

Engineering Antibacterial Activities and Biocompatibility of Hyperbranched Lysine-based Random Copolymers

Hui Liu^{a,b}, Xiao Liu^a, Yuan-Qiao Cao^{a,b}, Ming Liu^a, Miao-Miao Han^{a*}, and Sheng-Xiang Ji^{a,b*}

^a Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

^b University of Science and Technology of China, Hefei 230026, China

Electronic Supplementary Information

Abstract Antimicrobial peptides (AMPs) have been considered as an alternative to small molecule antibiotics since they are difficult to develop antimicrobial resistance. Hyperbranched polylysine (HPL), an AMP mimics, has gained attention due to its broad-spectrum antibacterial activities, but it also suffers from high toxicity. Here we report a facile strategy to engineer the toxicity of HPL by copolymerizing lysine (K) with a hydrophobic amino acid, e.g., alanine (A), tryptophan (W) or phenylalanine (F), to afford hyperbranched random copolymers. These copolymers have comparable antibacterial activities to HPL while their cytotoxicities and *in vivo* toxicities are lowered when the type and content of hydrophobic amino acid and the size of copolymers are optimized. The *G. mellonella* infection model demonstrates that the copolymers are effective against the *S. aureus* infection *in vivo*. The copolymers kill the bacteria through the disruption of cell membranes and the bacteria do not develop resistance to the copolymers.

Keywords Hyperbranched random copolymers; Lysine; Hydrophobic amino acid; Antibacterial activity; High selectivity

Citation: Liu, H.; Liu, X.; Cao, Y. Q.; Liu, M.; Han, M. M.; Ji, S. X. Engineering antibacterial activities and biocompatibility of hyperbranched lysine-based random copolymers. *Chinese J. Polym. Sci.* 2023, 41, 345–355.

INTRODUCTION

Antibiotics have saved countless lives since their development,^[1] however, the development of antimicrobial resistance (AMR) also poses threats to our health due to the abuse of antibiotics.^[2–5] In contrast to small molecule antibiotics, antimicrobial peptides (AMPs) kill microbes mainly by disrupting the cell membrane, which reduces the possibility to develop AMR.^[6,7] As a result, AMP is a viable alternative to small molecule antibiotics in the fight against superbug.^[8–10] However, the therapeutic application of AMP is limited by its systematic toxicity, lability to proteases, short half-life, and deleterious pro-inflammatory responses.^[9,11–13]

To overcome these restrictions, scientists are working to develop AMP-mimicking polymers that can retain antibacterial properties while offering excellent biocompatibility.^[14,15] Poly(amino acid)s (PAAs) have emerged as one of the promising AMP-mimicking candidates.^[16] Among various PAAs, α -poly(L-lysine) (α -PL) exhibits broad-spectrum antibacterial activities and can be synthesized by ring-opening polymerization of lysine *N*-carboxyanhydride (NCA), but it suffers from the high cytotoxicity.^[17–20] Efforts have been made to modu-

late the antibacterial activity and toxicity of polylysine by introducing hydrophobic amino acids. Zhou *et al.* first applied NCA polymerization to synthesize copolymers with lysine as the hydrophilic amino acid and alanine, phenylalanine or leucine as the hydrophobic amino acid.^[21] They found that the poly(lysine-*ran*-phenylalanine) (PKF) copolymers contained 40%–80% hydrophobic phenylalanine residue had higher antibacterial activity than polylysine, but the copolymer with the best antibacterial properties (P(K₁₀F₁₅)) possessed a high hemolytic activity. Zhou *et al.* studied the length effect of the phenylalanine block on the antibacterial activity of triblock copeptides, and found that K₁₀-b-F₂₀-b-K₁₀ had higher antibacterial activities against *E. coli* and *S. aureus* than K₁₀-b-F₁₀-b-K₁₀ and K₁₀-b-F₃₀-b-K₁₀ while the hemolytic toxicity of triblock copeptides increased with the increase of the phenylalanine block length.^[22] Lam *et al.* reported that structurally nanoengineered antimicrobial peptide polymers (SNAPPs) containing valine exhibited significantly greater antibacterial activity than those without valine, but they had non-negligible cytotoxicity and the selectivity, i.e., IC₅₀/MBC, of SNAPPs was only ~2.^[23] Chen *et al.* modified star-shaped poly(L-lysine) with tryptophan to give 6-armed PLL₂₀-g-indo_{0.2}. The cytotoxicity of 6-armed PLL₂₀-g-indo_{0.2} was reduced, but its antibacterial activity against *K. pneumoniae* and *S. aureus* also decreased.^[24]

In addition, ϵ -poly(L-lysine) (ϵ -PL) also has excellent antibacterial activities against both Gram-positive and Gram-neg-

* Corresponding authors, E-mail: hanmiaomiao@ciac.ac.cn (M.M.H.)

E-mail: sjj@ciac.ac.cn (S.X.J.)

Received July 18, 2022; Accepted August 23, 2022; Published online October 20, 2022

active bacteria and high biocompatibility, which plays an important role in many fields such as food preservation.^[17,25–30] The commercial production of ϵ -PL is overly reliant on *Streptomyces albulus*, which limits the expansion of the production capability.^[31] Compared to the linear analogues ϵ -PL and α -PL, hyperbranched polylysine (HPL) can be easily synthesized by one-pot thermal polymerization of lysine,^[32–34] and has been proved to exhibit excellent antibacterial activities.^[35] Although HPL has been studied as the carrier for gene delivery, its cytotoxicity is still a critical concern for its potential biomedical application.^[36–38] However, there has no study on the reduction of the toxicity of HPL. In this study, we synthesized hyperbranched random copolymers by copolymerization of lysine and a hydrophobic amino acid, e.g., alanine, tryptophan or phenylalanine, through facile thermal condensation to engineer the antibacterial activities and toxicities. The effect of the type and content of the hydrophobic amino acid on their cytotoxicities and antibacterial activities against Gram-positive bacteria and Gram-negative bacteria was investigated. Compared to HPL, hyperbranched random copolymers had reduced cytotoxicity, increased selectivity between bacteria and mammalian cells, and improved killing kinetics. In addition, all copolymers were effective in the treatment of *S. aureus*-infected *G. mellonella* larvae.

EXPERIMENTAL

Materials and Characterization

L-lysine, L-alanine, L-tryptophan, L-phenylalanine, ciprofloxacin, dimethyl sulfoxide (DMSO), glutaric dialdehyde and Triton X-100 were obtained from Aladdin Co., Ltd. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent and Trypsin-EDTA solution were obtained from Beijing Solarbio Science & Technology Co., Ltd. LIVE/DEAD Bacterial Staining Kit (BBcellProbe® N01/PI) were obtained from Bestbio Co., Ltd. Luria-Bertani Broth (LB), Mueller-Hinton Broth (MHB) and Luria-Bertani-Agar (LB-Agar) were purchased from Qingdao Hope biotechnology Co., Ltd. Fetal bovine serum (FBS) was purchased from Tianjin Kangyuan Biotechnology Co., Ltd. Roswell Park Memorial Institute (RPMI) 1640 Media and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco Co., Ltd. *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 25923) and Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) were purchased from Shanghai Luwei Technology Co., Ltd. NIH 3T3 mouse fibroblast cells were purchased from Procell Life Science & Technology Co., Ltd. *G. mellonella* larvae were purchased from Henan Jiyuan Baiyun Industry Co., Ltd. ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker AV500 NMR spectrometer with D₂O as the solvent. An Agilent gel permeation chromatography (GPC) was used to determine the molecular weights. The eluent was a buffer containing 280 mmol/L acetic acid and 20 mmol/L sodium acetate (pH 3.6) and was filtered with a 0.22 μ m pore size membrane before use. The eluent flow rate was 1.0 mL/min at 35 °C. The dynamic light scattering (DLS) technique was used to measure particle sizes in the Zetasizer Nano ZS90 by Malvern Panalytical. The morphologies of the microorganisms were characterized by field emission scanning electron microscopy (Zeiss, Merlin) at an accelerating voltage of 1 kV.

Synthesis and Characterization of Copolymers

A series of lysine-based hyperbranched random copolymers were synthesized by copolymerization of L-lysine with L-alanine, L-tryptophan or L-phenylalanine. A representative synthesis procedure is detailed for PKA10-1. L-lysine (1.316 g, 9 mmol) and L-alanine (0.089 g, 1 mmol) were completely dissolved in 5 mL of distilled water, and the mixture was deoxygenated using ultrapure nitrogen for 30 min. The solution was heated at 180 °C for 6 h under a nitrogen atmosphere. The final product was dissolved in ethanol and precipitated in ethyl acetate to yield a slightly yellow solid (1.236 g, 88%). By changing the feed ratio and reaction time of PKA, PKW or PKF, a series of copolymers could be obtained.

