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Ternary Nylon-3 Copolymers as Host-Defense Peptide Mimics: Beyond Hydrophobic and Cationic Subunits

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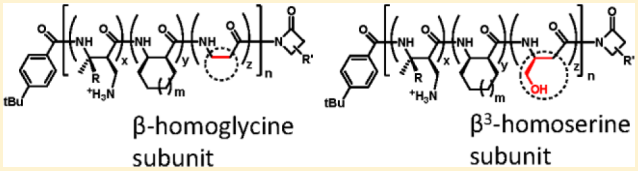
Supporting Information

ABSTRACT: Host-defense peptides (HDPs) are produced by eukaryotes to defend against bacterial infection, and diverse synthetic polymers have recently been explored as mimics of these natural peptides. HDPs are rich in both hydrophobic and cationic amino acid residues, and most HDP-mimetic polymers have therefore contained binary combinations of hydrophobic and cationic subunits. However, HDP-mimetic polymers rarely duplicate the hydrophobic surface and cationic charge density found among HDPs (Hu, K.; et al. *Macromolecules* **2013**, *46*, 1908); the charge and hydrophobicity are generally higher among the polymers. Statistical analysis of HDP sequences (Wang, G.; et al. *Nucleic Acids Res.* **2009**, *37*, D933) has revealed that serine (polar but uncharged) is a very common HDP constituent and that glycine is more prevalent among HDPs than among proteins in general. These observations prompted us to prepare and evaluate ternary nylon-3 copolymers that contain a modestly polar but uncharged subunit, either serine-like or glycine-like, along with a hydrophobic subunit and a cationic subunit. Starting from binary hydrophobic−cationic copolymers that were previously shown to be highly active against bacteria but also highly hemolytic, we found that replacing a small proportion of the hydrophobic subunit with either of the polar, uncharged subunits can diminish the hemolytic activity with minimal impact on the antibacterial activity. These results indicate that the incorporation of polar, uncharged subunits may be generally useful for optimizing the biological activity profiles of antimicrobial polymers. In the context of HDP evolution, our findings suggest that there is a selective advantage to retaining polar, uncharged residues in natural antimicrobial peptides.



INTRODUCTION

The innate immune response to bacterial infection includes the release of relatively short peptides that attack prokaryotic cells in preference to eukaryotic cells.[1] These "host-defense peptides" (HDPs) often have a net positive charge near neutral pH; Coulombic forces attract HDPs to bacterial cell surfaces, which generally bear a substantial net negative charge.[2−4] Once HDPs are localized on a cell surface, they are thought to disrupt membrane barrier function.[2,3] These disruptions appear to be associated with induction of membrane curvature, and the type of curvature involved requires certain proportions of lysines, arginines, and hydrophobic residues, which sets a specific range of cationic charge density and hydrophobicity for HDPs.[5] Many HDPs adopt α-helical conformations in interfacial environments, while others form small β-sheets or discrete tertiary structures enforced by internal disulfides.[4]

The variation in conformation, composition, and sequence evident among HDPs has led a number of groups to explore sequence-random copolymers as functional HDP mimics.[6−8] Such efforts are appealing from a practical perspective because the high cost of synthesizing peptides or other oligomers with discrete sequences would make it very difficult to use this type of molecule in real-world applications.[1b] HDP-mimetic copolymers are interesting from a fundamental perspective because their properties pose a question: why would evolution have elicited sequence-specific polypeptides for a task that can be accomplished in the absence of sequence control? One possible answer to this question is that HDPs perform functions in addition to bacterial membrane disruption.

The HDP-mimetic copolymers studied to date have been largely binary, containing one subunit that displays a positive charge on a side chain and another subunit that displays a hydrophobic side chain.[6−9] In addition, antimicrobial homopolymers have been constructed from subunits that contain both cationic and hydrophobic moieties.[10] This focus on hydrophobic and cationic components, however, is somewhat at odds with the considerable sequence variation among HDPs. Indeed, HDP-mimetic polymers typically display higher charge density and more extensive hydrophobic surface than do HDPs.[11] A high-fidelity mimic must simultaneously have lower cationic charge density and lower levels of hydrophobicity,

