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Article

Secondary Amine Pendant β-Peptide Polymers Displaying Potent Antibacterial Activity and Promising Therapeutic Potential in Treating MRSA-Induced Wound Infections and Keratitis

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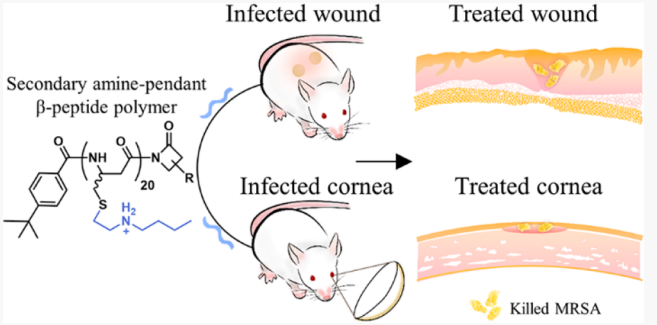
ABSTRACT: Interest in developing antibacterial polymers as synthetic mimics of host defense peptides (HPDs) has accelerated in recent years to combat antibiotic-resistant bacterial infections. Positively charged moieties are critical in defining the antibacterial activity and eukaryotic toxicity of HDP mimics. Most examples have utilized primary amines or guanidines as the source of positively charged moieties, inspired by the lysine and arginine residues in HDPs. Here, we explore the impact of amine group variation (primary, secondary, or tertiary amine) on the antibacterial performance of HDP-mimicking β-peptide polymers. Our studies show that a secondary ammonium is superior to either a primary ammonium or a tertiary ammonium as the cationic moiety in antibacterial β-peptide polymers. The optimal polymer, a homopolymer bearing secondary amino groups, displays potent antibacterial activity and the highest selectivity (low hemolysis and cytotoxicity). The optimal polymer displays potent activity against antibiotic-resistant bacteria and high therapeutic efficacy in treating MRSA-induced wound infections and keratitis as well as low acute dermal toxicity and low corneal epithelial cytotoxicity. This work suggests that secondary amines may be broadly useful in the design of antibacterial polymers.



INTRODUCTION

Human health is threatened by bacterial infections, such as skin ulcers, microbial keratitis, sinusitis, endocarditis, pneumonia, and urinary tract infections.[1,2] The rapid emergence of drug-resistant bacteria and the lack of effort to develop new antibiotics have put antibacterial treatment on the verge of the "post-antibiotics era",[3,4] where mortality rates for bacterial infections are predicted to be higher than for cancers.[4] There is an urgent need to develop new agents to address drug-resistant bacterial infections. Host defense peptides (HDPs) exert broad-spectrum antimicrobial activity[5−7] and generally do not induce bacteria to develop resistance, which has sparked significant interest in clinical application of HDPs.[8−13] However, the high cost of solid-phase peptide synthesis has hindered the application of HDPs and HDP analogs.

The shortcomings of HDPs and their peptide analogs have motivated researchers to explore amphiphilic copolymers as synthetic mimics of HDPs.[14−29] These HDP-mimicking polymers generally work by disrupting bacterial membranes, a mechanism that relies on global amphiphilicity and net positive charge of the polymer chains.[30−32] The cationic moieties in HDPs and HDP-mimicking polymers are believed to play a critical role in antibacterial performance because the initial contact between bacterial cells and HDPs or HDP-mimicking polymers is mediated by electrostatic interactions.[33−35] An analysis of the current antimicrobial peptide database (APD) in its third iteration,[36] APD3 (3257 peptides), for cationic residues showed an average content of 9.51% lysine, 5.88% arginine, and 2.17% histidine (Figure 1A). Natural HPDs do not employ secondary or tertiary amines to generate positive charge.

HDP-mimicking antimicrobial polymers with diverse backbones have been reported,[17,19,37−39] and primary amine and guanidine are the dominant sources of positively charged groups in these studies, a design choice that was presumably inspired by the presence lysine and arginine in HDPs.[37,40−49] One of the advantages of synthetic antimicrobial polymers vs HDPs is structural diversity, which encouraged us to explore antibacterial β-peptide polymers containing secondary amine or tertiary amine groups as the source of the positively charged

