

Polymer Chemistry

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Antimicrobial Amphiphilic Random Copolymers From Bio-Based Methacrylates: Effect of Chemical Composition on Activity and Selectivity

View Article Online
DOI: 10.1039/C5PY00298F

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1. Abstract

Antibiotic resistance is a growing global threat, prompting the development of novel synthetic antimicrobial agents. Many systems are inspired by antimicrobial peptides (AMPs), which structurally are cationic amphiphilic copolymers. To mimic this type of structure, using at the same time bio-based building blocks, in this study, we synthesized 15 amphiphilic random copolymers using amino acid-derived methacrylates with different side chains (Ala, Phe, Lys) as the cationic component, and bio-based tetrahydrogeranyl methacrylate (THGA) as the hydrophobic component via Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization, yielding copolymers with varied cationic/hydrophobic balances. The antimicrobial activity of these copolymers was evaluated against *E. coli* and *S. aureus*, whereas cytotoxicity using mouse fibroblasts (*L929*). The results indicate that Lys-based copolymers with higher cationic content, are the most active and selective. Activity against *S. aureus* was influenced by the hydrophobic-hydrophilic balance, showing a non-linear trend. The cytotoxicity of these polymers also increased with the rise in cationic content. Live/dead staining, SEM, and calcein leakage assays confirmed the membrane-disrupting effects of random copolymers containing Lys as the cationic component. Furthermore, leakage was observed in liposomes mimicking bacterial membranes at concentrations below the minimum inhibitory concentration (MIC). These findings highlight the potential of bio-based amphiphilic copolymers as selective antimicrobial agents, paving the way for a more sustainable design of such pharmaceutical products.



Keywords: Antimicrobial polymers, RAFT polymerization, Bio-based monomers, Amphiphilic copolymers, Membrane disruption

2. Introduction

Controlling the proliferation of harmful microorganisms, identifying pathogenic bacteria, and minimizing their impact on human life have long been focal points in research¹. This issue has become even more critical with the overuse of antibiotics, which has led to the emergence and rapid spread of antibiotic-resistant bacteria, posing a serious threat to global health^{2,3}. In response, researchers are actively seeking novel structural strategies to create the next-generation antimicrobial materials that combine high efficacy with excellent biosafety. These efforts extend to a diverse array of approaches, including metal nanoparticles^{4,5}, antimicrobial peptides^{6,7}, antimicrobial polymers⁸⁻¹¹, and emerging hybrid systems that integrates multiple mechanisms of action¹²⁻¹⁴. In the search for effective antibiotic alternatives, researchers are focused on identifying critical factors that enhance antimicrobial efficacy while minimizing cytotoxicity. Antimicrobial peptides (AMPs) represent a promising class of molecules, typically composed of 10-50 amino acid residues^{7,15}. Although this may be an oversimplification, the accepted antibacterial mechanism of AMPs is attributed to their amphiphilic nature, characterized by cationic charges, hydrophobic chains, and hydrophilic groups, that enables them to interact with bacterial membranes, disrupting cell integrity¹⁶. The positive charges allow AMPs to bind preferentially to the negatively charged components commonly found on bacterial surfaces, such as anionic lipids in the membranes or teichoic acids in Gram-positive bacteria^{17,18}. These electrostatic interactions promote selective accumulation on bacterial membranes over mammalian cells. In Gram-negative bacteria, AMPs often first interact with the outer membrane, which contains lipopolysaccharides (LPS), before reaching the inner membrane; in Gram-positive bacteria, although the absence of LPS allows direct access to the cytoplasmic membrane, the thick peptidoglycan layer can also constitute a substantial barrier to AMPs penetration^{19,20}. Consequently, the efficiency of membrane disruption and pore formation depends strongly on the specific AMPs and bacterial strain, and membrane destabilization remains a common mode of action²¹.