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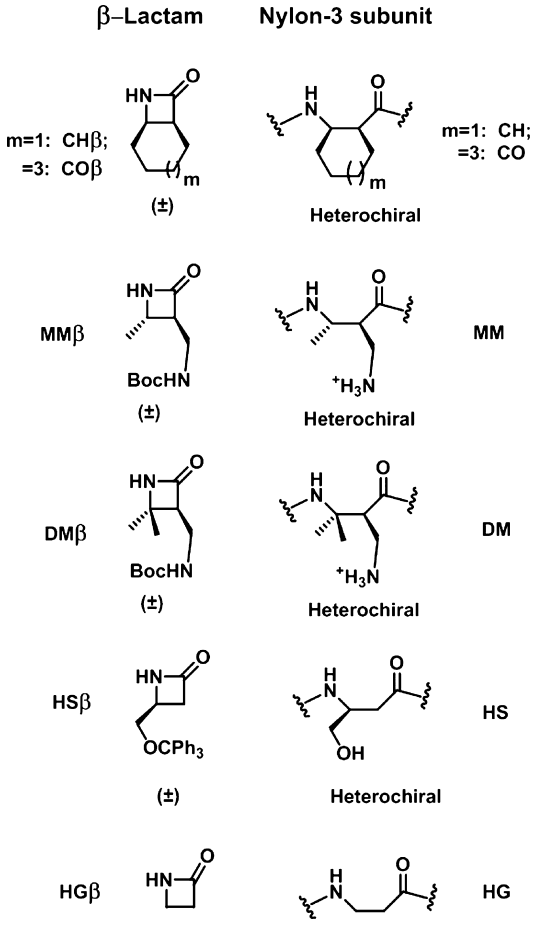
which is difficult to achieve with most binary cationic and hydrophobic subunit combinations. These considerations prompted us to examine three-component polymers containing cationic, hydrophobic, and "neutral" subunits as potential HDP mimics. Glycine is the single most common residue among known HDPs, even though Gly ranks behind Leu and Ala among known proteins.[12] Serine is quite common among HDPs, with a prevalence comparable to that found among proteins.[12] We synthesized and evaluated the biological activity profiles of ternary nylon-3 copolymers in which previously described hydrophobic and cationic subunits are supplemented by either a glycine-like or serine-like subunit.

RESULTS AND DISCUSSION

Polymer Synthesis and Characterization. The ternary nylon-3 polymers discussed here contain subunits from one of two β-lactams that have not previously been employed to generate antimicrobial materials (Scheme 1). One β-lactam bears no substituent and gives rise to a glycine-like subunit in polymer chains. This simplest of β-lactams is a derivative of β-alanine, which can also be designated β-homoglycine.[13] We designate this β-lactam **HGβ** and the resulting subunit **HG**. The other β-lactam, designated **HSβ**, gives rise to a β^3-homoserine subunit (**HS**) in polymer chains. The hydroxymethyl β-lactam precursor to **HSβ** has previously been synthesized in enantiopure form,[14] and we followed the reported route to generate racemic material, which was then trityl-protected to generate **HSβ**. Each ternary polymer contains a hydrophobic subunit, **CO** or **CH**, and a cationic subunit, **MM** or **DM**. The latter subunits bear a peripheral amino group that should be protonated and therefore positively charged at neutral pH. The necessary β-lactams, **COβ**, **CHβ**, **MMβ**, and **DMβ**, have been used previously to generate biologically active binary nylon-3 copolymers.[8] All of the chiral β-lactams were used in racemic form, and all of the ternary copolymers discussed here are therefore heterochiral mixtures.

Scheme 1. β-Lactams and Corresponding Subunits within the Nylon-3 Polymer Chain



Nylon-3 copolymers were synthesized via base-initiated anionic ring-opening polymerization (AROP) of β-lactams.[15] Reactions involving **HGβ** were conducted in *N*,*N*-dimethylacetamide (DMAc), and reactions involving **HSβ** were conducted in tetrahydrofuran (THF). *p*-*tert*-Butylbenzoyl chloride was included in all of the polymerizations; this acid chloride presumably reacts rapidly under AROP conditions to generate a mixture of *N*-acyl-β-lactams.[8,16,17] These imides then serve as co-initiators for the ring-opening polymerization process, leaving a *p*-*tert*-butylbenzoyl group at the N-terminus of each chain.[16,17] An imide derived from one of the three β-lactam starting materials remains at the C-terminus of each chain.