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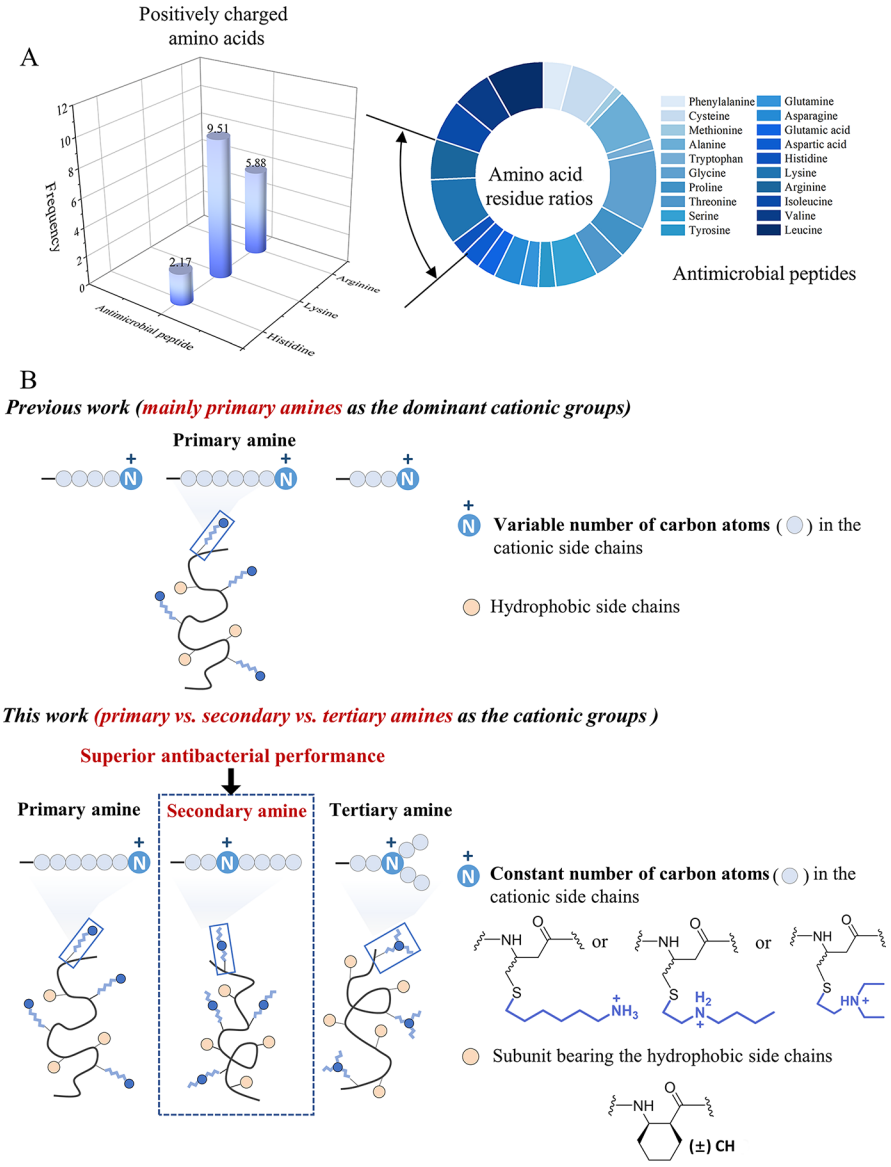

Figure 1. Design of β-peptide polymers bearing variable amine pendant groups. (A) Proportions of positively charged residues in antimicrobial peptides; data were collected from the APD3 by searching the "sequence information" as of July 2021 (https://aps.unmc.edu/statistic/statistics).[36] (B) Comparison of previous work and this work involving HDP-mimicking polymers with different amine groups as the source of cationic groups under physiological conditions.

moieties. A few previous studies of HDP-mimicking polymers have compared antibacterial effects across structures possessing varied amine side chains,[50−53] but variation in the number of alkyl carbon atoms within the cationic subunits has accompanied changes in the degree of substitution on the amino nitrogen in the prior work. Varying carbon number is expected to influence polymer hydrophobicity, a property that substantially affects both antibacterial activity and toxicity toward mammalian cells. In contrast, our structural design holds the number of carbon atoms constant in the amine-containing β-peptide side chains as the degree of substitution on the amino group is varied (Figure 1B). Therefore, our study provides a unique opportunity to isolate the impact of amino group substitution on biological activity profile, in the context of a consistent β-peptide polymer backbone. Our results indicate that side chains containing secondary amine groups are superior to analogs containing either primary or tertiary amine groups in terms of the biological performance of β-peptide polymers.

RESULTS AND DISCUSSION

To prepare β-lactams bearing side chains that contain amino groups with different degrees of substitution and identical numbers of carbon atoms, substitution reactions were conducted using a common iodo-β-lactam and different amino thiol reagents as nucleophiles. The primary and secondary amino groups were Boc-protected. β-Lactams with a pendant primary amine (PriN-β), secondary amine (SecN-β), or tertiary amine (TerN-β) were prepared as racemic compounds in 75%, 58%, and 88% yields, respectively (Figure 2A, Figures S1−S5). Ring-opening polymerization of each of these amine-bearing β-lactams was conducted, alone or in a 1:1 mixture with hydrophobic β-lactam CH-β (Figure S6), followed by treating the polymers with neat TFA to remove the protecting groups (for PriN-β and SecN-β). Using *tert*-

