam mixture containing 63% **MM**. We now report the effect of varying the polymer chain length on MIC against the four species in our bacterial panel and on MHC for this copolymer composition (Figure 4). The number of monomer units (horizontal axis of Figure 4) was estimated based on M_n determined via GPC for the Boc-protected polymers, using the known molecular weights of the *p*-(*tert*-butyl)benzoyl end group and the **MM** and **CHx** subunits as well as the β-lactam ratio employed in the polymerization reactions (63:37). Length control was only moderate for the smallest polymers, with PDI values of 1.2−1.4 for samples containing ~10 subunits. Larger polymers had PDI ≈ 1.15. It should be noted that polydispersity is intrinsically difficult to control for short polymer chains: if the absolute difference between M_w and M_n is similar for all lengths of a given polymer, the percentage difference will be smaller for higher molecular weights, and the ratio of M_w/M_n will be smaller as well. We believe that the biological activity data for polymers of varying length can be meaningfully compared despite the disparity in PDI.

Figure 4 reveals that antibacterial activities are not strongly affected by polymer length, with MIC for each of the four species varying within only one order of magnitude. There is no consistent trend among these variations. MIC for *E. coli*, for example, decreases slightly as length increases, while MIC against *S. aureus* increases with growing chain length. These deviations, though small, could imply that larger polymers have trouble penetrating the cell wall of Gram-positive bacteria such as *S. aureus* (as has been suggested for polynorbornenes[22]). Binding to lipopolysaccharide (LPS) of discrete all-L-α-peptide mimics has been observed and shown to prevent killing of Gram-negative bacteria.[36] A 60:40 **MM:CHx** copolymer has been shown to bind to both lipoteichoic acid (a key component of Gram-positive cell walls) and LPS.[27] Such interactions could hinder the polymer from reaching the inner membrane of a Gram-negative bacterium, which is presumably required for antibacterial activity.

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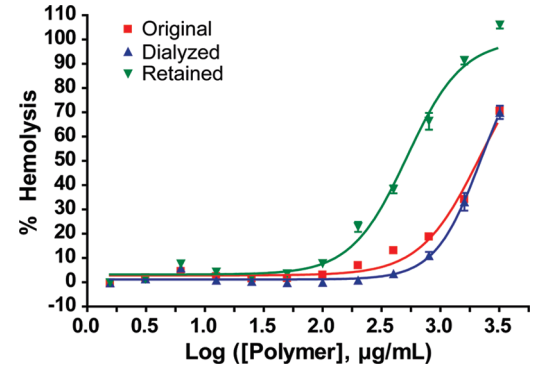


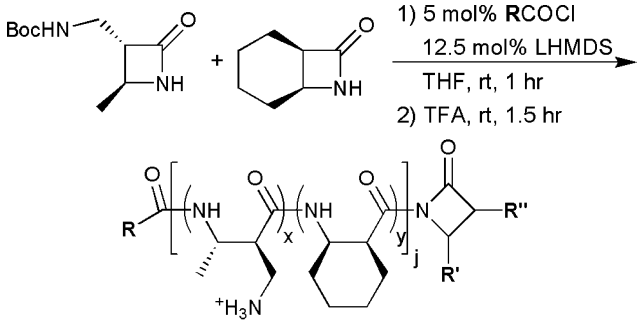
Figure 5. Hemolytic activity curves of a 63:37 **MM:CHx** *p*-(*tert*-butyl)benzoyl copolymer with an average length of 33 residues and PDI ≈ 1.10, dialyzed through a membrane with a molecular weight cutoff of 2000 Da.

Hemolytic activity is strongly influenced by polymer length, in contrast to the trends in antibacterial activity. Polymers containing an average of 10−30 subunits show very weak tendencies to induce hemoglobin release, with MHC values in the vicinity of 1000 μg/mL. These shorter polymers can be very selective, fully inhibiting bacterial growth at concentrations that are 10-fold to >100-fold lower, depending on the bacterium, than those required to produce detectable hemolysis. Above ~30 subunits, however, hemolytic activity increases precipitously, and ultimately MHC becomes lower than MIC for all of the bacteria.

The high sensitivity of hemolytic activity to polymer length was dramatically manifested in the case of three separately prepared polymer samples that were estimated to contain an average of 32 subunits. The MHC values of these polymers ranged from 800 to 12.5 μg/mL. The variation in hemolytic activity parallels the variation in polydispersity: PDI = 1.06 for the least hemolytic sample and PDI = 1.15 for the most hemolytic sample. In light of the length dependence described above, we hypothesize that the higher hemolytic activity of the more polydisperse samples results from the increased proportion of long polymer molecules in these samples.