Acid chloride:β-lactam ratios were chosen to generate average chain lengths of 18−35 subunits. The resulting materials were analyzed via gel-permeation chromatography (GPC) prior to removal of side-chain protecting groups. Polydispersity index (PDI) values were in the range 1.05−1.43 according to data from multiangle light scattering (MALS) and a refractive index detector. Boc and trityl side-chain deprotection was accomplished by treatment with trifluoroacetic acid (Figure 1). The structures of the ternary polymers are shown in Figure 2.

Biological Activities. A panel of four bacteria, including one Gram-negative species (*Escherichia coli*[18]) and three Gram-positive species (*Bacillus subtilis*,[19] *Staphylococcus aureus*,[20] and *Enterococcus faecium*[21]), was used to gauge the antibiotic properties of the ternary copolymers. Antibacterial activity was measured in terms of minimum inhibitory concentration (MIC), i.e., the lowest concentration of a polymer that fully inhibits bacterial growth. Evaluation of HDPs and synthetic analogues typically involves an assessment of prokaryotic cell versus eukaryotic cell selectivity based on a comparison of MIC values with the extent to which an agent disrupts red blood cell (RBC) membranes. The latter property was measured by monitoring the release of hemoglobin from RBCs as a function of agent concentration. We used HC_{10}, the concentration of a polymer that causes release of 10% of the hemoglobin from human RBCs, as our index of hemolytic activity.

The studies reported here are based largely on two previously reported binary nylon-3 copolymers, $\mathbf{MM_{60}CO_{40}}$ and $\mathbf{DM_{50}CH_{50}}$.[8c] Both polymers are active against the bacteria in our test panel (low MIC values), but both are also quite hemolytic (low HC_{10} values). These two binary polymers provide an excellent opportunity to determine whether incorporation of a third nylon-3 subunit, one that is neither cationic nor hydrophobic, can enable decoupling of the antibacterial activity from the hemolytic activity. In all previous studies by our group and others involving binary cationic−hydrophobic copolymers, diminishing the hydrophobic content necessarily meant increasing the cationic content and vice versa. Use of an **HG** or **HS** subunit, however, allows the hydrophobic content to be changed without affecting the overall cationic

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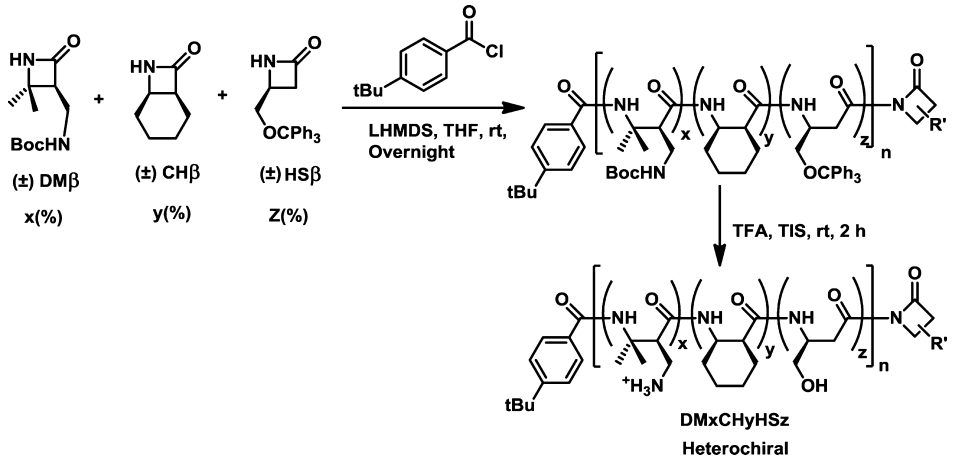

Figure 1. Synthesis of **DM** + **CH** + **HS** ternary copolymers. All of the other ternary copolymers discussed here were synthesized analogously.