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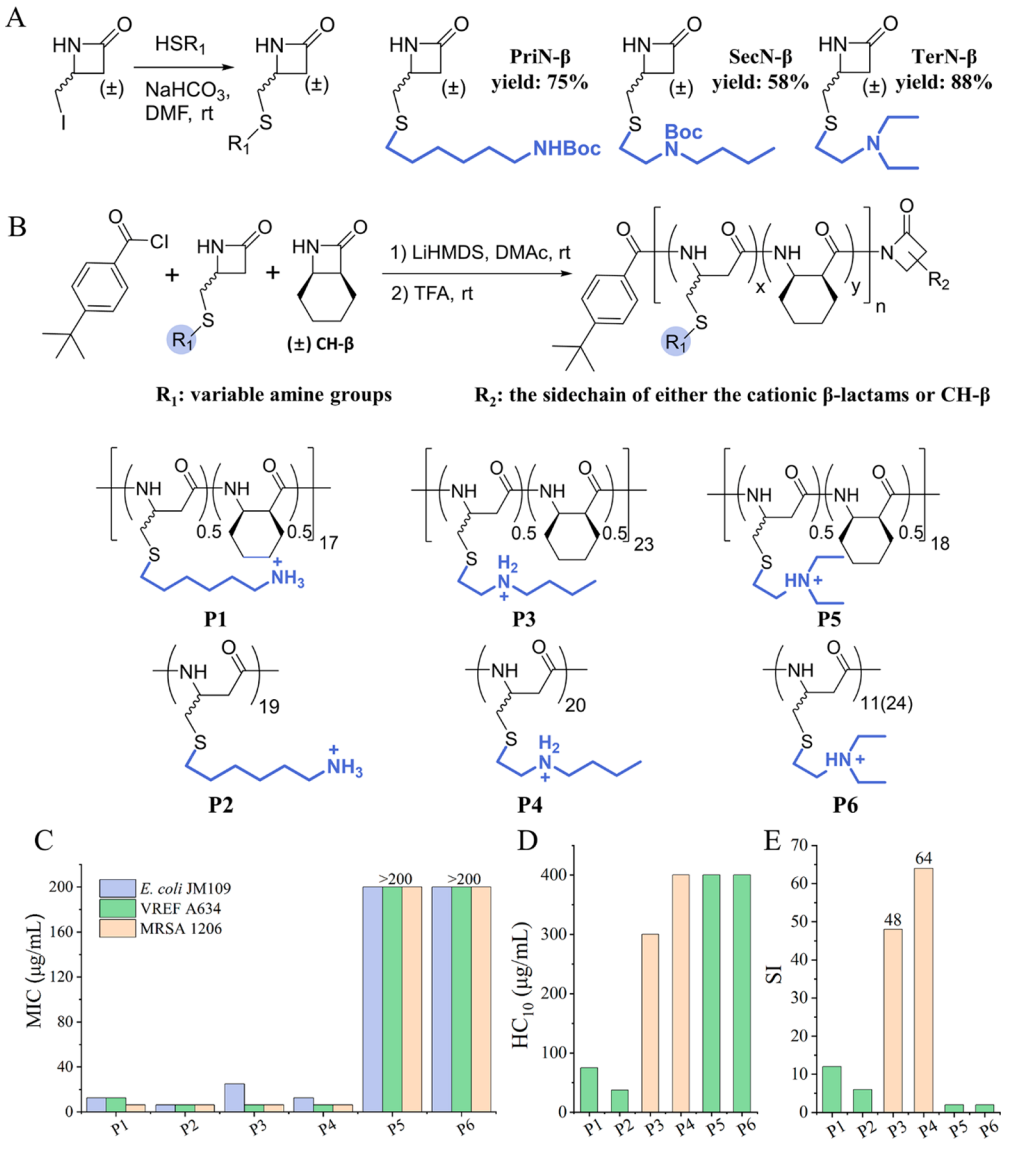

Figure 2. Synthesis of β-peptide polymers bearing variable amine pendant groups and their bacterial killing efficacy. (A) Synthesis of variable amine pendent β-lactams. (B) Synthesis of β-peptide polymers bearing variable amine functional groups, $x + y = 1$. DP values of all polymers were obtained from GPC analysis. The chain length of P6 was underestimated due to the limited solubility of P6 even when DMAc was the mobile phase for GPC. The DP value for P6 was independently obtained from NMR data; this value is provided in parentheses. (C) Antibacterial activity of polymers. (D) Hemolytic activity of polymers. (E) SI of polymers toward MRSA 1206, SI = MIC/HC_{10}.

butylbenzoyl chloride as the co-initiator for β-lactam polymerization introduced a *tert*-butylbenzoyl group to the N-terminus to generate one series of polymers, P1−P6 (Figure 2B), for biological function studies.

Each of these polymers, except P6, had an average degree of polymerization (DP) in the range 20−40, and they had narrow dispersities, 1.05−1.25, according to GPC analysis of the polymers bearing protected side chains for primary and secondary amine (Figure 2B, Figures S7, S9, S11, S13, S15, and S17). In addition, we characterized these polymers using proton NMR after side chain protecting groups were removed (Figure 2B, Figures S8, S10, S12, S14, S16, and S18). For polymers bearing an N-terminal *tert*-butylbenzoyl group, we could determine DP based on characteristic signals of the N-terminal group. For polymers P1−P5, DP determined from GPC data was similar to DP determined from NMR analysis. For P6 (the homopolymer of TerN-β), however, GPC suggested a much smaller average chain length (DP = 11) than that determined from NMR analysis (DP = 24). We believe that this discrepancy resulted from the low solubility of the high molecular weight portion of P6 in the GPC solvent. Because of this solubility problem, the dispersity of peptide polymer P6 was not calculated (Figures S17 and S18). In addition, NMR analysis of subunit composition within the peptide polymer chains indicated a nearly 1:1 ratio of the two residues for P1 and P3, as expected, but a higher proportion of the cationic subunit within P5 (Table S1).

The antibacterial activities of β-peptide polymers P1−P6 were initially assessed by determining the minimum inhibitory concentration (MIC) values against three bacteria, clinically isolated strains of MRSA 1206 and vancomycin-resistant *Enterococcus faecium* (VREF) along with a laboratory strain of *Escherichia coli* (*E. coli*). The binary copolymers bearing either primary amine or secondary amine groups, P1 and P3, showed strong antibacterial activities against these bacteria, with MIC values of 6.25−25 μg/mL. The homopolymers