To test this hypothesis, we prepared a 63:37 **MM:CHx** copolymer with PDI ≈ 1.10 that was estimated to contain an average of 33 residues and dialyzed a sample of it using a membrane with a 2000 Da cutoff. MALDI mass spectrometric analysis suggested that the material retained by the membrane had a higher average molecular weight than did the material that passed through the membrane (see Supporting Information). Thus, the largest members of the original polymer population appeared to have been depleted from the material that moved through the dialysis membrane. We compared the retained material, the material that moved through the membrane, and the original polymer sample in terms of antibacterial and hemolytic activities. The antibacterial activities of these three materials were indistinguishable (Table S8, Supporting Information). The hemolytic activity of the smaller molecular weight fraction (the material that moved across the dialysis membrane) was very slightly diminished relative to the original polymer, while the hemolytic activity of the larger molecular weight fraction (retained) was increased by a small but significant extent (Figure 5). These results support the hypothesis that higher molecular weight components of polydisperse samples in this length range can increase hemolytic activity, and the results

Scheme 2. Synthesis of **MM-CHx** Copolymers (x:y = 63:37) for the Study of the Effect of End-Group Lipophilicity on Biological Activity



C2: R = CH_3 **C8:** R = C_7H_{15} **C16:** R = $C_{15}H_{31}$
C3: R = C_2H_5 **C10:** R = C_9H_{19} **C18:** R = $C_{17}H_{35}$
C6: R = C_5H_{11} **C12:** R = $C_{11}H_{23}$

suggest that removing the larger members of a polymer population can improve the activity profile, at least to a modest extent.

Effects of Polymer End Group on Biological Activity. The impact of end-group hydrophobicity was explored with a series of polymers containing N-terminal linear alkanoyl units with incrementally varied length, from acetyl (C_2) to octadecanoyl (C_{18}). Each polymer was prepared from a β-lactam mixture containing 63% **MM** and 37% **CHx** (Scheme 2). These polymers typically contained an average of 27−35 subunits, as deduced from M_n data obtained via GPC for the Boc-protected precursors. PDI values were ~1.1. MALDI-TOF-MS confirmed the incorporation of the end groups. The increase in overall hydrophobicity engendered by the longer end groups, beginning with C_6, required use of DMSO rather than aqueous solution for preparation of polymer stock solutions. The MIC and MHC studies involving these polymers were conducted in buffers containing 4% DMSO; control experiments indicated that this additive does not influence the results.

Figure 6 summarizes antibacterial and hemolytic activities as a function of the number of carbon atoms in the N-terminal alkanoyl group. For each bacterial species a modest improvement in MIC is observed as tail length grows from C_2 to C_{12}.

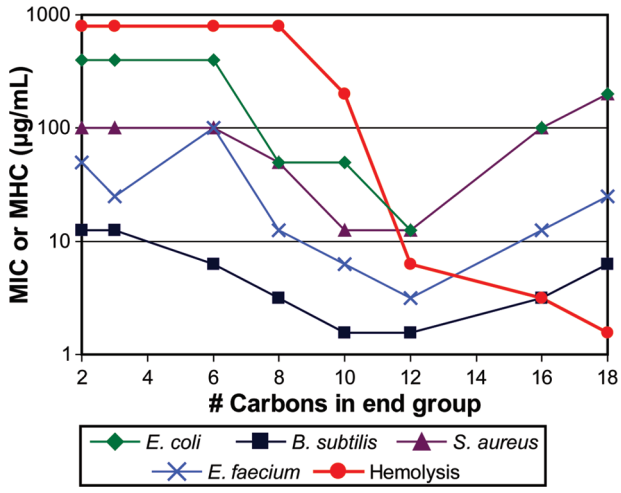


Figure 6. Dependence on acyl tail length of the biological profile of 63:37 **MM:CHx** polymers. Data points represent the median value of at least four independent antibacterial assays or three independent hemolytic assays. For tails containing ≥6 carbons, 4% DMSO was added to aid solubility. The lines simply connect the data points.

Further increases in tail length, to C_{16} or C_{18}, however, result in a diminution of antibacterial activity (higher MIC values). As indicated below, this latter effect may reflect self-association of the polymers with the most lipophilic N-terminal tails. Hemolytic activity is more strongly affected by variations in N-terminal tail length than is antibacterial activity, a trend that parallels the impact of polymer chain length on biological activity (Figure 4). Increasing tail length from C_2 to C_8 has no detectable effect on hemolytic activity; each of these polymers displays a very weak propensity to induce hemoglobin release from RBC. Further increases in N-terminal tail length, however, cause a substantial increase in hemolytic activity. In contrast to the MIC trend, the increase in hemolytic activity persists throughout the end group series; the C_{18} polymer is the most effective at inducing hemolysis, with MHC lower than all MIC values. The best selectivity in the alkanoyl series is seen with the C_8 end group, but this polymer is somewhat less effective as an antibacterial agent than is the 63:37 **MM:CHx** polymer with a *p*-(*tert*-butyl)benzoyl end group.

