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.
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 β3-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.

Nylon-3 copolymers were synthesized via base-initiated anionic ring-opening polymerization (AROP) of β-lactams.15 Reactions involving HGβ were conducted in NN-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-acetyl-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 multangle 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 coli18) and three Gram-positive species (Bacillus subtilis,19 Staphylococcus aureus,20 and Enterococcus faecium21), 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 HC10, 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, MMβCOβ and DMβCHβ.8c Both polymers are active against the bacteria in our test panel (low MIC values), but both are also quite hemolytic (low HC10 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
content or the overall charge to be altered without changing the net hydrophobicity.

Table 1 compares the biological activity profile of MM<sub>60</sub>CO<sub>40</sub> 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<sub>10</sub> 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 \(\beta\)-lactam proportions used for the synthesis of that polymer (i.e., the material designated MM<sub>60</sub>CO<sub>30</sub>HS<sub>10</sub> was prepared by copolymerization of a \(\beta\)-lactam mixture containing 60 mol % MM\(\beta\), 30 mol % CO\(\beta\), and 10 mol % HS\(\beta\)). In general, the \(\beta\)-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<sub>60</sub>CO<sub>30</sub>HG<sub>10</sub> and MM<sub>60</sub>CO<sub>25</sub>HG<sub>15</sub> are very similar to the binary polymer MM<sub>60</sub>CO<sub>40</sub> in terms of MIC and HC<sub>10</sub> values. Replacing most of the hydrophobic CO units with HG, as in MM<sub>60</sub>CO<sub>10</sub>HG<sub>30</sub>, 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.

**Table 1. Biological Activities of MM + CO + HG and MM + CO + HS Ternary Copolymers versus MM<sub>60</sub>CO<sub>40</sub>**

| polymer | \(M_n\)* | DP* | PDF* | MIC (\(\mu\)g/mL) | E. coli | B. subtilis | S. aureus | E. faecium | HC<sub>10</sub> (\(\mu\)g/mL)
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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 MM$_{60}$CO$_{30}$HS$_{10}$ is generally similar to 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 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, DM$_{50}$CH$_{40}$HG$_{10}$ and DM$_{50}$CH$_{40}$HS$_{10}$ display comparable antibacterial activities but significantly diminished hemolytic activity.
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, 1H NMR spectra, and GPC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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references