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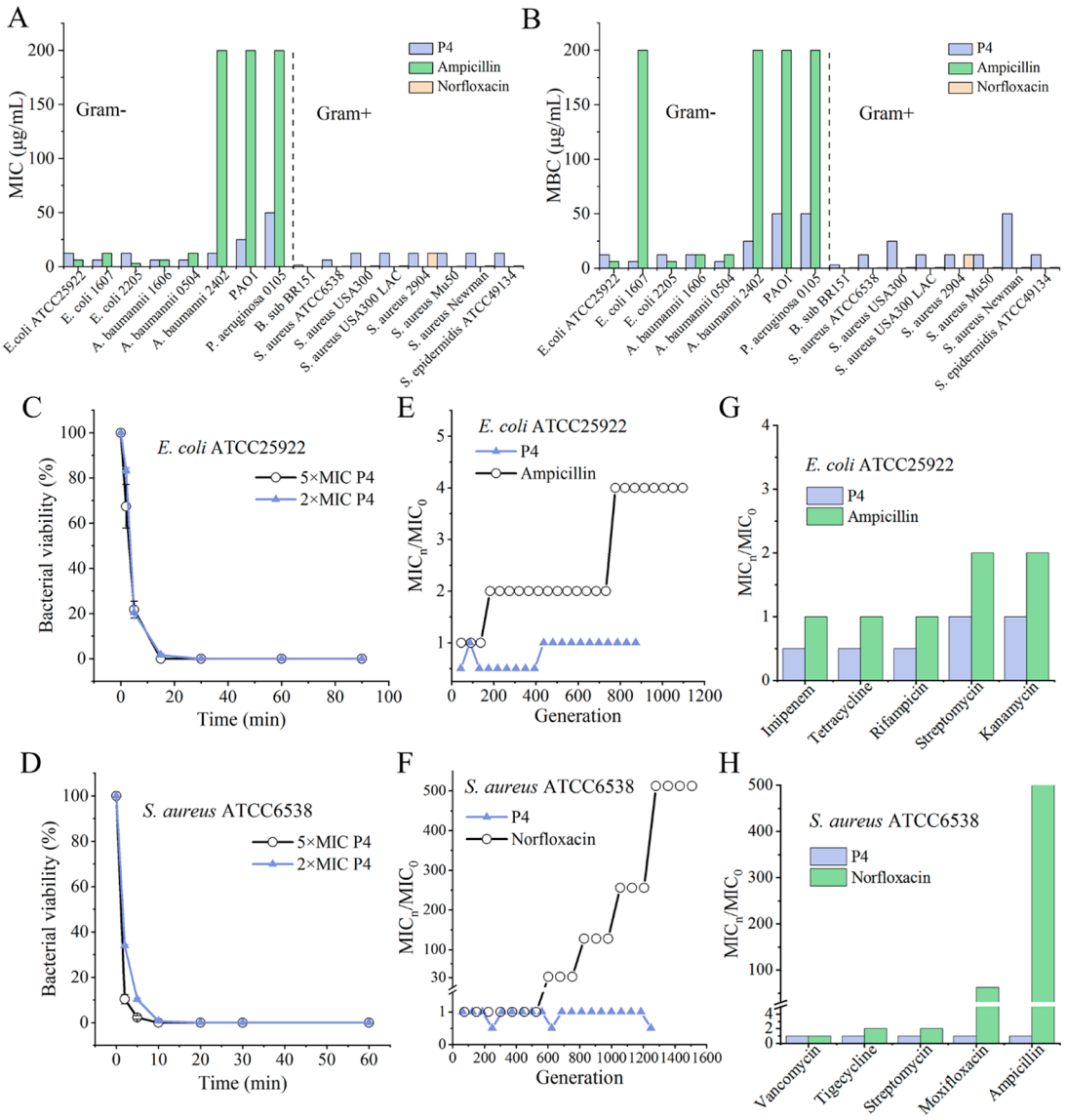

Figure 3. Antibacterial activity of secondary amine pendant β-peptide polymer P4. (A) MIC values of P4 against both Gram-negative and Gram-positive bacteria. (B) MBC values of P4 against both Gram-negative and Gram-positive bacteria. Bacterial killing kinetics of P4 against *E. coli* ATCC25922 (C) and *S. aureus* ATCC6538 (D) at concentrations of 2 × MIC and 5 × MIC, respectively. Antimicrobial resistance of P4 and norfloxacin against *E. coli* ATCC25922 (E) and *S. aureus* ATCC6538 (F). Cross-resistance between P4 and antibiotics against *E. coli* ATCC25922 treated with P4 at 1/2 × MIC concentration for 874 generations, or ampicillin at 0.5 × MIC concentration for 1097 generations (G) and against *S. aureus* ATCC6538 that was treated with P4 at 0.5 × MIC concentration for 1248 generations or norfloxacin at 0.5 × MIC concentration for 1506 generations (H).

bearing either primary amine or secondary amine groups, P2 and P4, also showed strong antibacterial activities against these bacteria, with MIC values of 6.25−12.5 μg/mL. P5 and P6, bearing tertiary amine groups, however, manifested low antibacterial activities (Figure 2C).

A different pattern of behavior was observed in hemolysis assays, based on the concentration necessary to induce 10% of lysis of human red blood cells (HC_{10}). The polymers containing primary amino groups were moderately hemolytic, but the polymers containing secondary or tertiary amino groups displayed little or no hemolytic activity up to a concentration of 400 μg/mL (Figure 2D). Selectivity index (SI) values were calculated as HC_{10}/MIC_{MRSA} and provide a measured prokaryote vs mammalian cell selectivity, with higher values corresponding to a more favorable activity profile. β-Peptide polymers P3 and P4, bearing secondary amino groups, displayed higher SI values than other polymers in our evaluation, which indicates the superiority of secondary amino groups in the side chain. The homopolymer P4, bearing secondary amino groups, displayed the highest SI value (64) as well as potent antibacterial activity (Figure 2C,E). Therefore, P4, bearing secondary amino groups, was selected as the optimal β-peptide polymer for the following studies.