With advancements in controlled polymerization techniques, particularly Reversible Deactivation Radical Polymerization (RDRP), such as Atom Transfer Radical Polymerization (ATRP) and Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization²²⁻²⁵, scientists were able to synthesize numerous amphiphilic antimicrobial polymers that mimic the above-mentioned functional and structural attributes of natural AMPs²⁶⁻³³. Recent investigations on synthetic polymers revealed that the molar mass plays a crucial role in determining their biocompatibility. In many reported literatures, particularly for linear polymers, an increase in molar mass is associated with heightened cytotoxicity³⁴⁻³⁶. To achieve an optimal amphiphilic balance, hydrophobic and cationic monomers are frequently utilized,



with hydrophilic neutral monomers sometimes included³⁷⁻³⁹. Importantly, the addition of these hydrophilic monomers has been demonstrated to significantly enhance biocompatibility. As mentioned above, it is commonly accepted that cationic groups facilitate the adsorption of polymers onto anionic bacterial membranes via electrostatic interactions, which has led to extensive research on various cationic moieties to better understand their structural implications for the overall biological activity⁴⁰⁻⁴³. On the other hand, hydrophobic groups are suggested to disrupt the integrity of bacterial and mammalian membranes, prompting researchers to screen various hydrophobic monomers (including differences in chain length and chemical structure) to improve selectivity^{37, 44-46}. Overall, both the type and density of cationic groups, as well as the nature of hydrophobic monomers, play crucial roles in determining antimicrobial activity and cytotoxicity^{39, 47, 48}. To address these requirements, cationic amino acid-functionalized monomers have been widely employed in antimicrobial polymer design, owing to their ability to partially reproduce key structural and functional features of AMPs⁴⁹⁻⁵¹. However, two critical limitations remain. First, although amino acid-based monomers have been increasingly used, most synthetic amphiphilic copolymers reproduce only a limited subset of AMPs compositional features, making it difficult to systematically disentangle the specific contributions of individual amino acid side chains to antimicrobial activity, selectivity, and cytotoxicity. In natural AMPs sequences, antimicrobial function arises from a complex interplay of multiple residues and motifs, which together govern membrane affinity, selectivity, and conformational stability⁵². Replicating this level of functional complexity in synthetic polymer systems remains a significant challenge. Second, the hydrophobic components in the majority of reported antimicrobial polymers are still based on fossil-derived monomers, which limits the sustainability of these materials^{37, 38, 44, 47}.

With the goal of proposing a more sustainable design for peptide-inspired amphiphilic polymers, in this study, we prepared polymers based on cationic and hydrophobic bio-based monomers. We converted amino acids into corresponding methacrylates, and explored the impact of amino acid side chain variations on the antimicrobial activity of amphiphilic polymers, utilizing amino acids with different side chains (Ala, methyl; Phe, aromatic; Lys, cationic) as the cationic components. Additionally, bio-based tetrahydrogeraniol (THG) was incorporated as the hydrophobic component, via modification into the corresponding methacrylate⁵³. Beyond its renewable origin, THG provides a moderate and well-balanced hydrophobicity, comparable to commonly used short-to-medium linear alkyl methacrylates (e.g., C6–C8), while its branched aliphatic structure helps avoid excessive hydrophobicity, which often significantly increases the cytotoxicity of polymers due to excessively long hydrophobic chains^{32, 54}. Through RAFT controlled polymerization, we successfully synthesized a library of 15 statistical amphiphilic random copolymers with varying ratios of cationic and hydrophobic components. The antimicrobial activity and cytotoxicity of these copolymers were evaluated against Gram-negative *E. coli*, Gram-positive *S. aureus*, and mouse fibroblast cells (*L929*). These findings contribute to a deeper understanding of amphiphilic antimicrobial polymers and may help develop agents with enhanced



antimicrobial efficacy and reduced cytotoxicity toward mammalian cells, starting from more sustainable building blocks.

Article Online
DOI: 10.1039/D6PY00298F

3. MATERIALS AND METHODS

Materials

2,2'-Azobisisobutyronitrile (AIBN), tetrahydrogeraniol (THG), triethylamine (TEA), Methacryloyl chloride, Boc-Ala-OH, Boc-Phe-OH, Boc-Lys (Boc)-OH·DCHA, *N,N'*-Dicyclohexylcarbodiimide (DCC), *N,N*-Dimethylpyridin-4-amine (DMAP), trifluoroacetic acid (TFA), 2-Hydroxyethyl methacrylate (HEMA), 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid, calcein, phosphatidylcholine (PC), sodium bicarbonate (NaHCO₃), anhydrous sodium sulfate (Na₂SO₄), sodium chloride (NaCl), potassium bisulfate (KHSO₄), hydrochloric acid (HCl), dimethylformamide (DMF), dichloromethane (DCM); dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Gibco. Cell Proliferation Kit XTT was purchased from Panreac Applichem, LIVE/DEAD BacLight Bacterial Viability Kit bought from Thermo Fisher Scientific (Catalog number L7012). The bacteria and cell were obtained from ATCC: *E. coli* (ATCC 25922); *S. aureus* (ATCC 6538); mouse fibroblast cells (*L929* (ATCC CCL-1)).