Effects of Subunit Identity on Biological Activity. We evaluated the impact of varying the subunits in our random copolymers on biological activity by replacing either **MM** or **CHx** with an analogous β-lactam in the ROP process (Figure 7). **CHx** replacements included the analogues containing a cyclopentyl (**CP**), cycloheptyl (**CHp**), or cyclooctyl (**CO**) ring. **MM** was replaced with **DM**, which bears an additional methyl substituent. All of these changes alter the net lipophilicity of the resulting polymers, since the analogous β-lactams differ from one another in the number of methylene units they contain. It is possible that these changes alter other polymer properties as well. For example, each ring size among **CP**, **CHx**, **CHp**, and **CO** will exert a distinctive constraint on the torsion angles available to the backbone C−C bonds in the corresponding polymer subunits. Thus, there should be differences in local folding propensities, and perhaps even global folding propensities, among the polymers containing these different cycloalkyl subunits, although these differences might be diminished, especially over larger length scales, by the heterochirality of these materials. Replacing **MM** with **DM** could exert a significant conformational effect as well, since polymers containing **DM** subunits have a quarternary carbon in the backbone while polymers containing **MM** subunits do not.

All polymers prepared for the subunit variation studies bear an N-terminal *p*-(*tert*-butyl)benzoyl group, and each is identified by the ratio of β-lactams used in the polymerization reaction. In each series the cationic:lipophilic subunit ratio was varied from 100:0 to 30:70. These variations were accomplished mostly in 10% increments, but in a few cases a finer series of variations was explored. Average chain lengths for these polymer samples were between 28 and 37 residues according to GPC analysis, with a few exceptions. The **MM-CO** series displayed average chain lengths in the range of 24−30 residues, and the most hydrophobic **MM:CHp** materials, 40:60 and 30:70, had average chain lengths of 44 and 69 residues, respectively. PDI values among the copolymers ranged from 1.04 to 1.18 with a few exceptions, the most significant of which were 40:60 and 30:70 **MM:CHp** (PDI = 1.28 and 1.39, respectively).

MIC values for the four bacteria and MHC values, as a function of the percent of the cationic unit (**MM** or **DM**), are summarized in Figure 8 for all five copolymer sets. There is a great deal of information in these graphs, but a few relatively simple trends can be perceived. We focus first on comparisons involving polymers that contain **MM** as the cationic subunit

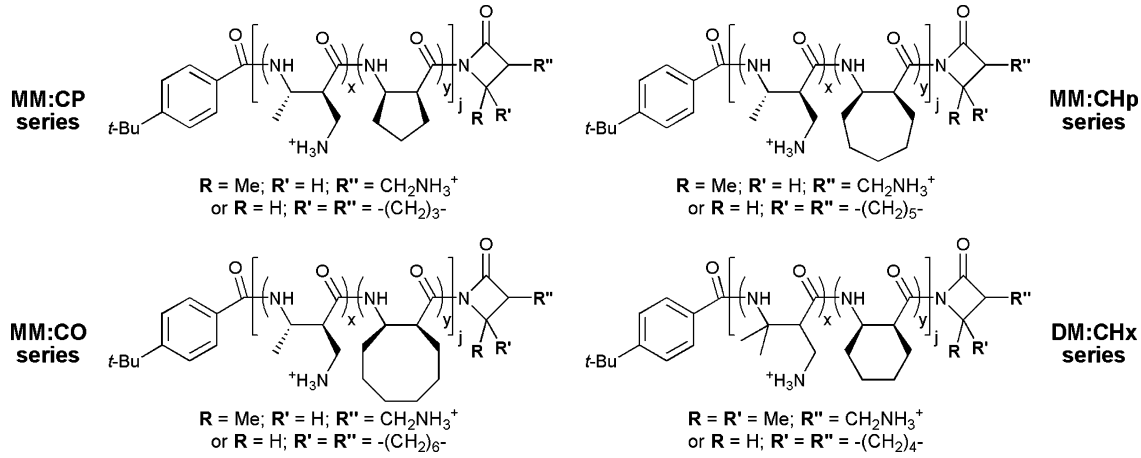


Figure 7. Polymers prepared to study the effect of monomer identity on biological profile.