We evaluated the hydrophobicity of homopolymers P2, P4, and P6, in which the subunits have identical numbers of carbon atoms in the side chains, using estimated octanol−water partition coefficients (log *P*). This approach is commonly used to measure the hydrophobicity of macromolecules.[54,55] Since these three homopolymers have the same N-terminal group (-*t*Bubz) and similar DP values of around 20, analysis of the log *P* values of the subunits should afford a reasonable evaluation of the hydrophobicities of corresponding

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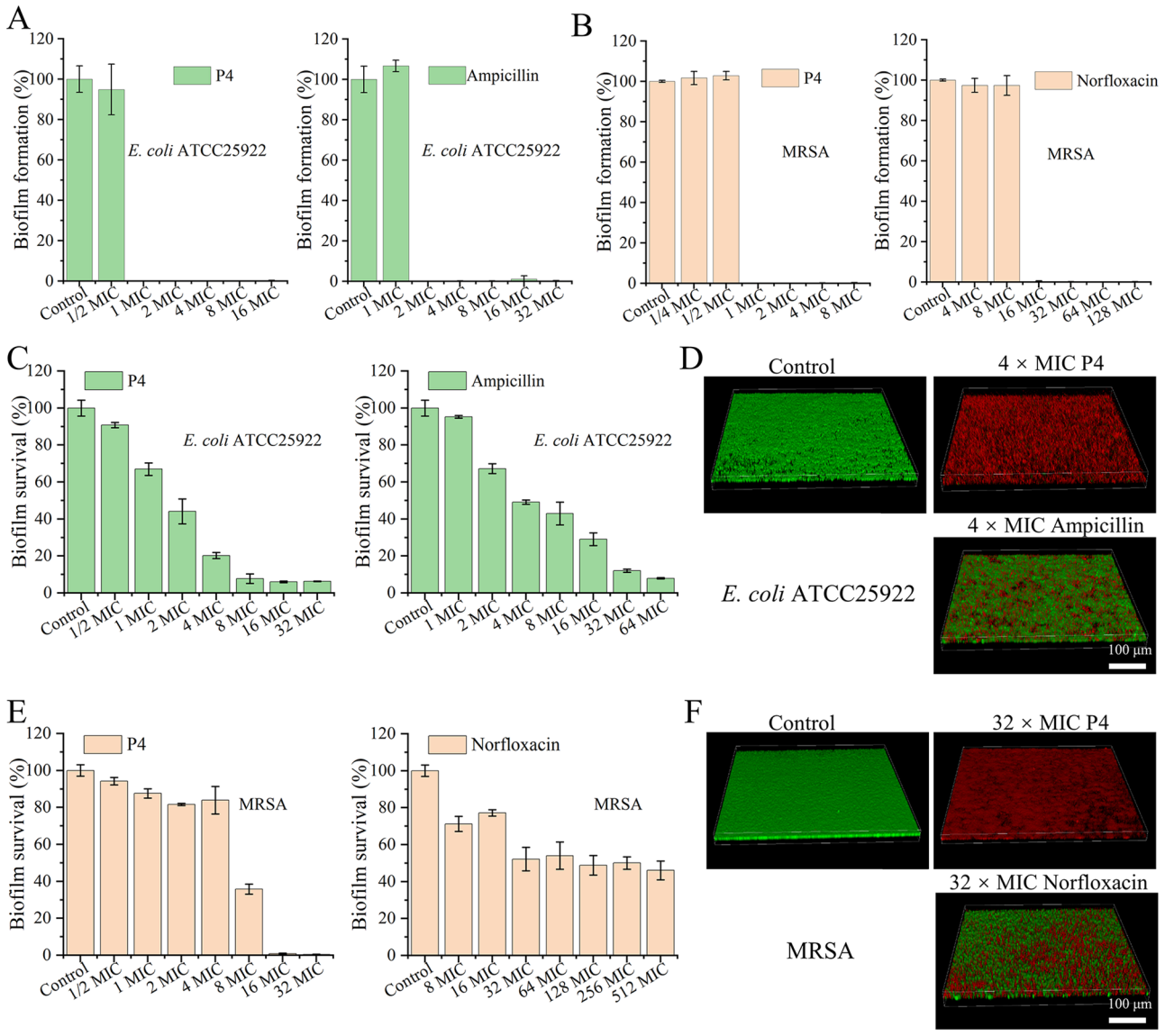

Figure 4. Biofilm inhibition and eradication ability of secondary amine pendant β-peptide polymer P4. Biofilm inhibition ability of P4, ampicillin, and norfloxacin against *E. coli* ATCC25922 (A) and MRSA (B). Biofilm eradication ability of P4, ampicillin, and norfloxacin against *E. coli* ATCC25922 (C) and MRSA (E). Fluorescent confocal imaging in eradicating mature biofilm of *E. coli* ATCC25922 with 8 × MIC concentration of P4 and ampicillin (E) and of MRSA with 32 × MIC concentration of P4 and norfloxacin (F).

homopolymers (P2, P4, and P6). We found that the three subunits have similar estimated log *P* values (Table S2). This observation is consistent with our experimental design, which required that the number of side chain carbon atoms be constant as the degree of nitrogen substitution was varied.

We explored the antibacterial activity of β-peptide polymer P4 against Gram-negative bacteria and Gram-positive bacteria, 8 strains each, including drug-resistant strains (methicillin-resistant *S. aureus* USA300, *S. aureus* USA300 LAC, *S. aureus* Newman, *S. aureus* Mu50; carbenicillin- and piperacillin-resistant *E. coli* ATCC25922; sulfamethoxazole- and tetracycline-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1), multidrug-resistant strains (*E. coli* 1607, *E. coli* 2205, *Acinetobacter baumannii* (*A. baumannii*) 1606, *A. baumannii* 0504, *A. baumannii* 2402, *P. aeruginosa* 0105, and *S. aureus* 2904), and drug-sensitive strains (*Bacillus subtilis* (*B. sub*) BR15, *S. aureus* ATCC6538, *Staphylococcus epidermidis* (*S. epidermidis*) ATCC49134). P4 showed potent activity against all bacteria, with MIC values in the range 6.25−50 μg/mL and the minimum bactericidal concentration (MBC) values in the range 6.25−50 μg/mL. In contrast, ampicillin, the antibiotic control for Gram-negative bacteria, was ineffective toward *A. baumannii* 2402, *P. aeruginosa* PAO1, and *P. aeruginosa* 0105 (Figure 3A,B).

