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Effects of Cyclic vs Acyclic Hydrophobic Subunits on the Chemical Structure and Biological Properties of Nylon-3 Copolymers

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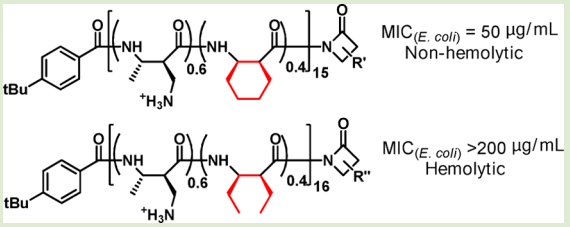
Supporting Information

ABSTRACT: Nylon-3 copolymers containing both hydrophobic and cationic subunits can mimic the activity profile of host-defense peptides, if subunit identity and proportion are carefully selected. These sequence- and stereo-random copolymers inhibit bacterial growth at relatively low concentrations, apparently via disruption of bacterial membranes, but they are relatively nondisruptive toward eukaryotic cell membranes (low hemolytic activity). In all previous examples, the hydrophobic subunits have contained cycloalkyl groups that incorporate the backbone Cα−Cβ bond. Here we have explored the effects of using analogous acyclic hydrophobic subunits. The results indicate that replacing cyclic with acyclic hydrophobic subunits has a modest influence on biological properties. This influence appears to arise from differences in subunit flexibility.



Increasing bacterial resistance to conventional antibiotics has drawn considerable attention toward the development of new types of antibacterial agents. Host-defense peptides are attractive in this regard because these natural antibiotics can display broad-spectrum activity, and resistance to these peptides seems to be difficult for bacteria to develop.[1] Host-defense peptides are very diverse in terms of sequence, but most feature a combination of hydrophobic and basic amino acid residues.[2] Many of these peptides appear to disrupt bacterial membranes.[1,3] A strong preference for prokaryotic vs eukaryotic cell membranes is typically observed, apparently as a result of Coulombic attraction between these cationic peptides and the net negative charge that is characteristic of prokaryotic cell surfaces.[4] Numerous synthetic peptides and related oligomers have been reported to display activity/selectivity profiles comparable to those of host-defense peptides;[5] however, the practical application of natural or designed peptides is limited by the cost associated with stepwise synthesis, which is necessary if amino acid sequence is to be controlled.

Some time ago we proposed that the membrane-selective activity profile of host-defense peptides could be mimicked with *sequence-random* copolymers containing both hydrophobic and cationic subunits if key physical characteristics, such as cationic:hydrophobic proportion and net hydrophobicity, were properly controlled.[6] Validation of this prediction would be useful because random copolymers are much easier to synthesize than are sequence-specific peptides. In recent years diverse cationic−hydrophobic copolymers have been evaluated for antimicrobial function;[7,8] earlier reports described antibacterial homopolymers but did not include comparisons with natural peptides.[9] Our efforts have focused on nylon-3 materials because the subunits are β-amino acid residues and the polymer backbone therefore bears an intrinsic similarity to that of conventional polypeptides (α-amino acid residues).[8] We have identified specific hydrophobic/cationic subunit identities and proportions that lead to substantial growth inhibition for several bacterial species but low propensity for eukaryotic membrane disruption, as monitored by lysis of red blood cells ("hemolysis"). Optimal nylon-3 copolymers (Figure 1) display biological activities comparable to those of helix-forming host-defense peptides.[8]

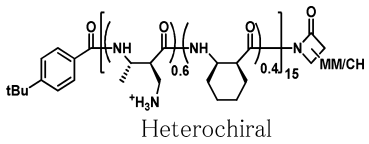


Figure 1. Representative nylon-3 copolymer containing subunits derived from β-lactams **MMβ** and **CHβ** (both racemic) in a 60:40 ratio. The polymer inhibits the growth of several bacteria at relatively low concentrations but exhibits only weak hemolytic activity.

All nylon-3 copolymers examined for antibacterial activity to date contain cycloalkane-based hydrophobic subunits.[8] Here we examine the effects of replacing these cyclic subunits with analogous acyclic subunits.