In vitro Antibacterial Activity

The antibacterial susceptibility testing of the polymers was carried out according to the modified standard of the Clinical Laboratory Standard Institute (CLSI) M07-A9. To obtain a 12 mg/mL stock solution, the polymers were dissolved in phosphate-buffered saline (PBS). The stock solution was then diluted using the double dilution method to obtain various concentrations of 6, 3, 1.5, 0.75, 0.375, 0.188, 0.094, 0.048 and 0.024 mg/mL. The bacteria were precultured in the LB broth at 37 °C to the mid-logarithmic phase and the suspensions were diluted to a concentration of 10⁶ CFU/mL. Then, 25 μ L of each diluted polymer solution was added to 96-well microplate, followed by adding 175 μ L of the bacteria suspension to reach the final polymer concentration of 1500, 750, 375, 188, 94, 48, 24, 12, 6 and 3 μ g/mL. The microplates were incubated at 37 °C for 18–22 h. The absorbance value at 600 nm (OD₆₀₀) was measured using an enzyme labeling instrument (Tecan Infinite F50). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of polymer that completely inhibits growth by more than 90%.

In vitro Hemolytic Activity

Fresh rabbit blood was collected in EDTA Vacutainer tubes and washed three times with PBS before erythrocyte was diluted to 2% with PBS. 175 μ L of the erythrocyte suspension and 25 μ L of the polymer solution were added to a 96-well plate and shaken for 1 h at 37 °C, followed by centrifugation for 8 min at 2000 $\times$ g. 0.8% Triton X-100 in PBS was used as a positive control, while PBS was used as a negative control. The OD value at 540 nm was measured and the hemolysis percentage was calculated using the equation:

$$\text{Hemolysis (\%)} = \frac{A_d - A_n}{A_p - A_n} \times 100\% \quad (1)$$

where A_d is the absorbance of the suspension of cells cultured with the polymer, A_n is the absorbance of the suspension of cells cultured with PBS as a negative control, and A_p is the absorbance of the suspension of cells cultured with Triton X-100 as a positive control. The HC₁₀ value is defined as the concentration at which the hemolysis percentage is 10%.

In vitro Cytotoxicity Assay

The *in vitro* cytotoxicity of polymers was evaluated by the MTT assay using mouse fibroblast cells (NIH 3T3). NIH 3T3 cells were cultured in DMEM containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin in a plastic culture dish at 37 °C and 5% CO₂. When cells adhered densely to the culture dish, the number of cells was adjusted to 10⁵ cells per mL with DMEM to

inoculate into a 96-well plate. After 18 h of culturing, the medium was immediately changed to 175 μ L of RPMI 1640 and 25 μ L of the polymer solution was added and the suspension was cultured for 24 h at 37 °C with 5% CO₂. Subsequently, 20 μ L of the MTT buffer was added to each well and the suspension was cultured for another 4 h. After removing the MTT medium, 200 μ L of DMSO was added into each well and the plate was shaken at 37 °C for 15 min. The OD value at 490 nm was measured and the cell survival ratio was calculated using the equation:

$$\text{Cell survival ratio (\%)} = \frac{A_d - A_n}{A_p - A_n} \times 100\% \quad (2)$$

where A_d is the absorbance of the suspension of cells cultured with the polymer, A_p is the absorbance of the suspension of cells cultured with PBS as a control, and A_n is the absorbance of DMSO as a blank control. The IC₂₀ value is defined as the concentration at which the cell survival ratio is reduced to 80%.

Time-kill Assay

The strains (*E. coli* and *S. aureus*) were precultured in the LB broth at 37 °C to the mid-logarithmic phase and then washed with PBS for three times. The bacteria suspensions were diluted to approximately 10⁶ CFU/mL with fresh MHB, and then were treated with the polymer at the concentration of 1×MIC. The group untreated with polymer served as a control. 20 μ L of the suspension was removed at 0, 0.5, 1, 2, 3, 4 and 6 h, and diluted to the appropriate concentration with PBS. 100 μ L of the solution was then streaked onto agar plates and incubated at 37 °C for 24 h. The Colony Forming Units (CFU) were counted and the dilution factor was recalculated.

Resistance Studies

The strains (*E. coli* and *S. aureus*) were cultured to the mid-logarithmic phase and diluted to 10⁶ CFU/mL. The bacteria suspension was treated with the polymer at the concentration of 1/2×MIC at 37 °C for 24 h and the MIC value was determined according to CSLI M07-A9. The strains were allowed to regrow and treated with the polymer for the subsequent passage. The MIC value was used to monitor the development of drug resistance over 12 passages. Ciprofloxacin was used as the control.

In vivo Toxicity

The *in vivo* toxicities of the polymers were implemented by *G. mellonella* larvae. All animal procedures were carried out in accordance with the published protocol.^[39] Healthy larvae (2–3 cm and 200–300 mg) were starved for 24 h prior to injection. 10 μ L of the polymer solution at the concentration of 30 mg/kg, 60 mg/kg, 100 mg/kg or 120 mg/kg was injected into the hemocoel of larvae via the last left proleg using the microsyringe. After injection, larvae were incubated for 96 h at 37 °C and 60% relative humidity in plastic containers and the number of dead larvae was counted every 24 h. The control group was treated with PBS.

The *G. mellonella* Infection Model

Healthy larvae were selected 24 h before being injected and then starved. *S. aureus* was cultured to mid-log growth, then washed twice with PBS and then diluted in PBS to different bacterial concentrations. 10 μ L of the bacterial suspension was injected into the hemocoel of larvae via the last left proleg using

the microsyringe. After 1 h, 10 μ L of polymer solution at a concentration of 60 mg/kg was injected into a different proleg. Two control groups were used: one was given PBS, and the other was given ciprofloxacin solution at a concentration of 20 mg/kg. The larvae were kept in the dark at 37 °C for 96 h after infection. When larvae did not respond to shaking, they were considered dead. The number of dead larvae was counted every 24 h. For each group of experiments, 30 larvae were injected.

Skin Irritation Test in Mice

Eight-week-old KM mice were shaved on the upper back (1 cm²) by an electrical razor and were divided into reasonable groups of three mice each. 200 μ L of the polymer solution with a concentration of 400 μ g/mL in PBS was smeared on their skin twice per day for seven days. The polymer was wiped off with distilled water after 15 min of exposure. PBS-treated mice and untreated mice were used as controls. The mice were sacrificed and the skins were harvested following the final topical administration. After being fixed in 4% formalin, the skin was embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) to assay the skin sensitivity.

Morphology of Bacteria

The strains (*E. coli* and *S. aureus*) were precultured in the LB broth at 37 °C to the mid-logarithmic phase and then washed with PBS for three times. The suspension was then treated with the polymer solution with the concentration of 4×MIC at 37 °C. After being treated for 4 h, the suspension was centrifugated and washed with PBS. The bacteria were fixed overnight with 2.5% of glutaraldehyde at 4 °C. The samples were then sequentially dehydrated with 30%, 50%, 60%, 70%, 80%, 90% and 100% aqueous ethanol solutions. Following the final wash, the bacteria were suspended in anhydrous ethanol and a drop of the suspension was transferred onto a silicon wafer. The cell morphology was imaged using field emission scanning electron microscope (FE-SEM) after sputtering the silicon wafers with gold layers.

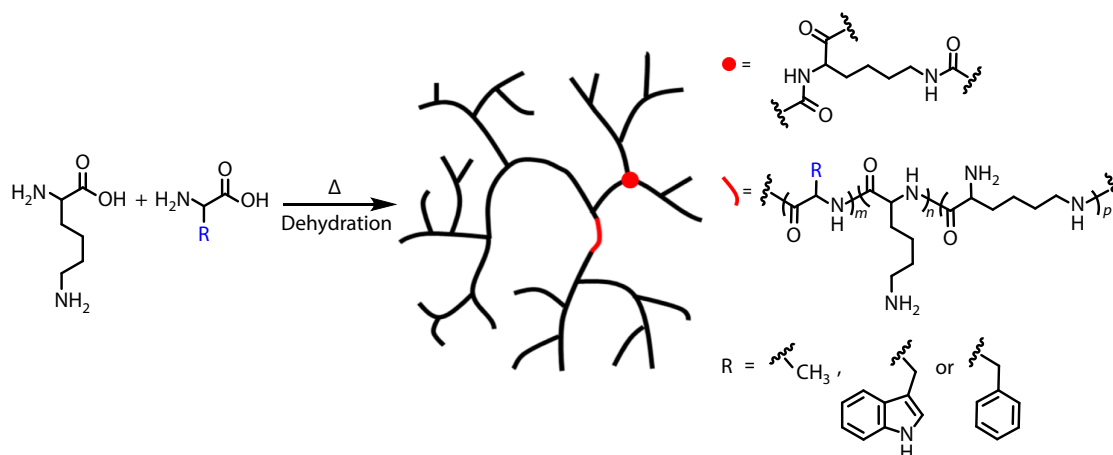
LIVE/DEAD BacLight™ Bacterial Viability Assay

Bacteria in the mid-logarithmic phase were incubated and diluted with PBS (*E. coli* for 10⁸ CFU/mL and *S. aureus* for 10⁷ CFU/mL). The suspension was then treated with the polymer with the concentration of 4×MIC at 37 °C for 4 h, washed with the saline, stained in the dark at 37 °C for 15 min with BBcellProbe® N01/PI, and then washed with the saline. 5 μ L of stained bacteria was dropped onto clean coverslip-covered slides and the cells were imaged with fluorescence microscopy.