We further characterized P4 in terms of killing kinetics, antibacterial resistance, and antibacterial cross-resistance with both *E. coli* (Gram negative) and *S. aureus* (Gram positive). At a concentration of 2 × MIC, P4 showed 98.5% killing of *E. coli* within 15 min and 99.4% killing of *S. aureus* within 10 min. At a concentration of 5 × MIC, P4 completely killed *E. coli* and *S. aureus* within 15 and 10 min, respectively (Figure 3C,D). To assess whether the P4 induces bacteria to develop resistance, bacteria were treated with P4 or the antibiotic control continuously at a concentration of 1/2 × MIC. Notably, P4 did not induce any resistance even after 800 passages with *E. coli* and 1200 passages with *S. aureus* (Figure S19 for the

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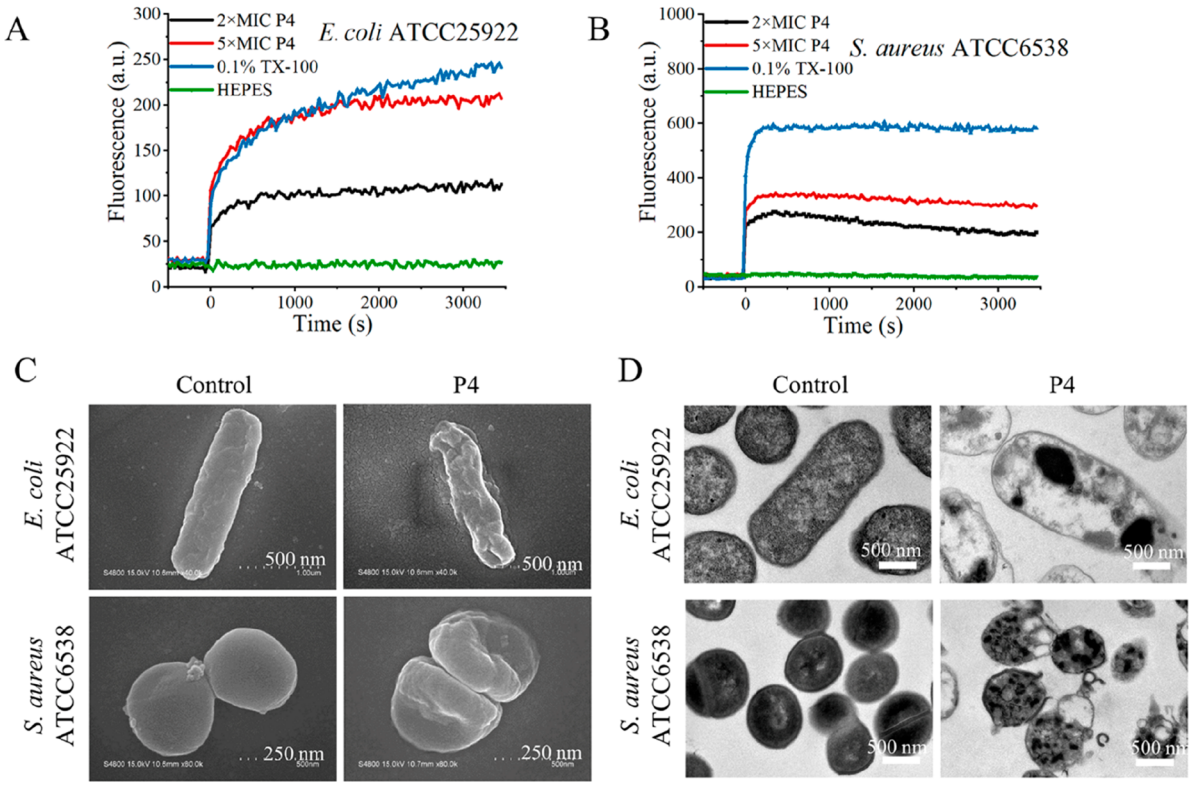

Figure 5. Antibacterial mechanism study of secondary amine pendant β-peptide polymer P4. Cytoplasmic membrane depolarization by P4 at 2 × MIC and 5 × MIC concentrations against *E. coli* ATCC25922 (inner membrane) (A) and *S. aureus* ATCC6538 (B). (C) SEM characterization on *E. coli* ATCC25922 and *S. aureus* ATCC6538 with and without P4 treatment at 2 × MIC concentration for 30 min. (D) TEM characterization on *E. coli* ATCC25922 and *S. aureus* ATCC6538 with and without P4 treatment at 4 × MIC for 6 h.

calculation on the number of passages). However, both *E. coli* and *S. aureus* acquired resistance toward the conventional antibiotics, ampicillin and norfloxacin, respectively (Figure 3E,F). In addition, we found that P4-treated *E. coli* and *S. aureus* did not show cross-resistance toward common antibiotics. In sharp contrast, we found that ampicillin-treated *E. coli* showed a moderate resistance toward streptomycin and kanamycin and that norfloxacin-treated *S. aureus* showed varying degrees of cross-resistance toward other antibiotics, including 500-fold resistance toward ampicillin (Figure 3G,H).