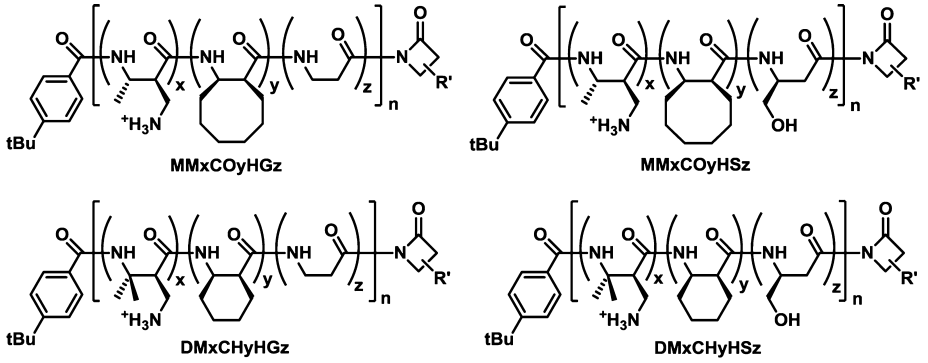

Figure 2. Structures of the ternary nylon-3 copolymers examined in this work. R′ represents the side chain from one of the starting β-lactams. All of the polymers are sequence-random and heterochiral.

Table 1. Biological Activities of MM + CO + HG and MM + CO + HS Ternary Copolymers versus $MM_{60}CO_{40}$

				MIC (μg/mL)[d]				
polymer	M_n[a]	DP[b]	PDI[c]	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*	HC_{10} (μg/mL)[e]
$MM_{60}CO_{40}$	4402	22	1.06	25	0.78	3.13	3.13	6.25−12.5
$MM_{60}CO_{30}HG_{10}$	5352	29	1.29	25	3.13	6.25	3.13	3.13
$MM_{60}CO_{25}HG_{15}$	5704	31	1.31	25	3.13	12.5	6.25	12.5
$MM_{60}CO_{10}HG_{30}$	5670	33	1.43	200	3.13	25	50	>400
$MM_{60}CO_{30}HS_{10}$	5441	25	1.07	25	1.6	6.25	3.13	50−100
$MM_{60}CO_{25}HS_{15}$	6296	28	1.27	200	1.6	6.25	6.25	100−200
$MM_{60}CO_{10}HS_{30}$	8873	35	1.05	>200	6.25	100	100	>400

[a]Number-average molecular weight. [b]Average degree of polymerization. [c]Polydispersity index based on GPC data obtained for polymers bearing Boc protecting groups on **MM** unit side chains and trityl protecting groups on **HS** unit side chains. [d]Minimum inhibitory concentration for bacterial growth. [e]Polymer concentration for 10% lysis of hRBCs.

content or the overall charge to be altered without changing the net hydrophobicity.

Table 1 compares the biological activity profile of $MM_{60}CO_{40}$ with the profiles of six new ternary copolymers, three in which varying proportions of the hydrophobic **CO** subunits have been replaced with **HG** subunits and three in which varying proportions of the **CO** subunits have been replaced with **HS** subunits. The HC_{10} values listed in Table 1 are supplemented by the full hemolysis data sets presented in Figure 3. The proportions indicated in the polymer designation are based on the β-lactam proportions used for the synthesis of that polymer (i.e., the material designated $MM_{60}CO_{30}HS_{10}$ was prepared by copolymerization of a β-lactam mixture containing 60 mol % **MM*β***, 30 mol % **CO*β***, and 10 mol % **HS*β***). In general, the β-lactam starting materials were fully consumed in the polymerization reactions. The data in Table 1 show that replacing a small proportion of the hydrophobic **CO** subunits with "neutral" **HG** units has relatively little effect on the biological activity profile: the ternary copolymers $MM_{60}CO_{30}HG_{10}$ and $MM_{60}CO_{25}HG_{15}$ are very similar to the binary polymer $MM_{60}CO_{40}$ in terms of MIC and HC_{10} values. Replacing most of the hydrophobic **CO** units with **HG**, as in $MM_{60}CO_{10}HG_{30}$, causes a stark decline in the hemolytic activity, but the antibacterial activity declines as well (i.e., the MIC value becomes higher). This observation is consistent with the general view that a minimum level of hydrophobicity is necessary for membrane-disruption activity.