RESULTS AND DISCUSSION

Synthesis and Characterization of Copolymers

To investigate the effect of the type of the hydrophobic amino acid on their bioactivities, a series of hyperbranched random copolymers containing lysine and a hydrophobic amino acid, e.g., alanine, tryptophan or phenylalanine, poly(lysine-co-alanine) (PKAx, where x is the mol% of alanine in the feed), poly(lysine-co-tryptophan) (PKW10, containing 10 mol% of tryptophan in the feed) and poly(lysine-co-phenylalanine) (PKF10, containing 10 mol% of phenylalanine in the feed), were successfully synthesized by one-step thermal condensation copolymerization (Scheme 1). These copolymers were



Scheme 1 Schematic of synthesis of hyperbranched random copolymers.

characterized by ^1H -NMR and ^{13}C -NMR spectroscopy in D_2O (Figs. S1–S3 in the electronic supplementary information, ESI). The actual contents of alanine, tryptophan or phenylalanine residues were determined by ^{13}C -NMR spectroscopy (Table S1 in ESI). The actual contents of alanine were about 3.2 mol% in PKA5, ~7.5 mol% in PKA10-1 to PKA10-4 and 12.5 mol% in PKA15. And PKW10 and PKF10 contained 8.0 mol% of tryptophan residue and 6.9 mol% of phenylalanine residue, respectively. Additionally, HPL was also synthesized as the control. The molecular weights of the copolymers were measured by gel permeation chromatography (GPC) with the HAc-NaAc buffer (pH 3.6) (Table S1 in ESI). The number-average molecular weight (M_n) of most copolymers was around 4.5 kg/mol, except for PKA10-3 and PKA10-4 which have molecular weights of 6.6 kg/mol and 8.0 kg/mol, respectively. The hydrodynamic diameters (D_{H}) of these copolymers in PBS (pH 7.4) were characterized by DLS (Fig. S4a in ESI). The D_{H} s for the most copolymers were ~4.0–4.7 nm, while the D_{H} increased from 4.3 nm for PKA10-1 to 19.3 nm for PKA10-4.

In vitro Antibacterial Activity

The antibacterial activities of each polymer against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *MRSA*) were evaluated by determining the minimal inhibitory concentration (MIC) using the micro broth dilution method (Table 1).^[40] In general, the majority of copolymers exhibited excellent antibacterial efficacy against both Gram-negative and Gram-positive bacteria, with

particularly effective antibacterial activity against *MRSA*, a Methicillin-resistant strain. Moreover, these copolymers were more efficient against Gram-positive bacteria than Gram-negative bacteria. This difference in antibacterial activity is due to the distinct membrane structures of the two types of bacteria since the membrane of Gram-negative bacteria has two layers, *i.e.*, the cytoplasmic membrane and the outer membrane, while Gram-positive bacteria have only one cytoplasmic membrane.^[41–43]

The effect of the hydrophobic content on the antibacterial activity was determined by varying the amount of alanine from 3.2 mol% to 7.5 mol% and 12.5 mol% in PKA5, PKA10-1 and PKA15, respectively. The D_{H} s of HPL, PKA5, PKA10-1 and PKA15 were similar in PBS to eliminate the size effect of polymers on the antibacterial activities. The antibacterial activities of the copolymers are strongly correlated to zeta potentials that are associated with the charge densities. The zeta potentials decreased from 36.2 mV for HPL to 25.6 mV for PKA15 with the increase of the alanine content (Fig. S4b in ESI). The antibacterial activity also had the same trend and decreased with the increase of the alanine content, *e.g.*, HPL exhibited activities against *S. aureus* with an MIC value of 24 $\mu\text{g/mL}$ and against *E. coli* with an MIC value of 48 $\mu\text{g/mL}$, while PKA15 had the corresponding MIC values of 48 $\mu\text{g/mL}$ and 375 $\mu\text{g/mL}$, respectively. The size effect on the antibacterial activity was determined using PKA10s. The D_{H} s varied from 4.3 nm for PKA10-1 to 19.3 nm for PKA10-4 while main-

Table 1 Antibacterial activities, hemolytic activities, cytotoxicities and selectivity index of polymers.

Polymer	MIC ($\mu\text{g/mL}$)				HC_{10}^a ($\mu\text{g/mL}$)	IC_{20}^b ($\mu\text{g/mL}$)	SI ^c	
	<i>E. coli</i> 8739	<i>P. aeruginosa</i> 9027	<i>S. aureus</i> 25923	<i>MRSA</i> 43300			<i>E. coli</i> 8739	<i>S. aureus</i> 25923
HPL	48	48	24	24	>1500	83.2	1.7	3.5
PKA5	188	94	24	94	>1500	651.7	3.5	27.2
PKA10-1	188	188	24	188	>1500	>1500	>8.0	>62.5
PKA10-2	48	48	12	94	>1500	706	14.7	58.8
PKA10-3	48	48	12	48	>1500	83.6	1.7	7.0
PKA10-4	48	24	12	24	>1500	26.9	0.6	2.2
PKA15	375	188	48	188	>1500	>1500	>4.0	>31.3
PKW10	48	94	24	24	>1500	184.3	3.8	7.7
PKF10	48	94	24	24	>1500	221.3	4.6	9.2

^a HC_{10} : the concentration at which 10% of red blood cells is lysed; ^b IC_{20} : the concentration at which cell viability reduced to 80%; ^c SI: $\text{IC}_{20}/\text{MIC}$.

taining a similar content of alanine, ~7.3 mol%–7.8 mol%, in PKA10s. The antibacterial activity increased with the increase of D_H , most notably the MIC value of PKA10 against MRSA decreased from 188 µg/mL for PKA10-1 to 24 µg/mL for PKA10-4. This may be due to the fact that copolymers with large particle sizes have higher zeta potentials, e.g., 31.5 mV for PKA10-1 versus 45.3 mV for PKA10-4. In general, the effect of the alanine content or the particle size of copolymers on antibacterial activities is highly dependent on the charge density which is evidenced by the change of zeta potentials. It has been well established that an increase in cationic charge density enhances the affinity for bacterial membranes through electrostatic interactions.^[44–46] Tew *et al.* also found that the antibacterial activity of poly(norbornene) against *S. aureus* increased with increasing the charge density while keeping the hydrophobic fraction constant, with MIC values decreasing from >200 µg/mL to 15 µg/mL.^[47] The introduction of alanine decreased the zeta potential of the polymer, which eventually leads to a decrease in antibacterial activity.

Modulation of the cationic charge and hydrophobicity of polymers is critical for the design of antibacterial polymers.^[48,49] Due to the low hydrophobicity of alanine, the antibacterial activity of poly(lysine-co-alanine) is mainly determined by the charge density. Therefore, we introduced phenylalanine and tryptophan, aromatic hydrophobic amino acids with a higher hydrophobicity index (0.81 and 1.2, respectively) than alanine (0.62), to explore the effect of hydrophobicity in lysine-based hyperbranched random copolymers, PKW10 and PKF10, on antibacterial activities.^[50,51] Phenylalanine and tryptophan are found in a variety of antibacterial polymers, and their presence significantly enhances the antibacterial activity of polymers.^[21,52,53] For comparison, the D_H s (~4.0–4.2 nm) of PKW10 and PKF10 were similar to that of PKA10-1 (4.3 nm), and the content of hydrophobic amino acid (~7.5 mol%, 8.0 mol%, 6.9 mol%) and zeta potentials (31.5 mV, 31.0 mV, and 26.4 mV) of PKA10-1, PKW10 and PKF10 were also close, which minimizes the effect of the micelle size, the content of hydrophobic amino acid, and zeta potentials on the antibacterial activities. The MIC values of PKA10-1, PKW10 and PKF10 revealed that the addition of tryptophan and phenylalanine increased the activities towards *P. aeruginosa*, *E. coli* and MRSA, and the MIC values were almost decreased by a factor of 2–8 compared to the addition of alanine, revealing the effect of hydrophobicity on the antibacterial properties. Similar results were found in linear P(KF) copeptides that exhibited antimicrobial activity over a wider range of hydrophobic amino acid content and lower MIC values than P(KA) copeptides.^[21] Kuroda *et al.* found that increasing the hydrophobicity of alkyl side chains in polymethacrylate copolymers enhanced their binding to lipid bilayers, which led to an increase in the antibacterial activity.^[54] It is noteworthy that the antibacterial activities of PKW10 and PKF10 were close to that of HPL, but PKW10 and PKF10 were less toxic. The similarity in the antibacterial activities between PKW10, PKF10 and HPL might be caused by the balance between the density of cations and the hydrophobicity.^[55,56] The cationic nature of the polymer has been reported to be a key factor for bacterial damage.^[57] HPL is a hydrophilic, cationic polymer and the introduction of the hydrophobic

amino acid moiety, e.g., tryptophan and phenylalanine, resulted in an increase in hydrophobicity but a decrease in zeta potential from 36.2 mV to 31.0 mV and 26.4 mV, respectively. These data suggest that modulation of hydrophobicity, charge density and hydrodynamic diameter can lead to polymers with higher antibacterial activity.