The ability of P4 to kill planktonic bacteria encouraged us to ask whether the β-peptide polymer could inhibit biofilm formation and eradicate the mature biofilm. These questions are important because biofilms represent a profound clinical challenge in tissue and implant-related microbial infections. P4 efficiently inhibited the formation of *E. coli* and MRSA (*S. aureus* USA300 LAC) biofilms at a concentration as low as 1 × MIC; two conventional antibiotics, ampicillin and norfloxacin, required 2 × MIC and 16 × MIC, respectively, to inhibit biofilm formation (Figure 4A,B). Eradicating a mature biofilm is even more difficult than inhibiting biofilm formation. P4 at 8 × MIC efficiently eradicated an *E. coli* biofilm (>92%); in contrast, ampicillin required 64 × MIC to achieve a similar effect. P4 completely eradicated an *S. aureus* biofilm at 16 × MIC, whereas norfloxacin was not effective even at 512 × MIC (Figure 4C,E). Confocal fluorescent imaging of antibacterial agent-treated mature biofilms showed that P4 effectively killed *E. coli* and MRSA within biofilms, at a concentration of 8 × MIC and 32 × MIC, respectively, which is superior to the performance of conventional antibiotics (Figure 4D,F).

The broad-spectrum antibacterial activities of β-peptide polymer P4 and the apparent inability of bacteria to develop resistance to this polymer inspired us to ask whether P4 kills bacteria via a membrane-disrupting mechanism, which is a common mode of action among HDPs.[1,56,57] Our studies indicated that P4 caused bacterial membrane depolarization, and the degree of membrane (inner membrane for *E. coli*) depolarization increased with increasing P4 concentration (Figure 5A,B). Interestingly, P4 had only a weak interaction with the outer membrane of *E. coli* (Figure S20), which was consistent with previous results with other β-peptide polymers.[58] Furthermore, we found bacteria released more reactive oxygen species (ROS) after being incubated with P4 relative to untreated bacteria, based on the use of ROS indicator 2′,7′-dichlorofluorescein diacetate. This measurement suggested that P4 may interact with the inner cell membrane and disrupt the aerobic respiratory electron transport chain involving proteins in the cell membrane, thereby reducing proton motive force and leading to the release of ROS as reported by others (Figure S21).[59,60] The morphological changes of P4-treated *E. coli* and *S. aureus* were studied by SEM. Both bacteria displayed the normal rod or spherical shape before treatment; in sharp contrast, bacterial envelopes were distorted and shrunken after the treatment of P4 at a concentration of 2 × MIC for 30 min (Figure 5C). Cytoplasmic leakage and membrane disruption were observed via TEM after the bacteria were incubated with P4 at a concentration of 4 × MIC for 6 h (Figure 5D).

We further evaluated the therapeutic potential of secondary amine pendent β-peptide polymer P4 with three *in vivo* model systems (Figure 6A). The first system tests for the possibility

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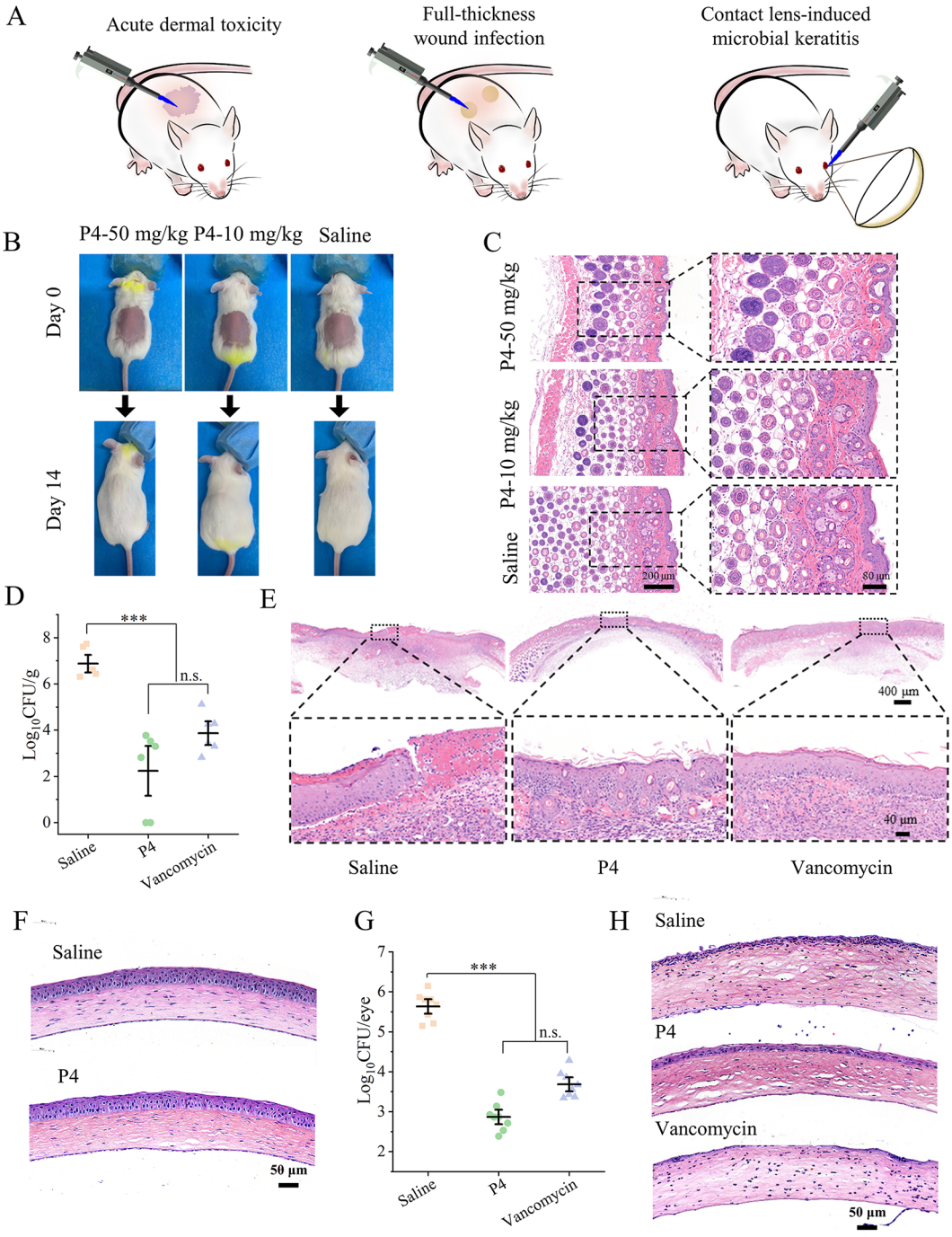