Nylon-3 materials are synthesized by anionic ring-opening polymerization of β-lactams;[10] use of an electrophilic co-initiator determines the group appended to the N-terminus of

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each polymer chain.[8,11] We have previously used β-lactams **DMβ** and **MMβ** to generate cationic subunits (after Boc deprotection) and **CHβ** and **COβ** to generate hydrophobic subunits.[8] For the present study β-lactams **HEβ**[12] and **OCβ**[13] (Figure 2) served as alternative sources of hydrophobic

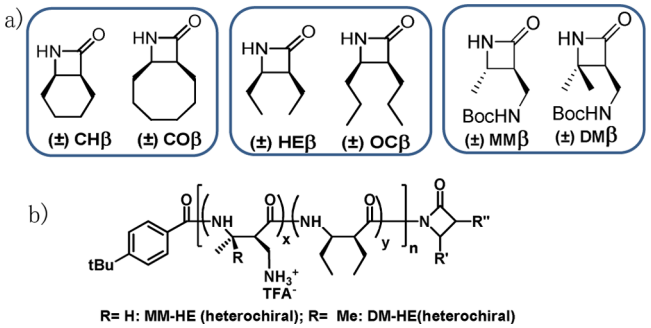

Figure 2. (a) β-lactams used in this study. (b) Random nylon-3 copolymer containing the *cis*-hexyl subunit.

subunits. **HEβ** and **OCβ** are analogues of **CHβ** and **COβ**, respectively, lacking the carbocycle; these β-lactams were prepared from the corresponding *cis*-alkenes. Each β-lactam was used in racemic form, and the resulting copolymers are therefore random in terms of both the sequence and stereochemistry. New polymers in the **DM+HE** and **DM+OC** series were prepared in THF.[8,11a,b] (Note: "**DMβ**" designates a β-lactam, while "**DM**" designates the resulting nylon-3 subunit.) Use of 5 mol % acid chloride co-initiator (relative to total β-lactam) provided materials with 20−30 subunit average chain length and polydispersity indices (PDIs) of 1.01−1.41.[14] Attempts to prepare **MM+HE** copolymers in THF, however, resulted in insoluble gels, and these materials were therefore synthesized in dimethylacetamide (DMAc). Attempts to prepare **MM+OC** copolymers were problematic in either THF or DMAc.

Antibacterial activities were initially assessed by measurement of minimum inhibitory concentrations (MIC) for four bacteria, *Escherichia coli*,[15] *Bacillus subtilis*,[16] methicillin-resistant *Staphylococcus aureus* (MRSA),[17] and vancomycin-resistant *Enterococcus faecium* (VRE).[18] Antibacterial activities for selected copolymers are summarized in Table 1. Figure 3 shows the

Table 1. Nylon-3 Copolymer Antibacterial Activities

	MIC[a] (μg/mL) of copolymers			
polymer	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*
$DM_{50}CH_{50}$	6.25	3.13	6.25	6.25
$DM_{50}HE_{50}$	25	6.25	25	25
$DM_{50}CO_{50}$	6.25	1.6	6.25	6.25
$DM_{50}OC_{50}$	100	6.25	25	25
$MM_{60}CH_{40}$	50	3.13	12.5	25
$MM_{60}HE_{40}$	>200	3.13	12.5	25

[a]MIC = Minimum inhibitory concentration for bacterial growth.

extent of hemolysis caused by selected copolymers as a function of their concentration. Direct comparisons between analogous polymers reveal that those containing cyclically constrained hydrophobic subunits (**CH** or **CO**) display somewhat more desirable properties than do those containing analogous acyclic hydrophobic subunits (**HE** or **OC**). Thus, for example, $DM_{50}CH_{50}$ shows moderately lower MIC values than does

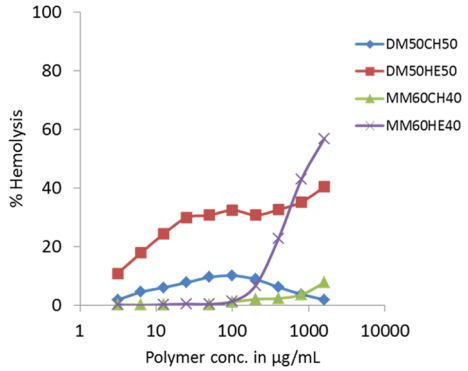

Figure 3. Hemolytic activity of nylon-3 copolymers.

$DM_{50}HE_{50}$, and the **CH**-containing copolymer induces less hemolysis at a given concentration than does the **HE**-containing polymer. A similar trend in antibacterial activities is observed for $DM_{50}CO_{50}$ vs $DM_{50}OC_{50}$, with the cyclic **CO** subunit providing lower MIC values than the acyclic **OC** subunit; however, both of these copolymers are highly hemolytic.[14] For the pair containing **MM**, in a 3:2 ratio with hydrophobic subunit **CH** or **HE**, the MIC values are identical for the three Gram positive bacteria, but the polymer containing **CH** is more active against *E. coli* (Gram negative) than is the polymer containing **HE**. In this case use of the cyclic **CH** subunit results in substantially less hemolytic activity than does use of the acyclic **HE** subunit.