In vitro Hemolytic Activity, Cytotoxicity and Selectivity of Copolymers

Hemolysis test is the gold standard for determining hemocompatibility which measures the ability of polymers to lyse red blood cells.^[58] The HC_{10} values were determined and defined as the lowest concentration corresponding to 10% lysis of the red blood cells (Fig. S5 in ESI).^[59] There is no observable difference in hemolytic activity between HPL and all hyperbranched random copolymers. All polymers exhibited low hemolytic activity even at the concentration of 1500 µg/mL and their hemolysis ratios were less than 10%, i.e., HC_{10} >1500 µg/mL, indicating superior hemocompatibility.

The *in vitro* cytotoxicity of polymers was further evaluated to determine their biocompatibility by the MTT assay using mouse fibroblast cells (NIH 3T3) (Table 1 and Figs. 1a–1c).^[60] Overall, all polymers affected the cell viability in a concentration-dependent manner when they were incubated with NIH 3T3 cells in a concentration range of 0–1500 µg/mL for 24 h. Since the cell viability was greater than 50% at 1500 µg/mL of the majority of copolymers, the IC_{20} value was determined to evaluate the cytotoxicity and defined as the concentration at which the cell viability was reduced to 80%.^[61,62] For the PKA10 series, the cytotoxicity increased with the increase of molecular weights or D_H s, and most notably the IC_{20} value decreased from >1500 µg/mL for PKA10-1 to 26.9 µg/mL for PKA10-4. A larger molecular weight or D_H leads to more hydrolysis of cellular phospholipids and subsequently to a greater degree of membrane disruption.^[61] In addition, the effect of the hydrophobic content on cytotoxicity was also tested for PKAs (HPL, PKA5, PKA10-1 and PKA15), and increasing the alanine content could reduce the cytotoxicity. The IC_{20} value is only 83.2 µg/mL for HPL, and increases to 651.7 µg/mL for PKA5 with the addition of only 3.2 mol% alanine. The IC_{20} value further increases to >1500 µg/mL for PKA15, exhibiting >20-fold decrease of the cytotoxicity compared to HPL. Analogously, the IC_{20} values of PKW10 and PKF10 were higher than that of HPL, representing that the addition of these amino acids also reduced the cytotoxicity of HPL polymers. This is consistent with the finding that modification of star-shaped poly(L-lysine) with tryptophan led to reduced cytotoxicity.^[24] They attribute this trend to the reduced electrostatic interaction of the polymer with the cell due to the reduction of the overall charge and hydrophilicity.

The mechanism of cytotoxicity caused by cationic polymers is not fully understood until now, and both the charge density and hydrophobicity affect the cytotoxicity of polymers.^[63,64] The initial polymer-cell interaction is mediated by electrostatic attraction,^[65] and cationic polymers appear to perturb the structure and function of cell membranes through the interactions with proteins and phospholipids in membranes.^[66,67] In addition, the increase of hydrophobicity in cationic polymers also results in increased interactions with mammalian cells, leading to increased cytotoxicity.^[64,68,69]

Our results revealed that the charge density was more dominant in controlling the antibacterial activities and cytotoxicity in PKAs when a less hydrophobic moiety, *e.g.*, alanine, was used, while the hydrophobicity also played a role in controlling the antibacterial activities and cytotoxicity in PKW and PKF when a more hydrophobic moiety, *e.g.*, tryptophan and phenylalanine, compared to alanine, was used.

The selectivity index (SI) was calculated using the ratio of IC_{20} to MIC (against *E. coli* and *S. aureus*) to evaluate the

biocompatibility and efficacy of copolymers (Table 1). In general, PKA10-1 and PKA10-2 had higher SI than HPL and PKA10-3 and PKA10-4. Particularly, PKA10-2 has relatively higher SI of 14.7 for *E. coli* and 58.8 for *S. aureus*. When tryptophan or phenylalanine were used as the hydrophobic amino acid, the SI of PKW10 and PKF10 to *E. coli* and *S. aureus* was also increased and slightly higher than that of HPL.

Bactericidal Kinetics Assay

Time-kill curves were employed to determine the antibacterial efficacies of HPL, PKA10-1, PKA10-2, PKW10 and PKF10 against *E. coli* and *S. aureus* with the concentration of $1 \times MIC$. As seen in Figs. 2(a) and 2(b) and Fig. S6 (in ESI), their antibacterial efficacies against *E. coli* and *S. aureus* are rather different. HPL, PKA10-1 and PKA10-2 exhibit a modest antibacterial activity against *E. coli* and about 2 log-CFU/mL reduction was achieved, while the addition of tryptophan or phenylalanine significantly increased the rate of bacterial elimination and 3 or 4 log-CFU/mL reduction was obtained within 6 h. Complete killing (>6 log-CFU/mL reduction) of *S. aureus* was observed within 2 h for these five polymers. The difference in killing efficiency between *E. coli* and *S. aureus* might be due to the difficulty of polymers in penetrating through the out-layer of the *E. coli* membrane.^[70] The kinetics results proved that these five polymers were bactericidal for *E. coli* and *S. aureus*.^[71]

Drug Resistance Studies

It is imperative to determine whether bacteria develop drug resistance to copolymers. *E. coli* and *S. aureus* were treated with HPL, PKA10-2 and ciprofloxacin at $1/2 \times MIC$ concentrations for 12 passages (Fig. 2c). *S. aureus* and *E. coli* developed resistance to ciprofloxacin at the fourth and sixth passage, respectively, and the MICs for *E. coli* and *S. aureus* both increased by 7-fold at the twelfth passage. In comparison, the MICs of HPL and PKA10-2 for *E. coli* and *S. aureus* did not change even after 12 passages, indicating that it is difficult to develop the resistance to HPL and PKA10-2 for *E. coli* and *S. aureus*.

In vivo Toxicity and the *G. mellonella* Infection Model

Larvae of *G. mellonella* have a similar innate immune response to mammals and involve both cellular and humoral defense.^[72] And *G. mellonella* larvae have previously been used as model hosts to study the virulence of *S. aureus* and the efficacy of various antimicrobial agents.^[73–77] Moreover, *G. mellonella* larvae are less expensive and easier to implement, and the use of larvae does not require ethical approval.^[78,79]

To investigate the *in vivo* antibacterial activity of these polymers, strains of *S. aureus* were propagated in larvae of *G. mellonella* to establish a model according to the reported procedure.^[39] The dose effect on the larval survival was investigated by injecting different amounts of HPL. The larval survival rate was 100% when the concentrations of HPL were 30 and 60 mg/kg, and was reduced to 70% and 60% at 100 mg/kg and 120 mg/kg of HPL, respectively (Figs. S7a and S7c in ESI). The toxicity of PKA10-2, PKW10 and PKF10 to *G. mellonella* larvae was also evaluated at the polymer concentrations of 60 and 120 mg/kg. All larvae survived at the dose of 60 mg/kg (Fig. S7b in ESI). Therefore, these four polymers are considered non-toxic to the larvae at the dose of 60 mg/kg. The survival rates of *G. mellonella* larvae in PKA10-2, PKW10 and PKF10 groups were higher than that in the HPL group at the

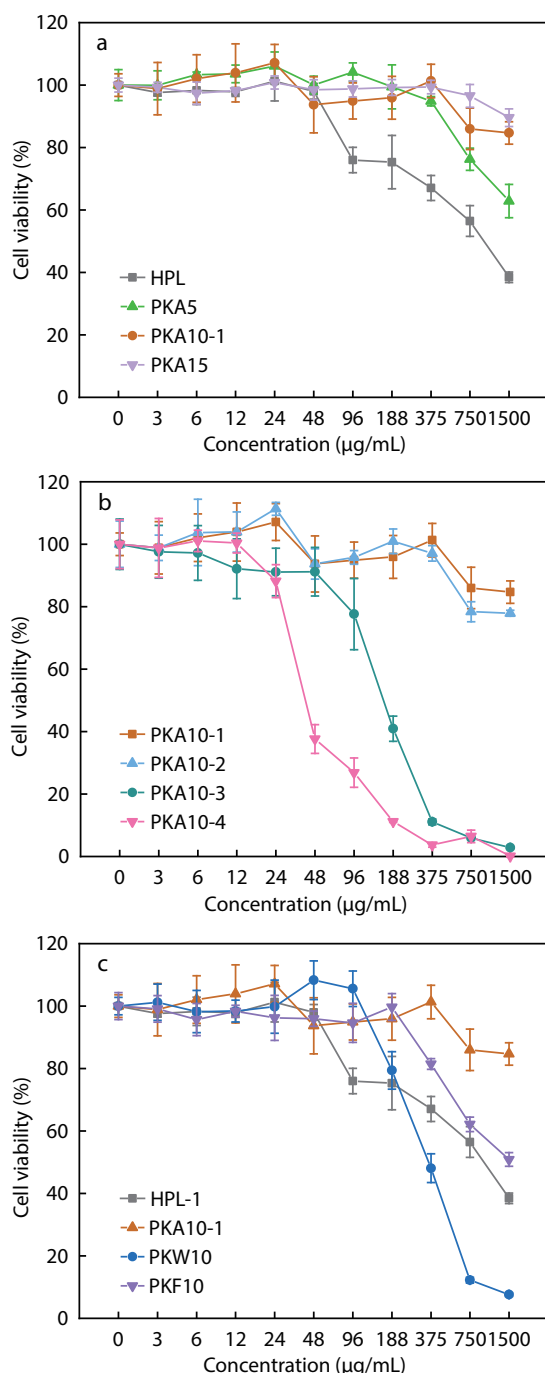


Fig. 1 The cell viability of NIH 3T3 fibroblast cells treated by polymers at different concentrations. (a) HPL, PKA5, PKA10-1 and PKA15; (b) PKA10s; (c) HPL, PKA10-1, PKW10 and PKF10.