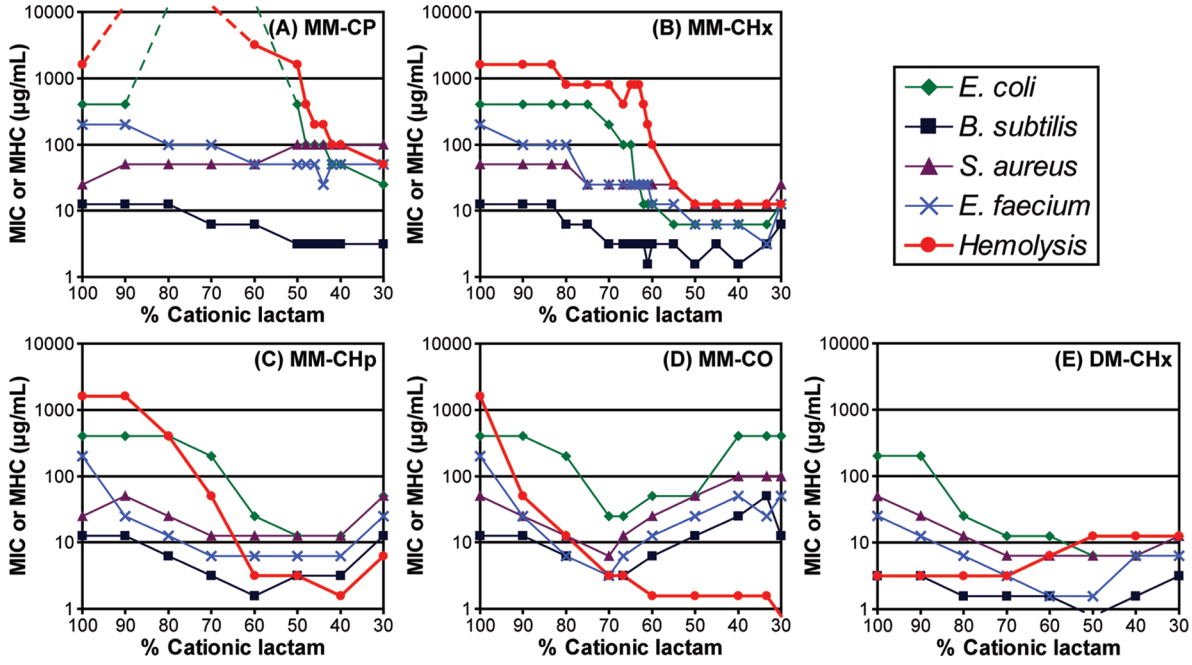


Figure 8. Biological activities of *p*-(*tert*-butyl)benzamide copolymers and their dependence on subunit identity. The lines merely connect the data points. (A) **MM-CP**; for 90:10−70:30 and 80:20−60:40 ratios, the MHC and MIC vs *E. coli*, respectively, were greater than the highest concentration used in the assay. (B) **MM-CHx**, reported previously.[23] (C) **MM-CHp**. (D) **MM-CO**; 4% DMSO was added for the 50:50−30:70 ratios to aid solubility. (E) **DM-CHx**; 4% DMSO was added for all to aid solubility.

and varying cycloalkyl groups in the lipophilic subunit, and then we compare the **MM-CHx** and **DM-CHx** polymers.

Comparison of the data sets in red among Figure 8A−D shows that hemolytic propensity generally increases as the cycloalkyl ring size increases. None of the **CP**-containing polymers displays a strong propensity to induce hemoglobin release (Figure 8A; MHC values mostly ≥100 μg/mL). A precipitous increase in hemolytic activity is seen within each of the four polymer sets as the lipophilic subunit proportion rises. The onset of this hemolytic activity increase occurs at a lower proportion of lipophilic subunits as the subunits themselves become more lipophilic, i.e., as we pass from **CHx** to **CHp** to **CO** (Figure 8B−D).[37] These trends suggest that increasing the overall lipophilicity of the polymer enhances hemolytic activity. A similar trend has been observed among α-peptides[12] and α/β-peptides.[5b]

The **CP** series is unique within the set of polymers with varying cycloalkyl ring size in that addition of small proportions of the lipophilic subunit (up to 30%) causes a *decrease* in hemolytic activity, relative to the **MM** homopolymer. This unusual effect is mirrored in the *E. coli* MIC data but not in the MIC data for any of the three Gram-positive bacteria. The origin of this behavior in polymers with low **CP** content is not clear, but these observations suggest that net lipophilicity is not the only factor that influences the ability to inhibit *E. coli* growth or to induce hemoglobin release from RBC. These nonmonotonic trends raise the possibility that more than one mechanism may underlie the inhibition of *E. coli* growth and hemolysis, with one mechanism favored for highly cationic agents and another favored for agents that contain both cationic and lipophilic subunits.

(37) The four hemolysis data sets from Figures 8A−D are presented in a single graph in the Supporting Information.

Table 1. Activity of Homopolymers of **MM** and **DM**

	MIC,[a] μg/mL				
polymer	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*	MHC,[b] μg/mL
MM	400	12.5	25	200	1600
DM	200	3.13	50	25	3.13

[a] MIC = minimum inhibitory concentration. [b] MHC = minimum hemolytic concentration.

The bacteria are generally less sensitive than RBC to changes in cycloalkyl ring size within the **MM**-containing copolymers, although sensitivity varies among bacterial species. (In the Supporting Information, the MIC data are grouped by bacterial species rather than by lipophilic subunit identity.) *E. coli*, the only Gram-negative species in our bacterial panel, displays the greatest sensitivity to differences among the polymers. *E. faecium* is the most sensitive to polymer differences among the Gram-positive species. For *S. aureus* and *B. subtilis*, MIC values among all four copolymer sets generally vary within only a factor of 10.