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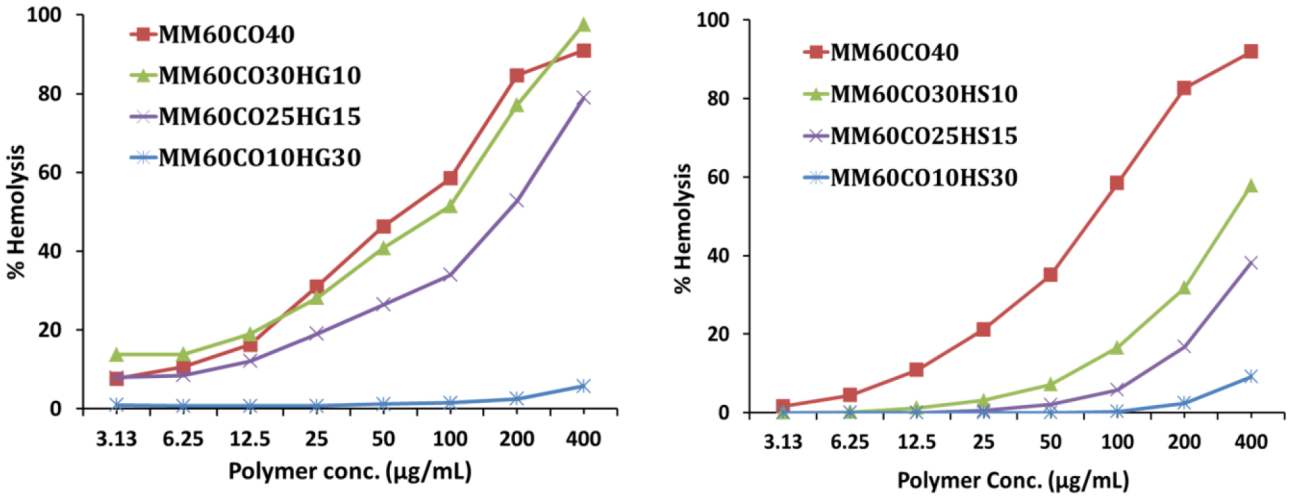

Figure 3. Hemolytic profiles of (left) **MM** + **CO** + **HG** and (right) **MM** + **CO** + **HS** ternary copolymers vs $\mathbf{MM_{60}CO_{40}}$.

Table 2. Biological Activities of DM + CH + HG and DM + CH + HS Ternary Copolymers versus $\mathbf{DM_{50}CH_{50}}$

				MIC (μg/mL)[d]				
polymer	M_n[a]	DP[b]	PDI[c]	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*	HC_{10} (μg/mL)[e]
$\mathbf{DM_{50}CH_{50}}$	4766	26	1.05	12.5	≤1.6	6.25	6.25	6.25
$\mathbf{DM_{50}CH_{40}HG_{10}}$	4341	24	1.25	12.5	3.13	12.5	12.5	25−50
$\mathbf{DM_{50}CH_{25}HG_{25}}$	5539	33	1.25	50	3.13	25	50	200
$\mathbf{DM_{50}CH_{40}HS_{10}}$	5731	28	1.09	25	1.6	12.5	6.25	50
$\mathbf{DM_{50}CH_{25}HS_{25}}$	7482	32	1.08	100	3.13	50	25	200
$\mathbf{DM_{40}CH_{50}HG_{10}}$	4383	26	1.21	6.25	3.13	12.5	12.5	12.5
$\mathbf{DM_{40}CH_{50}HS_{10}}$	5443	28	1.09	12.5	3.13	12.5	12.5	100

[a]Number-average molecular weight. [b]Average degree of polymerization. [c]Polydispersity index based on GPC data obtained for polymers bearing Boc protecting groups on **DM** unit side chains and trityl protecting groups on **HS** unit side chains. [d]Minimum inhibitory concentration for bacterial growth. [e]Polymer concentration for 10% lysis of hRBCs.