Why do nylon-3 copolymers prepared with cyclic β-lactam **CHβ** or **COβ** display different and somewhat more favorable biological properties relative to copolymers prepared with analogous acyclic β-lactam **HEβ** or **OCβ**? At least three factors could underly the activity variations, individually or in combination: (1) differences in subunit conformational propensity, (2) differences in subunit hydrophobicity, and/or (3) differences in subunit distribution along the copolymer chains. Within each cyclic−acyclic pair, **CH**−**HE** or **CO**−**OC**, overall subunit hydrophobicities are expected to be similar because the number of carbon atoms is identical; however, reverse-phase HPLC comparison of β-lactams indicates that **CHβ** is somewhat less hydrophobic than **HEβ**, which is consistent with the lower aqueous solubility of *n*-hexane relative to cyclohexane.[19] Lipophilic dye solubilization measurements indicated similar critical aggregation concentration (CAC) values for representative pairs of polymers in aqueous solution (identical hydrophobic:cationic proportion for each pair).[14] Since self-association of these copolymer chains in water is presumably driven by the hydrophobic effect, the similarity in CAC values suggests that replacing **CH** with **HE**, or **CO** with **OC**, does not lead to a large change in net copolymer hydrophobicity.

We have previously shown that variations in β-lactam structure can cause differences in polymerization reactivity, which leads to a compositional drift along the resulting copolymer chains.[11b] To assess whether variations in compositional drift arise from replacing **CHβ** with **HEβ** in a polymerization reaction, we monitored subunit incorporation into nylon-3 copolymers generated from **DMβ** + **CHβ** or **DMβ** + **HEβ** as a function of β-lactam conversion (Figure 4). The results suggest that both copolymers are subject to compositional drift and that the extent and nature of this drift differ between the two materials. For $DM_{50}CH_{50}$, analysis of remaining β-lactam after varying extents of polymerization

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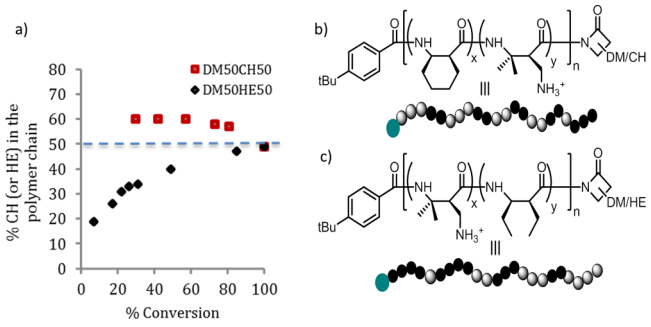

Figure 4. (a) Composition of copolymers (fraction derived from **CH*β*** or **HE*β***, as deduced by measuring residual *β*-lactam by GC at various extents of reaction, at room temperature) versus total conversion of the monomers; (b, c) cartoons meant to illustrate different subunit distributions in typical copolymer molecules that result from replacing the cyclic **CH** unit with the acyclic **HE** unit in the polymer backbone. The open balls represent **CH** or **HE** subunits, and the filled balls represent **DM** subunits. The green ball represents the N-terminal p-*t*-benzoyl group.

indicates that **CH*β*** is incorporated a little more readily than **DM*β*** into the growing polymer chains. Therefore, after complete conversion and side chain deprotection there should be a slightly higher incidence of hydrophobic subunits near the N-terminus relative to the C-terminus, and vice versa for the cationic subunits, for a typical polymer molecule. In contrast, the data for 1:1 copolymerization of **HE*β*** and **DM*β*** indicate that **DM*β*** is incorporated substantially more readily than **HE*β*** into growing polymer chains. In this case, after complete conversion and side chain deprotection there should be a preponderance of cationic subunits near the N-terminus and a corresponding preponderance of hydrophobic subunits near the C-terminus of the average polymer molecule. It should be noted that the chain ends of these nylon-3 copolymers are not functionally equivalent,[8d] since all N-termini bear a hydrophobic p-*t*-butylbenzoyl unit, derived from the co-initiator, while either a unit derived from **CH*β*** or a unit derived from **DM*β*** can occur at the C-termini. The data in Figure 4 suggest that differences in compositional drift between nylon-3 copolymers containing cyclic vs acyclic hydrophobic subunits could potentially contribute to variations in biological activity profiles among such materials.