The **MM** homopolymer displays weak to modest antibacterial activity, depending on the species, and MIC generally improves (becomes smaller) as lipophilic subunits are introduced. One exception to this trend has already been noted, involving *E. coli* MIC for copolymers with low **CP** content. Another exception is seen among the **CO**-containing copolymers for all four bacteria: MIC declines as **CO** content rises to ~30% (i.e., 70% **MM**), but then MIC rises with further increases in **CO** content. Thus, it appears that increasing lipophilicity up to a certain point promotes the inhibition of bacterial growth, but too much lipophilicity is counterproductive with respect to antibacterial activity. Interpretation of this trend in terms of net copolymer lipophilicity rather than some conformational effect specific to the cyclooctyl ring is supported by the MIC trends observed among **CHx**- and **CHp**-containing copolymers with the largest lipophilic proportions (right sides of Figure 8B and 8C). In both series, MIC values for all four bacteria are higher for 30% **MM** than for 40% **MM**. Since the cyclohexyl and cycloheptyl rings are less lipophilic than the cyclooctyl ring, it is not surprising that these "upturns" in MIC graphs occur at higher proportions of the lipophilic subunit relative to the upturn in the **CO** MIC graphs.

The homopolymers of **DM** and **MM** are generally comparable in antibacterial activity, with a significant advantage for **DM** vs **MM** observed only in the case of *E. faecium*. Nevertheless, the **DM** homopolymer is more hemolytic: the MHC is >100-fold lower than the MHC for the **MM** homopolymer (Table 1). These trends are consistent with others noted above in that making the polymer more lipophilic has a stronger effect on the propensity to induce hemoglobin release from RBC than on the ability to inhibit bacterial growth. Figure 8E shows that the **DM-CHx** copolymers containing ≥20% **CHx** display low MIC values against all four bacteria, but MHC values for these copolymers are low as well. The 50:50 **DM:CHx** copolymer is the most potent antibacterial agent among all the materials we examined.

Assessing Polymer Selectivity. Figure 9 shows how the concentration of one polymer, 63:37 **MM:CHx** with an N-terminal *p*-(*tert*-butyl)benzoyl group, an average of ~32 subunits, and PDI = 1.06, affects the growth of *E. coli* and the extent of hemoglobin release from RBC. The MIC is graphically identified based on the *E. coli* growth data, and the MHC is graphically identified based on the hemoglobin release data. The

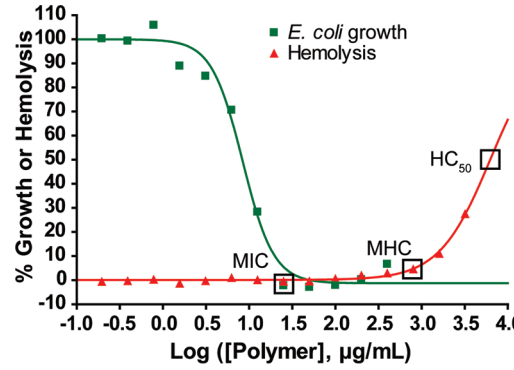


Figure 9. Activity curves for polymer 63:37 **MM:CHx** vs *E. coli* and hRBC. The MIC value vs *E. coli* and HC_{50} and MHC values vs hRBC are indicated.

Table 2. Selectivity of 50:50 **DM:CHx** *p*-(*tert*-Bu)-benzoyl Copolymer vs Each Bacterial Strain

	MIC,[a] μg/mL	HC_{50}/MIC	MHC/MIC
E. coli	6.25	17	2
B. subtilis	0.78	140	16
S. aureus	6.25	17	2
E. faecium	1.56	68	8
MHC[b]	12.5		
HC_{50}	106		

[a] MIC = minimum inhibitory concentration. [b] MHC = minimum hemolytic concentration.

HC_{50} estimate is derived from extrapolation of the curve[38] beyond the highest concentration examined, because only 27% hemoglobin release was observed at that concentration (3200 μg/mL). The MHC value is challenging to identify with precision because there appears to be a very low level of hemoglobin release (<5%) over a >10-fold range of polymer concentration. We found this behavior to be common, but not universal, among our polymers. In contrast, MIC values were generally less ambiguous with our polymers, as illustrated in Figure 9. The imprecision of MHC determination may explain why some researchers use HC_{50} as the metric of hemolytic activity; however, as illustrated by the present example, HC_{50} determination is inherently inaccurate for agents that have a low propensity to induce hemoglobin release, which are often the most interesting cases.

Selectivity for inhibition of bacterial growth relative to hemolysis is commonly quantified as HC_{50}/MIC[18−22] or MHC/MIC.[34c−e] Since HC_{50} is necessarily larger than MHC, use of HC_{50}/MIC will always lead to a numerically greater selectivity index than will MHC/MIC. Although selectivities may appear less impressive when expressed as MHC/MIC, this approach seems more sensible because the nature of the MIC, which indicates when a plateau in behavior is reached as a function of concentration, is more akin to the nature of the MHC than to the nature of the HC_{50}, which is the midpoint in behavior change as a function of concentration.