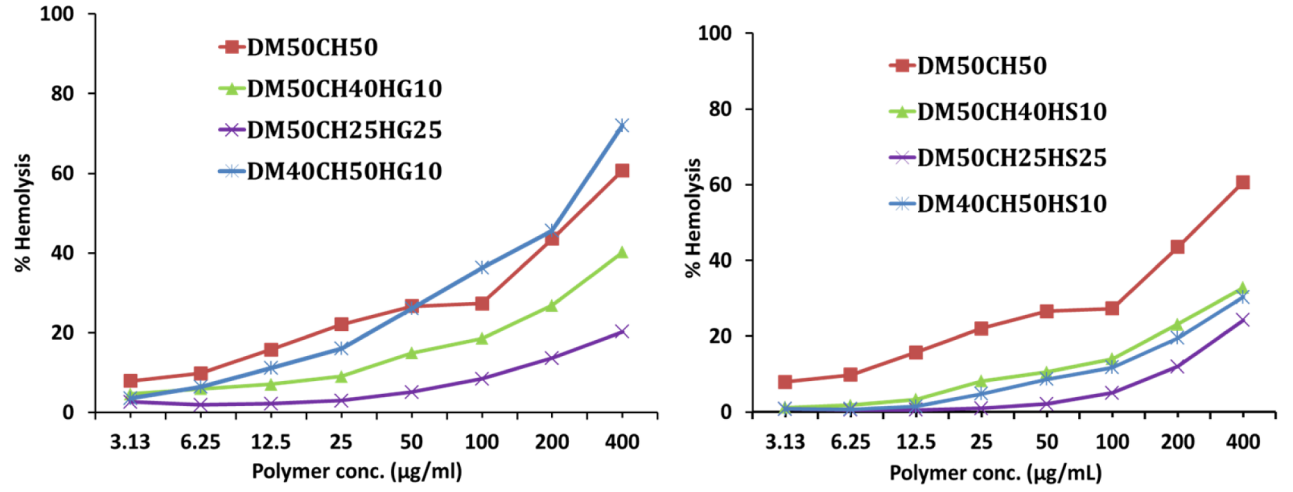

Figure 4. Hemolytic profiles of (left) **DM** + **CH** + **HG** and (right) **DM** + **CH** + **HS** ternary copolymers vs $\mathbf{DM_{50}CH_{50}}$.

The **HS** subunit is expected to be more hydrophilic than the **HG** subunit because of the pendant hydroxyl group in the former, and replacement of **CO** with **HS** exerts more interesting effects on the biological activity profile relative to replacement with **HG**. Thus, the ternary copolymer $\mathbf{MM_{60}CO_{30}HS_{10}}$ is generally similar to $\mathbf{MM_{60}CO_{40}}$ in terms of antibacterial activity, but the ternary copolymer is considerably less hemolytic than the binary prototype. The antibacterial activity erodes, however, with further replacement of **CO** subunits by **HS** subunits.

Table 2 presents the biological activity profiles for ternary copolymers related to the binary copolymer $\mathbf{DM_{50}CH_{50}}$; hemolytic assay results are shown in Figure 4. Replacing a small proportion of the hydrophobic **CH** units with either **HG** or **HS** has a similar impact: relative to the binary starting point, $\mathbf{DM_{50}CH_{40}HG_{10}}$ and $\mathbf{DM_{50}CH_{40}HS_{10}}$ display comparable antibacterial activities but significantly diminished hemolytic

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Table 3. Biological Activities of Representative Binary versus Ternary Nylon-3 Copolymers Having the Same Hydrophobic:Cationic Molar Ratio and Varied Content of the Third (Hydrophilic) Subunit HG or HS

				MIC (μg/mL)[d]				
polymer	M_n[a]	DP[b]	PDI[c]	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*	HC_{10} (μg/mL)[e]
$DM_{50}CH_{50}$	4766	26	1.05	12.5	≤1.6	6.25	6.25	6.25
$DM_{47.5}CH_{47.5}HG_5$	3558	20	1.20	12.5	≤1.6	12.5	12.5	400
$DM_{45}CH_{45}HG_{10}$	3174	18	1.24	25	≤1.6	12.5	25	400
$DM_{45}CH_{45}HS_{10}$	4943	25	1.12	25	1.6	12.5	12.5	200–400
$DM_{40}CH_{40}HS_{20}$	5539	26	1.05	50–100	3.13	25	50	200–400

[a]Number-average molecular weight. [b]Average degree of polymerization. [c]Polydispersity index based on GPC data obtained for polymers bearing Boc protecting groups on **DM** unit side chains and trityl protecting groups on **HS** unit side chains. [d]Minimum inhibitory concentration for bacterial growth. [e]Polymer concentration for 10% lysis of hRBCs.