Monomers Synthesis

Tetrahydrogeranyl methacrylate, THGA⁵³: In a three-neck 500 mL round-bottomed flask equipped with a rubber seal, a dropping funnel and a magnetic stirrer, were added THG (9.50g, 0.06 mol), TEA (6.07g, 0.06mol) and DCM (180 mL). The mixture was placed in an ice bath and cooled with stirring for 2 hours. Subsequently, methacryloyl chloride (8.15 g, 0.09 mol) was added dropwise through a dropping funnel. After the addition was complete, the mixture was stirred in ice bath for 1 hour and stirred at ambient temperature for 24 hours. Then, the mixture was suction filtered and the mixture was washed several times with brine (150 mL) and deionized water (150 mL). The organic phase solvent was removed by rotary evaporation to obtain a light yellow transparent viscous liquid (12.48 g, 92 % yield). ¹H NMR (400 MHz, CDCl₃) δ 6.09 (dt, *J* = 2.0, 1.0 Hz, 1H), 5.54 (p, *J* = 1.6 Hz, 1H), 4.25 – 4.11 (m, 2H), 1.94 (dd, *J* = 1.6, 1.0 Hz, 3H), 1.70 – 1.66 (m, 1H), 1.55 – 1.48 (m, 3H), 1.34 – 1.10 (m, 6H), 0.89 (dd, *J* = 20.5, 6.6 Hz, 9H).

¹³C NMR (101 MHz, CDCl₃) δ 167.56, 136.56, 125.11, 63.28, 39.17, 37.11, 35.47, 29.92, 27.93, 24.60, 22.66, 22.58, 19.57, 18.31.

Boc-Amino acid-HEMA⁵⁵: In brief, for the synthesis of Boc-Ala-HEMA, Boc-Ala-OH (26.43 mmol, 5.0 g) was dissolved in 100 mL of anhydrous DCM in a 250 mL two-neck round-bottom flask equipped with a magnetic stir bar, and the solution was purged with anhydrous N₂ for 20 minutes. Under a N₂ atmosphere, DCC (29.07 mmol, 6.00 g) was dissolved in 30 mL of DCM and added dropwise with vigorous stirring. After the addition, DMAP (1.32 mmol, 0.16 g) was added. The reaction flask was then



transferred to an ice-water bath, and HEMA (26.43 mmol, 3.44 g) was added dropwise. The mixture was stirred in the ice bath for 30 minutes and then stirred at room temperature for 24 hours. The reaction mixture was filtered, and the filtrate was washed sequentially with 0.1 N HCl (4 × 200 mL), saturated NaHCO₃ (4 × 200 mL), and brine (2 × 100 mL). The filtrate was dried over anhydrous Na₂SO₄. The solvent was removed by rotary evaporation to yield a colorless viscous liquid, identified as Boc-Ala-HEMA (91 % yield).

The synthesis methods for Boc-Phe-HEMA (93 % yield) and Boc-Lys(Boc)-HEMA (95 % yield) are identical. Notably, for the purchased Boc-Lys(Boc)-OH·DCHA salt, a pre-treatment was required. The precursor was first dissolved in DCM, the solution was then extracted three times with cold KHSO₄ aqueous solution. The organic layer was dried over Na₂SO₄, filtered, and the solvent was removed under reduced pressure to obtain the free Boc-Lys(Boc)-OH acid.

Boc-Ala-HEMA:

¹H NMR (400 MHz, CDCl₃) δ 6.13 – 6.11 (m, 1H), 5.59 (p, *J* = 1.6 Hz, 1H), 4.46 – 4.40 (m, 1H), 4.39 – 4.31 (m, 4H), 1.94 (dd, *J* = 1.6, 1.0 Hz, 3H), 1.44 (s, 12H).

¹³C NMR (101 MHz, CDCl₃) δ 173.15, 167.03, 153.62, 135.81, 126.16, 125.99, 80.23, 62.82, 62.16, 50.14, 28.32, 28.28, 28.26, 18.59, 18.22.

Boc-Phe-HEMA:

¹H NMR (400 MHz, DMSO) δ 7.37 – 7.13 (m, 5H), 6.02 (s, 1H), 5.71 – 5.63 (m, 1H), 4.38 – 4.03 (m, 4H), 2.95 (dd, *J* = 13.8, 5.1 Hz, 1H), 2.83 (dd, *J* = 13.8, 9.9 Hz, 1H), 1.85 (t, *J* = 1.3 Hz, 2H), 1.34 – 1.04 (m, 9H).