concentration of 120 mg/kg (Fig. S7c in ESI), e.g., 93.3% for PKA10-2, 83.3% for PKW10, 90% for PKF10 and 60% for HPL, respectively. And the statistical analysis indicated that the sur-

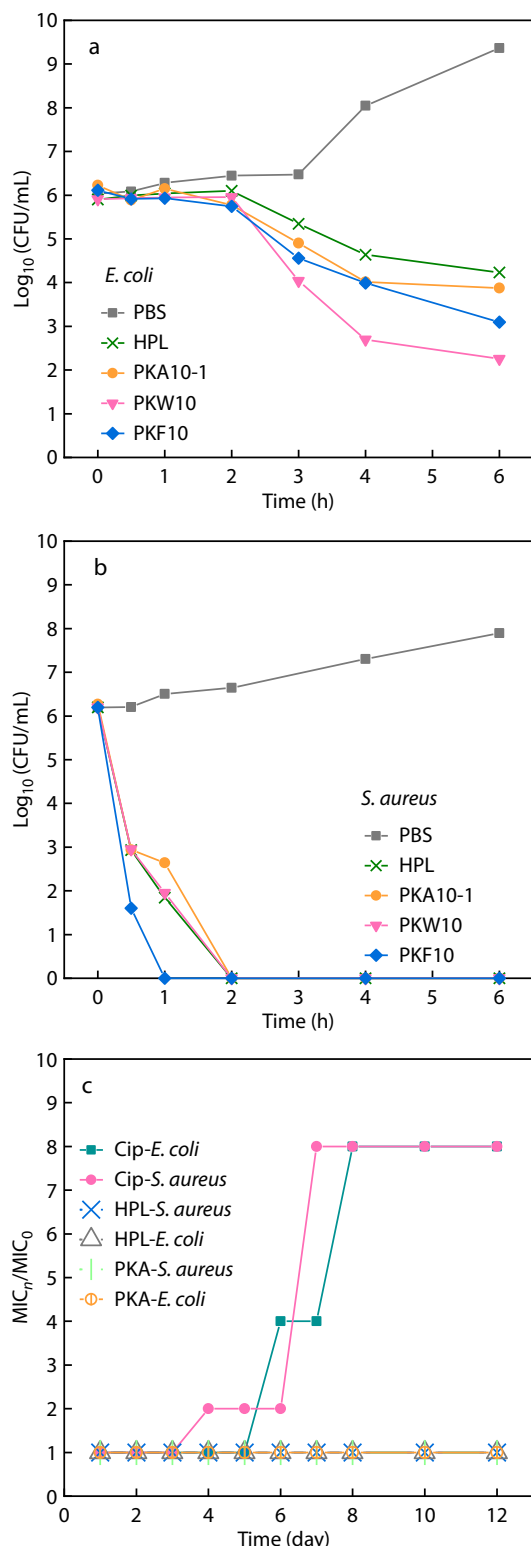


Fig. 2 Time-kill kinetics of polymers (1×MIC) against (a) *E. coli* and (b) *S. aureus*; (c) Antibacterial resistance test of HPL, PKA and Ciprofloxacin against *E. coli* and *S. aureus*.

vival rates of copolymers were statistically different from those of HPL ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively). These results demonstrated that the introduction of these three amino acids could reduce the *in vivo* toxicity in *G. mellonella* larvae.

The inoculum dose of *S. aureus* also affected the larval survival rate and the survival rate was reduced with the increasing inoculum (Fig. S7d in ESI). Compared to uninfected control, the larvae had a survival rate of 90% at 96 h when treated with the lowest inoculum of 10^6 CFU/mL, and the survival rate reduced to 80%, 70% and 0% at 10^7 , 10^8 and 10^9 CFU/mL of *S. aureus*, respectively. The inoculum dose of 5×10^8 CFU/mL of *S. aureus* was selected for further studies. The larvae were infected by *S. aureus* (ATCC 25923, 5×10^8 CFU/mL) for 1 h and then treated by injecting 60 mg/kg of HPL, PKA10-2, PKW10, and PKF10 or 20 mg/kg of ciprofloxacin and the larval survival curves within 96 h are plotted in Fig. 3. The survival rates of the larvae treated with all these polymers were above 70% and the four treated groups were significantly different from untreated PBS controls ($p < 0.0001$). The results showed that all polymers could effectively treat the *S. aureus* infected larvae *in vivo*. Moreover, the *in vivo* therapeutic effect of all copolymers was superior to that of HPL, and PKA10-2 and PKW10 even had similar *in vivo* therapeutic efficacy to ciprofloxacin.

The Skin Irritation Tests (SIT)

The skin irritation of these polymers is evaluated on mice to determine whether copolymers cause inflammatory irritation of the mice skin (Fig. S8 in ESI). Compared with the PBS-treated skin, H&E staining results showed no inflammatory infiltration, acanthosis, interstitial edema and parakeratosis in any of the skin treated with HPL, PKA10-2, PKW10 or PKF10. The results showed that these polymers did not cause skin inflammatory reactions.

Antibacterial Mechanism

To investigate the potential mechanism, the morphology of bacteria was imaged by SEM and the membrane integrity of bacteria was studied by the LIVE/DEAD bacterial viability assay. The morphologies of *E. coli* and *S. aureus* treated and untreated with copolymers are shown in Fig. 4. The untreated *E. coli* has a

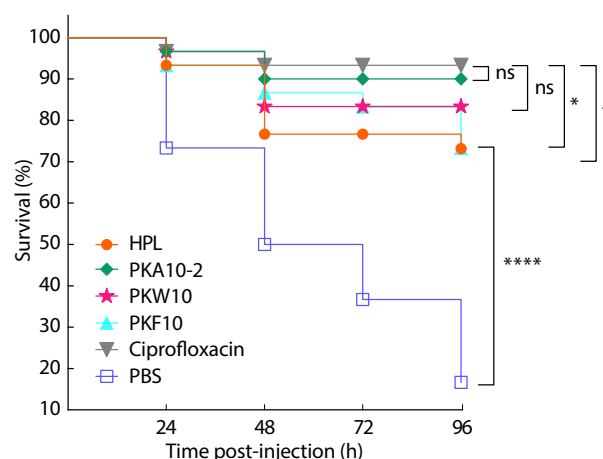


Fig. 3 Survival of *G. mellonella* larvae infected with *S. aureus* strain ATCC 25923 and treated with 60 mg/kg of polymer or 20 mg/kg of ciprofloxacin at 1 h post-infection. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 30$.

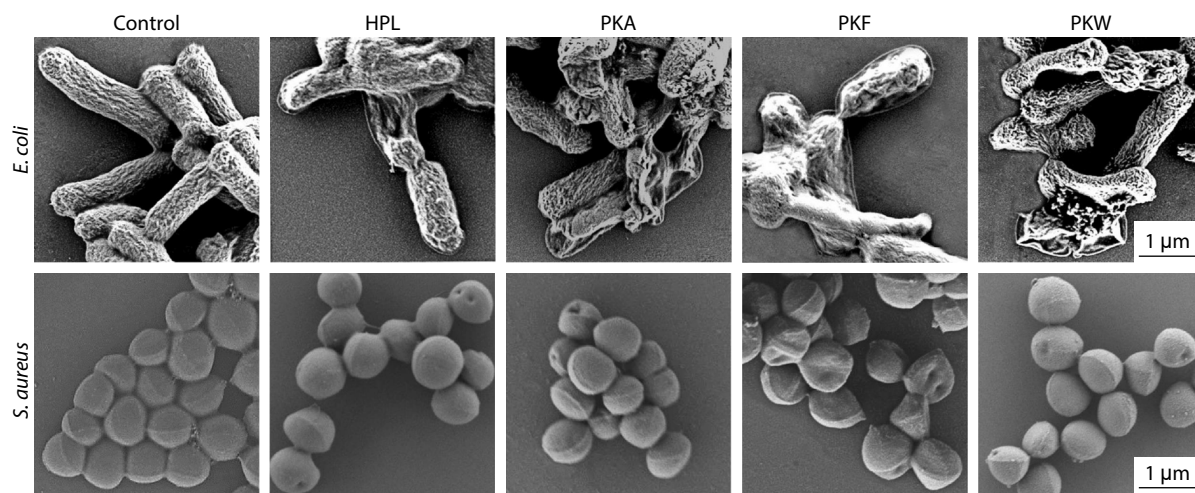


Fig. 4 SEM images of *S. aureus* and *E. coli* treated with polymers (HPL, PKA10-2, PKF10 and PKW10) and PBS (control).