Figure 6. *In vivo* antibacterial activity of secondary amine pendant β-peptide polymer P4. (A) Illustration of acute dermal toxicity, full-thickness wound infection, and contact lens-induced microbial keratitis models in mice. (B) Representative appearance of the skin in acute dermal toxicity analysis after treatment with P4. (C) Representative histological analysis of acute dermal toxicity after treatment P4 for 3 days by H&E staining. (D) CFU of MRSA in wound treated with saline, P4, and vancomycin in the full-thickness wound infection model. (E) Representative histological analysis of infected wound tissue on day 7 postoperation by H&E staining. (F) Representative histological analysis of corneal toxicity by H&E staining. (G) CFU of MRSA in cornea treated with saline, P4, and vancomycin in the keratitis model. (H) Representative histological analysis (H&E staining) of infected mice cornea after antibacterial treatment. * represents $p < 0.05$, ** represents $p < 0.01$; *** represents $p < 0.001$; not significant (n.s.) represents $p > 0.05$.

that the polymer might cause acute dermal toxicity. The other two systems, involving MRSA-infected full-thickness wound infection or MRSA-infected keratitis, examine whether the polymer can promote recovery from infection. After P4 was applied at a dose of 50 mg/kg or 10 mg/kg to a hairless patch (~2 cm^2) on the backs of mice, new fur grew and covered the back completely after 14 days. Similar behavior was observed when saline was applied (Figure 6B). Moreover, no obvious inflammatory responses were observed in P4-treated skin tissue samples. The epidermal structure was intact, and the structure of each tissue layer was clear (Figure 6C). These results indicated that P4 is safe for topical application.

In a MRSA-infected full-thickness wound infection model in mice, P4 was evaluated using vancomycin as the positive

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control and saline as the negative control. After the wound was infected with MRSA for 24 h, P4 was administered to the wound topically every 4 h for a total of 12 h. Four h after the last administration of P4, the bacterial load in the wound tissue had a 4.6-log reduction compared with that in the saline control group. P4 was even more potent than vancomycin, which caused only a 3.0-log bacteria reduction (Figure 6D). After 7 days of treatment, hair covered the wound sites again, and intact epidermis was observed in the H&E staining images of P4- and vancomycin-treated wounds. In contrast, in the saline-treated wounds, tissue necrosis appeared in the epidermis and dermis layer, and amorphous eosinophils formed by damaged cells were observed (Figure 6E).

We further evaluated the therapeutic potential of P4 using a MRSA-infected keratitis model in mice. For safety assessment, we administered P4 to the uninfected mice cornea using the same dose as in the keratitis treatment, and no apparent defects in the corneal epithelium were found. This observation indicated that P4 is compatible with the corneal epithelium and underlying stroma and is safe to be used for the treatment of keratitis (Figure 6F). We then grew a MRSA biofilm on the contact lens (Figure S22) and placed the contact lens on the pretreated cornea surface to establish MRSA infected keratitis. At 12 h postimplantation, contact lenses were removed, and the infected cornea was treated with P4 eye drops every 5 min during the first hour and every 30 min during the next 7 h. We examined the bacteria load in the eyeballs 30 min after the last dose. P4 treatment significantly lessened MRSA corneal infection by reducing 2.8-log of bacteria in the wound, compared to the saline-treated control group. P4 was even more effective than vancomycin, which reduced only 1.9-log of bacteria compared to the saline-treated group (Figure 6G). Histopathological analysis revealed corneal alterations caused by MRSA infection, which involved severe inflammatory infiltration in the saline-treated cornea. In contrast, P4-treated animals showed an obvious reduction of inflammatory infiltration and regeneration of corneal epithelium, comparable to the vancomycin treated group (Figure 6H). These studies demonstrated that P4 is safe and effective in treating MRSA-infected keratitis.

CONCLUSION

Cationic moieties have been widely recognized as critical components of the amphiphilic structure in HDPs and HDP mimics. These positively charged molecules are assumed to be drawn to bacteria membranes via the electrostatic interactions. Currently, primary amine and guanidine groups are the major units employed to confer positive charges on HDP mimics, inspired by the presence of lysine and arginine in HDPs. The studies reported here were intended to determine whether other amine groups can be as effective as, or even more effective than, primary amine groups in constructing HDP mimics. Our experimental strategy was designed to compare side chains containing primary vs secondary vs tertiary amine groups with minimum variation in hydrophobicity, which contrasts with previous attempts to make this comparison.[50−53] The data show clearly that the homopolymer bearing secondary amines as source of the cationic group (P4) has the best performance. P4 displays potent efficacy against drug-resistant bacteria and low toxicity. We were unable to observe development of bacterial resistance to P4, which is consistent with the membrane-disruption mechanism proposed for HDP mimics. Studies conducted *in vivo* revealed potent therapeutic efficacy of P4 for the treatment of MRSA-induced wound infections and keratitis. This work suggests an important new guideline for development of HDP mimics with optimal antimicrobial function and therapeutic potential.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c10659.

Monomer and polymer synthesis, characterization, bioassay (PDF)

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Notes

The authors declare no competing financial interest.

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