The impact of the way hemolytic activity is defined is illustrated by the comparison shown in Table 2 for data obtained with a 50:50 **DM:CHx** polymer (see Figure 8E). As noted above, this material shows the strongest antibacterial activity

(38) The hemolysis curve is the result of six independent experiments run in triplicate (i.e., 18 data sets), averaged, and curve fit in GraphPad Prism 4 using a sigmoidal dose response function (variable slope) with the top of the curve constrained to 100%. The *E. coli* curve was generated similarly using four duplicate experiments (8 data sets).

among the nylon-3 polymers studied to date. MHC for this material is 6.25 μg/mL, while HC_{50} is 106 μg/mL. Table 2 shows the MIC values determined with this polymer for the four bacteria and the selectivities calculated for each bacterium as either HC_{50}/MIC or MHC/MIC. The polymer appears to be rather selective when the former parameter is employed but nonselective when the latter is employed.

The way one calculates selectivity for inhibition of bacterial growth relative to induction of hemoglobin release from RBC becomes important when comparisons are made. This point can be illustrated in the context of a recent paper from Tew et al., who reported antibacterial and hemolytic activities for amphiphilic polymers prepared via ring-opening polymerization of oxanorbornene derivatives.[22] One polymer was reported to display a selectivity of >533, based on MIC for *S. aureus* and HC_{50}-derived hemolytic activity. The authors concluded that this selectivity is "unprecedented".[22a] In their discussion of related results, Tew et al. noted that our preliminary report on antibacterial nylon-3 polymers described a maximum selectivity of 32 for *S. aureus* vs hemolysis and characterized this result as "far from optimal";[22a] these results were obtained for the 63:37 **MM:CHx** polymer with the *p*-(*tert*-butyl)benzoyl end group. However, the numerical comparison of selectivities implied by this discussion, >533 vs 32, is not meaningful because the nylon-3 derivative selectivity was calculated as MHC/MIC while the poly(oxanorbornene) selectivity was calculated as HC_{50}/MIC. Use of comparable parameters to assess the two polymers leads to the conclusion that their selectivies are similar. For example, HC_{50}/MIC >128 for the nylon-3 polymer and *S. aureus*, which indicates that the selectivity of this material and the best poly(oxanorbornene) (>533) are not distinguishable by this criterion. (Only lower limits are available in each case because neither polymer reached 50% hemoglobin release at the maximum concentration tested.) The MHC for the best poly(oxanorbornene) is $\leq$50 μg/mL, according to the available data.[39] If we conservatively use 50 μg/mL, then MHC/MIC = 13 for this polymer, which shows that selectivity in this case is no better than the selectivity previously reported for the 63:37 **MM:CHx** nylon-3 copolymer (MHC/MIC = 32).

Polymer Self-Association. The data reported above show that increasing the overall lipophilicity of nylon-3 polymers generally makes them more effective at inhibiting bacterial growth and at inducing hemoglobin release from RBC. Such trends have been previously observed among host-defense peptides and synthetic α-peptide analogues,[12,33,34c,d,40] oligomers intended to mimic these peptides[6,34e] and amphiphilic polymers.[21,22] In some cases, however, we observe that increasing nylon-3 lipophilicity causes a *decrease* in bacterial growth inhibition (i.e., an increase in MIC), although this effect is not seen in hemolytic activity. The two most striking examples of this trend were observed for lengthening the alkanoyl end group at the N-terminus of 63:37 **MM:CHx** copolymers (Figure 6) and for increasing the lipophilic subunit proportion in **MM:CO** copolymers (Figure 8D). One potential explanation for this behavior is that the more lipophilic polymers self-associate, and the self-associated state is less effective at inhibiting bacterial growth than is a nonassociated state.[33] Hodges et al.[34c,d] as well as Meng and Kumar[40] postulated that self-association of helix-forming α-peptides can hinder antibacterial activity if it is more difficult for the associated form than for a monomer to pass through the dense cell wall en route to the bacterial membrane. Papo and Shai have shown that the ability of a self-associating α-peptide to reach the bacterial membrane is indeed correlated with antibacterial efficacy.[36] We tested the hypothesis that the decline in antibacterial activity among highly hydrophobic polymers arises from polymer self-association by evaluating the abilities of selected polymers to solubilize a lipophilic fluorescent dye in aqueous solution.

Lipophilic dye solubilization is commonly used to determine the critical micelle concentration (CMC) of detergents, that is, the minimum concentration necessary for detergent molecules begin to self-associate to form micelles.[41] These measurements involve combining aqueous solutions that contain incrementally increasing surfactant concentrations with a water-insoluble dye and then determining the amount of dye that has been solubilized in each sample via either absorbance or fluorescence (depending on the dye). The CMC is the concentration at which the onset of dye uptake occurs. Among our polymers, the shape and size of the aggregates is likely to vary widely due to the high variation of size and sequence of the molecules; therefore, we express our results as a critical aggregation concentration (CAC), rather than the more commonly used CMC.