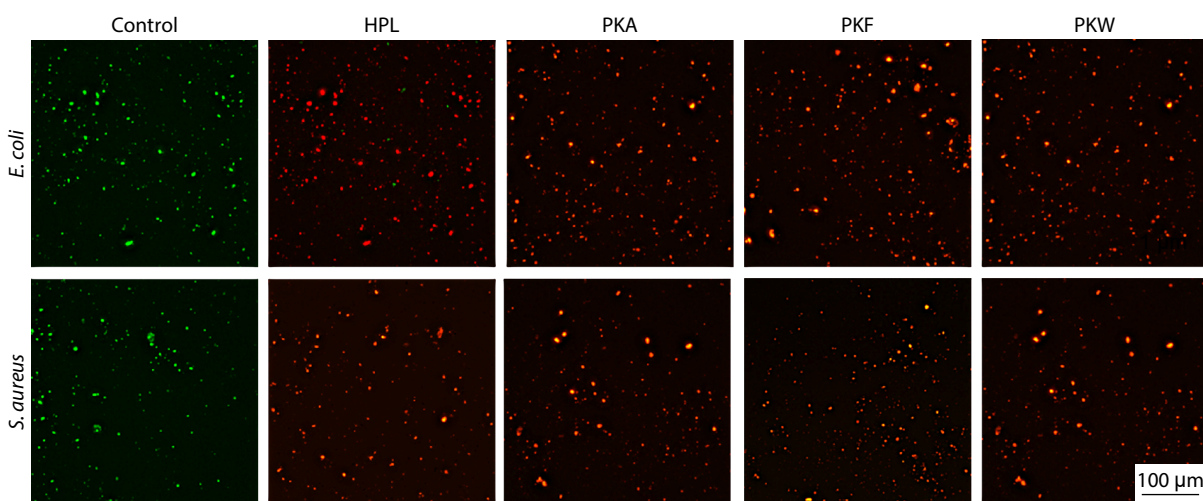


Fig. 5 Fluorescence images of stained *E. coli* and *S. aureus* under the treatment of PBS control, HPL, PKA10-2, PKF10 and PKW10.

regular rod shape and *S. aureus* shows a spherical shape, both with intact surfaces, while *E. coli* and *S. aureus* treated with HPL, PKA10-2, PKW10 or PKF10 exhibit distinct morphological changes. The cell surfaces of *S. aureus* are no longer smooth and some of them have indentations, while the morphology of *E. coli* is significantly changed and most of cells are even collapsed or destroyed, indicating that cell membranes had been damaged.

To further investigate the damage of cell membranes in *E. coli* and *S. aureus* induced by these copolymers, the LIVE/DEAD bacterial viability assay was performed. N01/PI was used to stain the bacteria treated with copolymers. N01, the green dye, can pass through the cell membrane of live bacteria, while PI could only pass through the damaged cell membranes and bind to the nucleic acid to produce red fluorescence.^[80,81] As shown in Fig. 5, both bacteria tested in the absence of polymers exhibit green fluorescence, while *E. coli* and *S. aureus* treated with copolymers exhibit strong red fluorescence, indicating that the cell membrane integrity of both bacteria was disrupted.

The antibacterial mechanism of hyperbranched random copolymers against bacteria was proposed and illustrated in Fig. 6. The cationic lysine residues are electrostatically attrac-

ted to the cell surfaces with negative charges,^[17,26] and the hydrophobic amino acid (alanine, tryptophan or phenylalanine) residues facilitate the insertion of copolymers into the cell membrane. The membrane integrity is then disrupted by copolymers, leading to the lysis of bacteria.^[82–85] The membrane integrity is then disrupted by copolymers, leading to the lysis of bacteria. Since changes in the membrane integrity

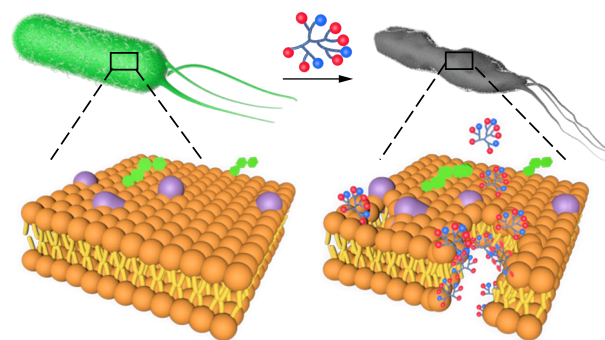


Fig. 6 Schematic illustration of the proposed mechanism of copolymers with *E. coli*.

are unlikely to cause bacterial mutations, this non-specific killing mechanism reduces the development of resistance to the copolymer.

CONCLUSIONS

In summary, a series of hyperbranched random copolymers containing lysine and a hydrophobic amino acid, *e.g.*, alanine, tryptophan, or phenylalanine, were successfully synthesized by one-pot thermal copolymerization. All copolymers had excellent blood compatibility ($HC_{10} > 1500 \mu\text{g/mL}$), while their antibacterial activities and cytotoxicity were dependent on the type and content of hydrophobic amino acid, *i.e.*, the charge density and hydrophobicity, and the hydrodynamic diameter. The antibacterial activities and cytotoxicities of PKAs both decreased with the increase of the alanine content (or the decrease of the charge density), but the cytotoxicities were more sensitive to the alanine content than the antibacterial activities. In particular, PKA10-2 had comparable antibacterial activities to HPL, while its cytotoxicity was significantly lower than HPL. Under the optimized condition, PKW10 and PKF10 also showed similar antibacterial activities to HPL, but were less toxic than HPL. The charge density was more dominant in controlling the antibacterial activities and cytotoxicity of the copolymers, while the hydrophobicity also played a role in controlling the antibacterial activities and cytotoxicity in PKW and PKF. The *in vivo* toxicity and antibacterial efficacy studies showed that PKA10-2, PKW10 and PKF10 were less toxic than HPL and had excellent efficacy in the treatment of *S. aureus* infections in the *G. mellonella* infection model, and the antibacterial efficacy of PKA10-2 and PKW10 approached that of ciprofloxacin. The bacterial morphology studies and LIVE/DEAD bacterial viability assay demonstrated that the copolymers killed bacteria by disrupting their membranes, which eliminates the possibility of inducing drug resistance.

NOTES

The authors declare no competing financial interest.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-022-2859-1>.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (No. 51973212) and Department of Science and Technology of Jilin Province (Nos. 20210203119SF and 20210203173SF).

REFERENCES

- Hutchings, M. I.; Truman, A. W.; Wilkinson, B. Antibiotics: past, present and future. *Curr. Opin. Microbiol.* **2019**, *51*, 72–80.
- Broughton, C. E.; Van Den Berg, H. A.; Wemyss, A. M.; Roper, D. I.;

- Rodger, A. Beyond the discovery void: new targets for antibacterial compounds. *Sci. Prog.* **2016**, *99*, 153–182.
- Shen, S.; Huang, Y.; Yuan, A.; Lv, F.; Liu, L.; Wang, S. Electrochemical regulation of antibacterial activity using ferrocene-containing antibiotics. *CCS Chem.* **2021**, *3*, 129–135.
- Duval, R. E.; Grare, M.; Demore, B. Fight against antimicrobial resistance: we always need new antibacterials but for right bacteria. *Molecules* **2019**, *24*, 9.
- Blaskovich, M. A. T. The fight against antimicrobial resistance is confounded by a global increase in antibiotic usage. *ACS Infect. Dis.* **2018**, *4*, 868–870.
- Luong, H. X.; Thanh, T. T.; Tran, T. H. Antimicrobial peptides—advances in development of therapeutic applications. *Life Sci.* **2020**, *260*, 118407.
- Xie, X.; Gao, B.; Ma, Z.; Liu, J.; Zhang, J.; Liang, J.; Chen, Z.; Wu, L.; Li, W. Host–guest interaction driven peptide assembly into photoresponsive two-dimensional nanosheets with switchable antibacterial activity. *CCS Chem.* **2021**, *3*, 1949–1962.
- Cruz, J.; Ortiz, C.; Guzman, F.; Fernandez-Lafuente, R.; Torres, R. Antimicrobial peptides: promising compounds against pathogenic microorganisms. *Curr. Med. Chem.* **2014**, *21*, 2299–2321.
- Hancock, R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
- Da Costa, J. P.; Cova, M.; Ferreira, R.; Vitorino, R. Antimicrobial peptides: an alternative for innovative medicines. *Appl. Microbiol. Biot.* **2015**, *99*, 2023–2040.
- Wang, J. J.; Dou, X. J.; Song, J.; Lyu, Y. F.; Zhu, X.; Xu, L.; Li, W. Z.; Shan, A. S. Antimicrobial peptides: promising alternatives in the post feeding antibiotic era. *Med. Res. Rev.* **2019**, *39*, 831–859.
- Kawano, Y.; Jordan, O.; Hanawa, T.; Borchard, G.; Patrulea, V. Are antimicrobial peptide dendrimers an Escape from ESKAPE. *Adv. Wound Care* **2020**, *9*, 378–395.
- Ciornei, C. D.; Sigurdardottir, T.; Schmidtchen, A.; Bodelsson, M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. *Antimicrob. Agents Chemother.* **2005**, *49*, 2845–2850.
- Hartlieb, M.; Williams, E. G. L.; Kuroki, A.; Perrier, S.; Locock, K. E. S. Antimicrobial polymers: mimicking amino acid functionality, sequence control and three-dimensional structure of host-defense peptides. *Curr. Med. Chem.* **2017**, *24*, 2115–2140.
- Salas-Ambrosio, P.; Tronnet, A.; Verhaeghe, P.; Bonduelle, C. Synthetic polypeptide polymers as simplified analogues of antimicrobial peptides. *Biomacromolecules* **2021**, *22*, 57–75.
- Shen, W.; He, P.; Xiao, C.; Chen, X. From antimicrobial peptides to antimicrobial poly(alpha-amino acid)s. *Adv. Healthc. Mater.* **2018**, *7*, 1800354.
- Shima, S.; Matsuoka, H.; Iwamoto, T.; Sakai, H. Antibacterial action of epsilon-poly-L-lysine. *J. Antibiot.* **1984**, *37*, 1449–1455.
- Katchalski, E.; Grossfeld, I.; Frankel, M. Poly-condensation of alpha-amino acid derivatives. 3. Poly-lysine. *J. Am. Chem. Soc.* **1948**, *70*, 2094–2101.
- Katchalski, E.; Bichowskislomnitzki, L.; Volcani, B. E. The action of some water-soluble poly-alpha-amino acids on bacteria. *Biochem. J.* **1953**, *55*, 671–680.
- Mayandi, V.; Xi, Q. X.; Goh, E. T. L.; Koh, S. K.; Toh, T. Y. J.; Barathi, V. A.; Fazil, M.; Chalasani, M. L. S.; Varadarajan, J.; Ting, D. S. J.; Beuerman, R. W.; Chan, L. W.; Agrawal, R.; Barkham, T. M. S.; Zhou, L.; Verma, N. K.; Lakshminarayanan, R. Rational substitution of epsilon-lysine for alpha-lysine enhances the cell and membrane selectivity of pore-forming melittin. *J. Med. Chem.* **2020**, *63*, 3522–3537.
- Zhou, C. C.; Qi, X. B.; Li, P.; Chen, W. N.; Mouad, L.; Chang, M. W.;