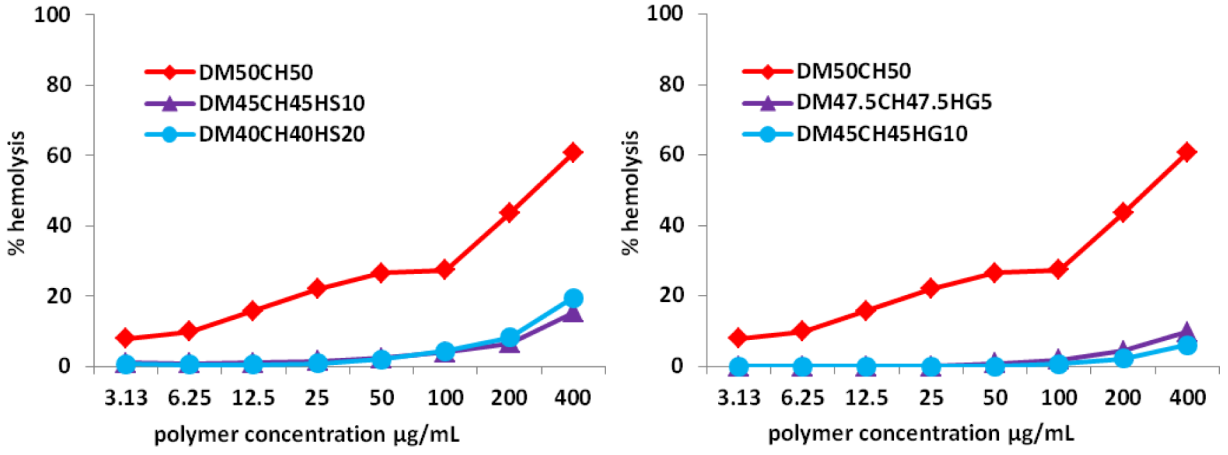

Figure 5. Hemolytic profiles of (left) **DM** + **CH** + **HS** and (right) **DM** + **CH** + **HG** ternary copolymers vs $DM_{50}CH_{50}$.

activities. Increasing the extent of hydrophobic unit replacement to generate $DM_{50}CH_{25}HG_{25}$ or $DM_{50}CH_{25}HS_{25}$ causes a substantial decline in antibacterial activity. In this series, we explored ternary copolymers in which a subset of the cationic units is replaced with either **HG** or **HS**. This effort was motivated by the prior observation that the **DM** homopolymer is quite hemolytic.[8c] For these modifications, **HS** provides a more interesting outcome than **HG**. Both $DM_{40}CH_{50}HG_{10}$ and $DM_{40}CH_{50}HS_{10}$ are relatively similar to $DM_{50}CH_{50}$ in terms of antibacterial profile, but $DM_{40}CH_{50}HS_{10}$ is significantly less hemolytic than the binary copolymer, while $DM_{40}CH_{50}HG_{10}$ is comparable to the binary copolymer in hemolytic activity.

The results summarized in Table 2 suggest that improvements in the biological activity profile (i.e., increases in HC_{10} without major changes in MIC) can be achieved by replacing small amounts of either the hydrophobic or the cationic subunit in $DM_{50}CH_{50}$ with neutral **HG** or **HS** subunits; therefore, we examined another set of ternary copolymers in which small amounts of *both* **DM** and **CH** subunits were replaced (Table 3 and Figure 5). This approach proved to be effective; the polymer $DM_{47.5}CH_{47.5}HG_5$ appears to have the most favorable profile among all of the ternary polymers examined in this study.

CONCLUSIONS

We have explored a new, biologically inspired approach to creating sequence-random copolymers that mimic the antibacterial/hemolytic activity profile characteristic of host-defense peptides. In contrast to previous efforts focused on binary copolymers in which one subunit is hydrophobic and the other cationic, we have examined ternary copolymers in which hydrophobic and cationic components are augmented by "neutral" components, i.e., components that are neither charged nor hydrophobic. On the basis of amino acid residue prevalence among HDPs, we selected two new subunits, one glycine-like (**HG**) and one serine-like (**HS**). Starting from previously known binary hydrophobic–cationic nylon-3 copolymers that display strong antibacterial activities but are also highly hemolytic, we have shown that partial replacement of hydrophobic subunits, cationic subunits, or both can lead to a decline in an unfavorable property (hemolysis) while a desirable property (antibacterial activity) is maintained. These findings suggest that the exploration of ternary copolymers may be useful for other efforts to optimize synthetic biomaterial performance.

ASSOCIATED CONTENT

Supporting Information

Materials and instrumentation, polymer synthesis, bioassays of polymers, dose–response curves, ^{1}H NMR spectra, and GPC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): S.H.G. and B.W. are co-inventors on a patent covering nylon-3 copolymers.

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