Table 3 summarizes results obtained with the **MM:CO** copolymers using the fluorescent dye 1,6-diphenylhexatriene.[41] These experiments were carried out in using the same Tris-buffered saline solution that was used in our hemolysis assays. For the most lipophilic members of this series the polymer solutions contained 4 vol % DMSO, because the polymer stock solution had to be prepared in DMSO. The most heavily cationic members of this series, with subunit ratios of 70:30 or higher, did not cause detectable dye solubilization at the highest polymer concentration evaluated (3200 μg/mL). However, for more lipophilic members of this series, with **MM:CO** ratio of 60:40 or lower, dye solubilization was detected at polymer concentrations of 90−150 μg/mL. Interestingly, the polymer composition at which dye solubilization becomes detectable is similar to the composition at which increasing the proportion of the lipophilic **CO** subunit causes a drop in antibacterial activity. These trends should not necessarily overlay perfectly because the solvents are different (Tris-buffered saline vs bacterial growth medium). Nevertheless, the parallel between these two behaviors as a function of polymer composition suggests that the decrease in antibacterial activity among the most lipophilic polymers arises from polymer self-association and may reflect difficulties encountered by polymer aggregates in traversing the bacterial cell wall.[34c,d,40]

Table 4 summarizes the results obtained with 63:37 **MM:CHx** polymers bearing N-terminal alkanoyl groups of increasing lipophilicity. The trends in this series are comparable to those seen among the **MM:CO** copolymers in that only the most lipophilic **MM:CHx** polymers are capable of solubilizing the dye. Thus, for end groups containing up to 10 carbons, no solubilization is detected up to the maximum polymer concentration examined (3200 μg/mL), but dye solubilization is detected for members of this series containing 12-, 16-, or 18-carbon end groups, at polymer concentrations in the 100−160 μg/mL range. The ability to inhibit bacterial growth declines as end group length increases from 12 to 16 to 18 carbons. Neither Table 3 nor Table 4 shows a decline in hemolytic activity among the most hydrophobic polymers. Since erythrocytes lack a cell wall, approach of larger polymer aggregates

(39) We used data from the Supporting Information of ref 22a.
(40) Meng, H.; Kumar, K. *J. Am. Chem. Soc.* **2007**, *129*, 15615–15622.
(41) Chattopadhyay, A.; London, E. *Anal. Biochem.* **1984**, *139*, 408–412.

Table 3. Activity of **MM-CO** Copolymers Compared to Their Observed Critical Aggregation Concentrations (CAC)

MM:CO ratio	MIC,[a] μg/mL *E. coli*	MHC,[b] μg/mL	CAC, μg/mL
90:10	400	12.5	>3200
80:20	200	6.25	>3200
70:30	25	3.13	>3200
60:40	50	1.56	150
50:50	50[c]	1.56	90
40:60	400[c]	1.56	90

[a] MIC = minimum inhibitory concentration. [b] MHC = minimum hemolytic concentration. [c] Four percent DMSO was added to aid solubility.

Table 4. Activity of **MM-CHx** Copolymers with Alkanoyl End Groups of Varying Length Compared to Observed Critical Aggregation Concentrations (CAC)

acyl tail length	MIC,[a] μg/mL *E. coli*	MHC,[b] μg/mL	CAC, μg/mL
2	400	800	>3200
3	400	800	>3200
6	400[c]	800	>3200
8	50[c]	800	>3200
10	50[c]	200	>3200
12	12.5[c]	6.25	160
16	100[c]	3.13	120
18	200[c]	1.56	105
p-(*t*-Bu)C_6H_4	25	800	>3200

[a] MIC = minimum inhibitory concentration. [b] MHC = minimum hemolytic concentration. [c] Four percent DMSO was added to aid solubility.

to the membrane may not be hindered relative to approach of smaller unassociated nylon-3 molecules; Hodges et al. offered an analogous argument regarding the hemolytic activity of highly hydrophobic α-peptides.[34c,d] Nonmonotonic effects on antibacterial activity have been observed upon increasing the length and therefore the hydrophobicity of alkyl appendages on quaternary vinylpyridinium polymers[16a,b] and quaternary vinylpyridinium-methacrylate ester copolymers,[16e] with optimal activity observed for intermediate hydrophobicity.

Random Copolymers vs Sequence-Specific Peptides. The results reported here show that random copolymers based on a peptide-like backbone and containing both lipophilic and cationic subunits can display a profile of biological activities comparable to that of discrete, naturally evolved peptides. Many of these host-defense peptides are believed to adopt a specific conformation in the biologically active form, which leads to spatial segregation of lipophilic and cationic side chains.[12,13] Such segregation appears to be crucial for the membrane-disruption mode of action that is thought to underlie the antibacterial effects of many host-defense peptides. Our nylon-3 polymers are unlikely to adopt regular conformations, in contrast to the peptide prototypes, because the polymers are heterogeneous in terms of subunit sequence and subunit stereochemistry. We hypothesize that most or all molecules within the polymer mixture are nevertheless able to achieve global amphiphilicity by adopting *irregular* conformations in which cationic and lipophilic surfaces are well segregated, and these conformations enable membrane disruption. The sequence- and stereoheterogeneity of the copolymers make it difficult or impossible to apply the spectroscopic techniques that we used previously to establish the global amphiphilicity of β- and α/β-peptide antimicrobial agents.[5b] We do not have direct evidence of global segregation of lipophilic and cationic side chains in the biologically active polymer conformations, but extensive prior evidence of such behavior in host-defense peptides themselves and discrete oligomers intended to mimic these peptides suggests by extrapolation that achieving global amphiphilicity is functionally important for our random copolymers as well.