- Leong, S. S. J.; Chan-Park, M. B. High potency and broad-spectrum antimicrobial peptides synthesized via ring-opening polymerization of α -amino acid-*N*-carboxyanhydrides. *Biomacromolecules* **2010**, *11*, 60–67.
- 22 Zhou, X.; Su, X.; Tan, Z.; Zhou, C. Synthesis of triblock amphiphilic copolypeptides with excellent antibacterial activity. *Eur. Polym. J.* **2018**, *106*, 175–181.
- 23 Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H.; Chen, Y. Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G. Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat. Microbiol.* **2016**, *1*, 16162.
- 24 Chen, Y. F.; Lai, Y. D.; Chang, C. H.; Tsai, Y. C.; Tang, C. C.; Jan, J. S. Star-shaped polypeptides exhibit potent antibacterial activities. *Nanoscale* **2019**, *11*, 11696–11708.
- 25 Chen, S. J.; Huang, S. T.; Li, Y.; Zhou, C. C. Recent advances in epsilon-poly-L-lysine and L-lysine-based dendrimer synthesis, modification, and biomedical applications. *Front. Chem.* **2021**, *9*, 14.
- 26 Hyldgaard, M.; Mygind, T.; Vad, B. S.; Stenvang, M.; Otzen, D. E.; Meyer, R. L. The antimicrobial mechanism of action of epsilon-poly-L-lysine. *Appl. Environ. Microbiol.* **2014**, *80*, 7758–7770.
- 27 Zou, Y. J.; He, S. S.; Du, J. Z. Epsilon-poly(L-lysine)-based hydrogels with fast-acting and prolonged antibacterial activities. *Chinese J. Polym. Sci.* **2018**, *36*, 1239–1250.
- 28 Shi, C.; He, Y.; Feng, X. B.; Fu, D. H. Epsilon-polylysine and next-generation dendrigraft poly-L-lysine: chemistry, activity, and applications in biopharmaceuticals. *J. Biomater. Sci., Polym. Ed.* **2015**, *26*, 1343–1356.
- 29 Liu, X.; Guo, J. W.; Liu, Y. D.; Liu, M.; Liu, H.; Han, M. M.; Ji, S. X. Antibacterial thermoplastic polyurethane/PL-DOS composite films. *Chinese J. Polym. Sci.* **2021**, *39*, 1020–1028.
- 30 Ye, R.; Xu, H.; Wan, C.; Peng, S.; Wang, L.; Xu, H.; Aguilar, Z. P.; Xiong, Y.; Zeng, Z.; Wei, H. Antibacterial activity and mechanism of action of ϵ -poly-L-lysine. *Biochem. Biophys. Res. Commun.* **2013**, *439*, 148–153.
- 31 Shukla, S. C.; Singh, A.; Pandey, A. K.; Mishra, A. Review on production and medical applications of ϵ -polylysine. *Biochem. Eng. J.* **2012**, *65*, 70–81.
- 32 Harada, K. Thermal homopolymerization of lysine and copolymerization with neutral and acidic amino acids. *Bull. Chem. Soc. Jpn.* **1959**, *32*, 1007–1008.
- 33 Harada K.; W., F. S. Characterization of thermal polymers of neutral α -amino acids with dicarboxylic amino acids or lysine. *Arch. Biochem. Biophys.* **1965**, *109*, 49–56.
- 34 Scholl, M.; Kadlecova, Z.; Klok, H. A. Dendritic and hyperbranched polyamides. *Prog. Polym. Sci.* **2009**, *34*, 24–61.
- 35 Liu, X.; Yang, Y.; Han, M.; Guo, J.; Liu, H.; Liu, Y.; Xu, J.; Ji, S.; Chen, X. Guanlylated hyperbranched polylysines with high *in vitro* and *in vivo* antifungal activity. *Adv. Healthc. Mater.* **2022**, 2201091.
- 36 Kadlecova, Z.; Rajendra, Y.; Matasci, M.; Baldi, L.; Hacker, D. L.; Wurm, F. M.; Klok, H. A. DNA delivery with hyperbranched polylysine: a comparative study with linear and dendritic polylysine. *J. Controlled Release* **2013**, *169*, 276–288.
- 37 Kadlecova, Z.; Baldi, L.; Hacker, D.; Wurm, F. M.; Klok, H. A. Comparative study on the *in vitro* cytotoxicity of linear, dendritic, and hyperbranched polylysine analogues. *Biomacromolecules* **2012**, *13*, 3127–3137.
- 38 Thompson, M.; Scholz, C. Highly branched polymers based on poly(amino acid)s for biomedical application. *Nanomaterials* **2021**, *11*, 23.
- 39 Ramarao, N.; Nielsen-Leroux, C.; Lereclus, D. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. *JoVE* **2012**, *70*, 4392.
- 40 Balouiri, M.; Sadiki, M.; Ibensouda, S. K. Methods for *in vitro* evaluating antimicrobial activity: a review. *J. Pharm. Anal.* **2016**, *6*, 71–79.
- 41 Fischer, W. Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. *Med. Microbiol. Immunol.* **1994**, *183*, 61–76.
- 42 Beveridge, T. J. Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* **1999**, *181*, 4725–4733.
- 43 Li, P.; Zhou, C.; Rayatpisheh, S.; Ye, K.; Poon, Y. F.; Hammond, P. T.; Duan, H.; Chan-Park, M. B. Cationic peptidopolysaccharides show excellent broad-spectrum antimicrobial activities and high selectivity. *Adv. Mater.* **2012**, *24*, 4130–4137.
- 44 Lu, C.; Quan, G. L.; Su, M.; Nimmagadda, A.; Chen, W. D.; Pan, M.; Teng, P.; Yu, F. Y.; Liu, X.; Jiang, L.; Du, W. Y.; Hu, W.; Yao, F.; Pan, X.; Wu, C. B.; Liu, D. J.; Cai, J. F. Molecular architecture and charging effects enhance the *in vitro* and *in vivo* performance of multi-arm antimicrobial agents based on star-shaped poly(L-lysine). *Adv. Ther.* **2019**, *2*, 11.
- 45 Yeaman, M. R.; Yount, N. Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **2003**, *55*, 27–55.
- 46 Nam, S. Y.; Lee, J.; Shin, S. S.; Yoo, H. J.; Yun, M.; Kim, S.; Kim, J. H.; Lee, J. H. Antibacterial and cytotoxic properties of star-shaped quaternary ammonium-functionalized polymers with different pendant groups. *Polym. Chem.* **2022**, *13*, 1763–1773.
- 47 Al-Badri, Z. M.; Som, A.; Lyon, S.; Nelson, C. F.; Nusslein, K.; Tew, G. N. Investigating the effect of increasing charge density on the hemolytic activity of synthetic antimicrobial polymers. *Biomacromolecules* **2008**, *9*, 2805–2810.
- 48 Palermo, E. F.; Kuroda, K. Structural determinants of antimicrobial activity in polymers which mimic host defense peptides. *Appl. Microbiol. Biot.* **2010**, *87*, 1605–1615.
- 49 Li, P.; Poon, Y. F.; Li, W.; Zhu, H.-Y.; Yeap, S. H.; Cao, Y.; Qi, X.; Zhou, C.; Lamrani, M.; Beuerman, R. W.; Kang, E.-T.; Mu, Y.; Li, C. M.; Chang, M. W.; Jan Leong, S. S.; Chan-Park, M. B. A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. *Nat. Mater.* **2011**, *10*, 149–156.
- 50 Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C.; Wilcox, W. Hydrophobic moments and protein-structure. *Faraday Symp. Chem. Soc.* **1982**, *17*, 109–120.
- 51 Van den Bergen, G.; Stroet, M.; Caron, B.; Poger, D.; Mark, A. E. Curved or linear? Predicting the 3-dimensional structure of α -helical antimicrobial peptides in an amphipathic environment. *FEBS Lett.* **2020**, *594*, 1062–1080.
- 52 Su, X.; Zhou, X.; Tan, Z.; Zhou, C. Highly efficient antibacterial diblock copolypeptides based on lysine and phenylalanine. *Biopolymers* **2017**, *107*, 23041.
- 53 Ramamourthy, G.; Park, J.; Seo, C.; Vogel, H. J.; Park, Y. Antifungal and antibiofilm activities and the mechanism of action of repeating lysine-tryptophan peptides against *Candida albicans*. *Microorganisms* **2020**, *8*, 22.
- 54 Kuroda, K.; Caputo, G. A.; DeGrado, W. F. The role of hydrophobicity in the antimicrobial and hemolytic activities of polymethacrylate derivatives. *Chem. Eur. J.* **2009**, *15*, 1123–1133.
- 55 Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389–395.
- 56 Lienkamp, K.; Madkour, A. E.; Kumar, K. N.; Nüsslein, K.; Tew, G. N. Antimicrobial polymers prepared by ring-opening metathesis polymerization: manipulating antimicrobial properties by organic counterion and charge density variation. *Chem. Eur. J.* **2009**, *15*, 11715–11722.
- 57 Chen, L. C.; Kung, S. K.; Chen, H. H.; Lin, S. B. Evaluation of zeta potential difference as an indicator for antibacterial strength of low molecular weight chitosan. *Carbohydr. Polym.* **2010**, *82*, 913–919.
- 58 Henkelman, S.; Rakhorst, G.; Blanton, J.; van Oeveren, W. Standardization of incubation conditions for hemolysis testing of