¹³C NMR (101 MHz, CDCl₃) δ 171.69, 167.03, 155.04, 135.87, 135.80, 129.26, 128.55, 127.07, 126.23, 79.95, 62.94, 62.17, 54.35, 38.27, 28.26, 18.25.

Boc-Lys(Boc)-OH free acid:

¹H NMR (400 MHz, CDCl₃) δ 5.22 (s, 1H), 4.63 (s, 1H), 4.29 (s, 1H), 3.12 (d, *J* = 6.5 Hz, 2H), 1.79 (d, *J* = 49.5 Hz, 2H), 1.44 (d, *J* = 3.5 Hz, 22H).

¹³C NMR (101 MHz, CDCl₃) δ 176.22, 156.31, 155.81, 80.06, 79.35, 53.25, 40.06, 31.97, 29.51, 28.40, 28.32, 22.37.

Boc-Lys(Boc)-HEMA:

¹H NMR (400 MHz, CDCl₃) δ 6.12 (dd, *J* = 2.2, 1.1 Hz, 1H), 5.60 (p, *J* = 1.6 Hz, 1H), 4.47 – 4.23 (m, 5H), 3.09 (d, *J* = 7.4 Hz, 2H), 1.94 (dd, *J* = 1.6, 1.0 Hz, 3H), 1.68 (d, *J* = 8.2 Hz, 2H), 1.43 (s, 23H).

¹³C NMR (101 MHz, CDCl₃) δ 172.57, 167.00, 156.03, 135.81, 126.19, 79.89, 62.83, 62.17, 53.24, 40.01, 32.26, 29.56, 28.40, 28.29, 22.42, 18.23.

Synthesis of Boc-Protected Random Copolymers

Typically, Boc-Ala-HEMA and THGA (in varying ratios, totaling 10 mmol) were dissolved in 5 mL of 1,4-dioxane, followed by the addition of the RAFT chain transfer agent 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (5% of the total monomer amount, 0.5 mmol)



and the initiator AIBN (8 mg, 0.05 mmol). The mixture is purged with nitrogen for 15 minutes and then stirred at 70°C. After 18 hours, the reaction is terminated by cooling the vial in an ice bath. The copolymer is isolated by evaporating the dioxane under reduced pressure, dissolving the residue in dichloromethane, and precipitating twice in an excess of n-hexane (or in a 9:1 ethanol-water mixture for polymers with high THGA content) to remove unreacted impurities. The sample was then dried overnight in a vacuum oven at 40°C, yielding a light-yellow solid copolymer.

Deprotection of Copolymers

The Boc-protected random copolymer (1 g) is combined with TFA (1 mL)⁵⁵. After stirring for 30 minutes, TFA was removed by purging with nitrogen. The remaining residue was dissolved in water, impurities were removed using a 3500 Da dialysis bag for 3 days. The copolymer solution in the dialysis bag was then lyophilized using freeze dryer to obtain a light, fluffy, white fibrous copolymer. NMR analysis was used to determine the removal of Boc protective groups.

¹H NMR Spectroscopy

NMR spectroscopy was used to analyze all the Boc-protected and de-protected copolymers to analyze polymer composition and conversion. Analysis was performed to determine the mole percent of THGA (MP_{THGA}), degree of polymerization (DP), and number-average molecular weight (M_n). All experiments were performed on a Agilent 400 MHz WB NMR spectrometer. All experiments were run with a gas flow across the probes at 535 L h⁻¹ with sample spinning and at a temperature of 25 °C.

Size exclusion chromatography (SEC)

The copolymers were also characterized by SEC analysis to measure the M_n; weight-average molecular weight (M_w) and dispersity (Đ) using DMF (containing 0.01 M LiBr) as eluent in a Viscotek GPCmax instrument equipped with a model 302 TDA detector based on refractive index and intrinsic viscosity and two columns (Agilent Technologies-PolarGel-L and M, 8 μm, 30 cm) at a flow rate of 1.0 mL min⁻¹ and a temperature of 50 °C. A universal calibration curve was prepared using narrow dispersion Poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories). Polymer solutions of 2 mg mL⁻¹ in DMF were prepared in the eluent and filtered through 0.45 μm filters prior to injection.