To test the hypothesis that differences in compositional drift between $\mathbf{DM_{50}CH_{50}}$ and $\mathbf{DM_{50}HE_{50}}$ contribute to activity differences, we synthesized copolymers designated $\mathbf{rDM_{50}HE_{50}}$ by introducing **HE*β*+DM*β*** monomer mixtures in two portions, with differing monomer proportions, to the polymerization reaction.[14] The initial monomer mixture was enriched in **HE*β***, either 2:1 or 3:1, to diminish the predominance of **DM** near the N-termini of the polymer chains; the second monomer batch had the opposite proportion, so that the overall 1:1 subunit proportion was maintained. The $\mathbf{rDM_{50}HE_{50}}$ and $\mathbf{DM_{50}HE_{50}}$ polymers displayed similar biological activity profiles,[14] which suggests that variations in subunit distribution along the $\mathbf{DM_{50}HE_{50}}$ vs $\mathbf{DM_{50}CH_{50}}$ polymer chains do not have a strong influence on the antibacterial and hemolytic activities of these nylon-3 materials.

As a further examination of functional differences between nylon-3 copolymers containing constrained vs flexible subunits, we evaluated the activities of $\mathbf{DM_{50}CH_{50}}$ and $\mathbf{DM_{50}HE_{50}}$ against several pathogenic bacteria including *E.coli* (CFT073; uropathogenic)[20] and *P. aeruginosa* (PA1066; cystic fibrosis isolate), in terms of both MIC and minimum bactericidal activity (MBC) (Table 2). Both polymers were bactericidal at

Table 2. Bactericidal Activity of $\mathbf{DM_{50}CH_{50}}$ vs $\mathbf{DM_{50}HE_{50}}$

	$\mathbf{DM_{50}CH_{50}}$		$\mathbf{DM_{50}HE_{50}}$	
bacterium	MIC (*μ*g/mL)	MBC[a] (*μ*g/mL)	MIC (*μ*g/mL)	MBC[a] (*μ*g/mL)
E. coli (CFT073)	25	50	50	50
S. enterica (LT2)	50	100	50	100
B. cereus (ATCC14579)	100	100	100	100
P. aeruginosa (PA01)	25	25−50	50	50−100
P. aeruginosa (PA1066)	25	50	50	100

[a]MBC = Minimum bactericidal concentration.

or near the concentration necessary to inhibit growth (i.e., the MIC). In these tests there were no significant differences between polymers with the cyclic and acyclic subunits, in contrast to the modest differences manifested in Table 1. Moreover, the MIC values in these cases are somewhat higher than those in Table 1. In this regard, it should be noted that the Gram negative bacterium in Table 1 is a laboratory strain of *E. coli*, while clinically isolated bacteria, which tend to be hardier than laboratory strains, were used for Table 2. In addition, differences in composition of the growth medium and time of incubation could account for differences in the MIC values in the two tables.[14] We found that none of the bacteria represented in Table 2 was susceptible to growth inhibition by the host-defense peptide Magainin 2.[14]

Antibacterial random copolymers are of potential utility because they are much easier to prepare than are sequence-specific peptides. Although subunit sequence is not controlled in a random copolymerization reaction, the properties of the resulting materials can be modulated by changing subunit identity and proportion. Previous efforts to vary hydrophobic subunit properties in nylon-3 copolymers have focused on altering side chains, e.g., adding or subtracting carbon atoms to tune hydrophobicity or modifying the nature of the cationic group.[8] Here we have evaluated a different type of polymer tailoring, involving the use of cyclic vs acyclic hydrophobic subunits. Our results indicate that a cyclic hydrophobic subunit (**CH** or **CO**) can confer improved properties relative to an analogous acyclic subunit (**HE** or **OC**). In principle, this trend could arise from (1) alterations in subunit distribution along the polymer chains, (2) changes in subunit hydrophobicity, and/or (3) changes in local backbone flexibility as a result of replacing cyclic with acyclic hydrophobic subunits; however, experiments described above suggest that the first two factors do not exert strong effects on biological activity profiles. Thus, changes in local backbone flexibility between cyclic and acyclic hydrophobic nylon-3 subunits seem to have the largest influence on polymer properties.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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