- biomaterials. *Mater. Sci. Eng., C* **2009**, 29, 1650–1654.
- 59 Ong, Z. Y. I.; Yang, C.; Cheng, W.; Voo, Z. X.; Chin, W.; Hedrick, J. L.; Yang, Y. Y. Biodegradable cationic poly(carbonates): effect of varying side chain hydrophobicity on key aspects of gene transfection. *Acta Biomater.* **2017**, 54, 201–211.
 - 60 Chen, J.; Jiao, Z. X.; Lin, L.; Guo, Z. P.; Xu, C. N.; Li, Y. H.; Tian, H. Y.; Chen, X. S. Polylysine-modified polyethylenimines as siRNA carriers for effective tumor treatment. *Chinese J. Polym. Sci.* **2015**, 33, 830–837.
 - 61 Monnery, B. D.; Wright, M.; Cavill, R.; Hoogenboom, R.; Shaunak, S.; Steinke, J. H. G.; Thanou, M. Cytotoxicity of polycations: relationship of molecular weight and the hydrolytic theory of the mechanism of toxicity. *Int. J. Pharm.* **2017**, 521, 249–258.
 - 62 Huang, M.; Khor, E.; Lim, L. Y. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. *Pharm. Res.* **2004**, 21, 344–353.
 - 63 Colomer, A.; Pinazo, A.; Garcia, M. T.; Mitjans, M.; Vinardell, M. P.; Infante, M. R.; Martinez, V.; Perez, L. pH-sensitive surfactants from lysine: assessment of their cytotoxicity and environmental behavior. *Langmuir* **2012**, 28, 5900–5912.
 - 64 Uppu, D.; Samaddar, S.; Hoque, J.; Konai, M. M.; Krishnamoorthy, P.; Shome, B. R.; Haldar, J. Side chain degradable cationic-amphiphilic polymers with tunable hydrophobicity show *in vivo* activity. *Biomacromolecules* **2016**, 17, 3094–3102.
 - 65 Choksakulnimitr, S.; Masuda, S.; Tokuda, H.; Takakura, Y.; Hashida, M. *In vitro* cytotoxicity of macromolecules in different cell culture systems. *J. Controlled Release* **1995**, 34, 233–241.
 - 66 Morgan, D. M.; Clover, J.; Pearson, J. D. Effects of synthetic polycations on leucine incorporation, lactate dehydrogenase release, and morphology of human umbilical vein endothelial cells. *J. Cell Sci.* **1988**, 91, 231–238.
 - 67 Morgan, D. M.; Larvin, V. L.; Pearson, J. D. Biochemical characterisation of polycation-induced cytotoxicity to human vascular endothelial cells. *J. Cell Sci.* **1989**, 94, 553–559.
 - 68 Frederiksen, N.; Hansen, P. R.; Zabicka, D.; Tomczak, M.; Urbas, M.; Domraceva, I.; Björkling, F.; Franzyk, H. Alternating cationic-hydrophobic peptide/peptoid hybrids: influence of hydrophobicity on antibacterial activity and cell selectivity. *ChemMedChem* **2020**, 15, 2544–2561.
 - 69 Lee, J.; Kang, D.; Choi, J.; Huang, W.; Wadman, M.; Barron, A. E.; Seo, J. Effect of side chain hydrophobicity and cationic charge on antimicrobial activity and cytotoxicity of helical peptoids. *Bioorg. Med. Chem. Lett.* **2018**, 28, 170–173.
 - 70 Lienkamp, K.; Kumar, K. N.; Som, A.; Nüsslein, K.; Tew, G. N. “Doubly selective” antimicrobial polymers: how do they differentiate between bacteria. *Chem. Eur. J.* **2009**, 15, 11710–11714.
 - 71 Konaté, K.; Mavoungou, J. F.; Lepengué, A. N.; Aworet-Samseny, R. R. R.; Hilou, A.; Souza, A.; Dicko, M. H.; M'Batchi, B. Antibacterial activity against β -lactamase producing Methicillin and Ampicillin-resistants *Staphylococcus aureus*: fractional Inhibitory Concentration Index (FICI) determination. *Ann. Clin. Microbiol. Antimicrob.* **2012**, 11, 18.
 - 72 Kavanagh, K.; Reeves, E. P. Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev.* **2004**, 28, 101–112.
 - 73 Desbois, A. P.; Coote, P. J. Wax moth larva (*Galleria mellonella*): an *in vivo* model for assessing the efficacy of antistaphylococcal agents. *J. Antimicrob. Chemother.* **2011**, 66, 1785–1790.
 - 74 Gibreel, T. M.; Upton, M. Synthetic epidermicin NI01 can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2013**, 68, 2269–2273.
 - 75 Brackman, G.; Cos, P.; Maes, L.; Nelis, H. J.; Coenye, T. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* **2011**, 55, 2655–2661.
 - 76 Peleg, A. Y.; Monga, D.; Pillai, S.; Mylonakis, E.; Moellering, R. C.; Eliopoulos, G. M. Reduced susceptibility to vancomycin influences pathogenicity in *Staphylococcus aureus* infection. *J. Infect. Dis.* **2009**, 199, 532–536.
 - 77 Gao, W.; Chua, K.; Davies, J. K.; Newton, H. J.; Seemann, T.; Harrison, P. F.; Holmes, N. E.; Rhee, H. W.; Hong, J. I.; Hartland, E. L.; Stinear, T. P.; Howden, B. P. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog.* **2010**, 6, 100094.
 - 78 Peleg, A. Y.; Jara, S.; Monga, D.; Eliopoulos, G. M.; Moellering, R. C., Jr.; Mylonakis, E. *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrob. Agents Chemother.* **2009**, 53, 2605–2609.
 - 79 Tsai, C. J. Y.; Loh, J. M. S.; Proft, T. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* **2016**, 7, 214–229.
 - 80 Zhang, X. X.; Yang, L.; Lu, X. Q.; Lv, Y.; Jiang, D.; Yu, Y.; Peng, Z.; Dong, Z. H. Characterization and property of dual-functional Zn-incorporated TiO₂ micro-arc oxidation coatings: the influence of current density. *J. Alloys Compd.* **2019**, 810, 151893.
 - 81 Ren, L.; Chen, J. X.; Lu, Q.; Wang, C. B.; Han, J.; Huang, K.; Pan, X. N.; Wu, H. Construction of high selectivity and antifouling nanofiltration membrane *via* incorporating macrocyclic molecules into active layer. *J. Membr. Sci.* **2020**, 597, 117641.
 - 82 Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. *Nat. Rev. Microbiol.* **2005**, 3, 238–250.
 - 83 Takahashi, D.; Shukla, S. K.; Prakash, O.; Zhang, G. Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity. *Biochimie* **2010**, 92, 1236–1241.
 - 84 Ganewatta, M. S.; Tang, C. Controlling macromolecular structures towards effective antimicrobial polymers. *Polymer* **2015**, 63, A1–A29.
 - 85 Bechinger, B.; Gorr, S. U. Antimicrobial peptides: mechanisms of action and resistance. *J. Dent. Res.* **2017**, 96, 254–260.