In the most favorable cases, the polymers mimic host-defense peptides in causing potent inhibition of bacterial growth but showing little tendency to disrupt eukaryotic cell membranes. Well-established structure−activity relationships among natural host-defense peptides and related synthetic α-peptides are mirrored among the random nylon-3 copolymers. Thus, for example, as the polymers are made more hydrophobic (by increasing the lipophilic:cationic subunit ratio, increasing the intrinsic hydrophobicity of subunits, or adding increasingly hydrophobic units to one end of the polymer chain), they tend to become less selective for bacterial growth inhibition relative to hemolysis. Similar trends have been reported among α-peptides.[3,12,33,34c] It has been noted that antimicrobial activity (but not hemolytic activity) can decline with increasing hydrophobicity, if net hydrophobicity is high,[5] and we observe a comparable trend among our polymers. This behavior among α-helical peptides has been correlated with peptide self-association;[33,34c,d,36,40] analogously, our results suggest that sufficient hydrophobicity to cause a decline in antibacterial activity leads also to polymer aggregation.

There has been considerable debate regarding the mechanism by which natural peptides and synthetic analogues disrupt biological membranes, and it seems possible that the mechanism can change as a function of the nature of the membrane and/or the sequence of the peptide. The "barrel-stave" model[42] postulates that small numbers of peptide molecules self-assemble in a specific manner within the membrane to form discrete pores. The "carpet" model,[43] in contrast, postulates that peptides combine with lipids to form mixed micelles, which leads to a profound disruption of bilayer structure. The "toroidal pore" model[44] is in some ways intermediate between the other two hypotheses, since the pores can vary in size. Discrimination between prokaryotic and eukaryotic cell membranes by antimicrobial α-peptides has recently been suggested to arise from a difference in the manner in which such peptides disrupt these two types of membranes, with a carpet mechanism operational for prokaryotes and a barrel-stave mechanism operational for eukaryotes.[13,34c,d]

Given the compositional and stereochemical heterogeneity of our polymers, we propose that the formation of discrete polymer-lined pores in a bilayer, as dictated by the barrel-stave model, is improbable, because it is difficult to see how a heterogeneous mixture of molecules could self-assemble into a discrete pore structure. It also seems unlikely that the mechanistic discrimination hypothesis could explain the high prokaryote vs eukaryote selectivity manifested by some of our nylon-3 copolymers. Therefore, our results suggest that a carpet-like mechanism, possibly involving variably sized toroidal pores, is more likely to explain selectivity for bacterial membranes relative to RBC membranes observed for some of our polymers. This conclusion raises the possibility that α-peptide selectivity does not require a cell-based difference in mode of membrane disruption.

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The antibacterial behavior of host-defense peptides and synthetic analogues is often interpreted solely in terms of direct and disruptive interactions between these molecules and bacterial membranes (barrel-stave, toroidal pore, and carpet mechanisms). However, an indirect mechanism of disruption is possible as well: cationic peptides could induce natural autolysis systems in bacteria.[45] Autolytic enzymes degrade the bacterial cell wall as part of the "membrane stress response." Under most conditions these enzymes are held in check by natural inhibition mechanisms. Activation of autolytic enzymes by host-defense peptides or analogues could ultimately cause the barrier function of the bacterial membrane to be compromised.[46]

Mor and co-workers recently reported synthetic oligoacyllysines that act by different mechanisms based on differences in size: larger, more cationic oligomers interact more strongly with the membrane to cause lysis, whereas smaller oligomers penetrate the membranes without disrupting them and are able to interact with DNA.[7b] It is possible that subpopulations of different sizes within our polymer mixtures exert toxic effects on bacteria via different mechanisms.

Conclusions

The structure−activity relationships documented here encourage a broadening of thought regarding the ways in which molecules can compromise bilayer integrity, beyond the concepts that have been developed via extensive study of host-defense peptides and analogous discrete oligomers. In addition to basic insights, this work and related studies[22] raise the possibility that antibacterial copolymers might ultimately be useful. Copaxone, a polymeric multiple sclerosis medication, shows that copolymers can be successful for biomedical applications. Large-scale preparation of host-defense peptides requires stepwise synthesis, which is costly. In contrast, random copolymer synthesis is a one-pot process (in our case, a subsequent deprotection step is required). Thus, the nylon-3 antibacterial agents are much more readily available than comparable α-peptides.

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Supporting Information Available: Materials and instrumentation, polymer characterization data, CAC measurements, and supplementary biological data. This material is available free of charge via the Internet at http://pubs.acs.org.

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