Dynamic light scattering (DLS) and Zeta potential (ζ)

For those polymers that can self-assembled aggregates in aqueous solution, a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) instrument equipped with a HeNe laser was used with a wavelength of λ = 633 nm and a scattering angle of 173°. The data was computed by cumulant fit analysis and the hydrodynamic diameter size (D_h) was calculated by implementing the Stokes–Einstein equation by the software. The ζ was determined from the electrophoretic mobility of the particles by applying the Smoluchowski equation. All polymers were measured at a concentration of 1 mg mL⁻¹ in PBS (solutions were filtered using 0.22 μm pore size filter). The final collected data of the size, and ζ of the particles is the average of three consecutive measurements of the same sample.

Transmission electron microscopy (TEM)



The morphology of the nanoscale aggregates was analyzed by TEM using an Philips CM120 operating at 120 keV. Images were recorded using a Gatan model UltraScan 4000SP CCD camera under low-dose conditions. All polymers were measured at a concentration of 0.1–0.5 mg mL⁻¹ in PBS (using 0.22 μm pore size filter) and 10 μL of the polymer solution was placed on a carbon-coated copper grid (Quantifoil 3.5/1, QuantifoilMicro Tools, Jena, Germany). Then samples were negatively stained with 2% uranyl acetate, and the excess solution was blotted off and dried at room temperature for a few minutes before measurement.

Zone of Inhibition

Briefly, a single colony of microorganisms was picked from the agar plate, inoculated into Mueller–Hinton broth (50 mL) and cultured at 37 °C for 12 h. Fresh colonies were then collected by centrifugation (6500 rpm, 5min) and washed three times with PBS to remove the remaining culture medium. Then the microbial solution was diluted to 1.0 x 10⁸ CFU mL⁻¹ with PBS, 100 μL were taken and spread evenly on the agar plate, 10 μL of random copolymers (10 mg mL⁻¹ in PBS or (10 % DMSO in PBS was used as solvent for insoluble samples)) with the same content were added to different positions of the plate, and then the plate was incubated at 37°C for 18 hours; the antibacterial activity was evaluated by measuring the inhibition zone.

The minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test

Random copolymers were initially dissolved in PBS to achieve stock concentrations of 20 mg mL⁻¹. For polymers that were insoluble in PBS, PBS containing up to 10% DMSO was used to ensure complete dissolution. Serial 2-fold dilutions of polymers were prepared from stock solutions in PBS and transferred to the 96-well sterile round-bottom polypropylene plate for a final concentration of 7.8–2000 μg mL⁻¹ (10 μL per well). PBS and 10 %DMSO in PBS were used as solvent controls. Bacteria were grown overnight (~18 hours) in MHB at 37 °C, fresh colonies were then collected by centrifugation (6500 rpm, 5min) and washed three times with PBS to remove the remaining culture medium. Then the microbial solution was diluted to 4 x 10⁵ CFU mL⁻¹ (Count and the culture diluted using the Bürker-Türk Counting Chambers) with MHB. The bacterial suspension (90 μL per well) was transferred to a 96-well sterile round-bottom polypropylene plate. Then the polymer solution was then to a final concentration of 7.8–2000 μg mL⁻¹ (10 μL per well). Plates were sealed with parafilm and incubated for 18 hours at 37 °C without shaking. MIC was defined as the lowest concentration of polymers to completely inhibit bacterial growth, as indicated by lack of turbidity.

After the MIC test, the MBC test is carried out directly. For each sample, from the concentration where no bacterial growth was observed to the concentration where turbid growth occurred, 10 μL of each test concentration was spotted on an agar plate and incubated at 37°C for 24 hours. The lowest concentration where no bacteria survived was defined as the MBC.

Cytotoxicity Assay



The Cell Proliferation Kit XTT^{56,57} (Panreac Applichem) was used to assess in vitro cytotoxicity using mouse fibroblast cells (*L929*). *L929* were grown in complete medium (RPMI 1640 (Gibco), until reaching subconfluency at 37 °C and 5% CO₂ in a cell culture incubator (Eppendorf CellXpert C170i). A 96-well cell culture plate was used to seed the cells (10⁴ cells/well). After 24 h, the growth medium was replaced with RPMI 1640 medium consisting of different polymer concentrations, two-fold serial dilutions were performed starting from 5000 µg mL⁻¹ and or without polymer (with PBS-treated wells as negative control). Cell viability was normalized to the negative control and defined as 100%. After a further 24 hours of incubation in the incubator (polymer continued to expose cells), 50 µl of reaction solution (from the kit) was added to each well and the plate was placed in the incubator for 2 hours. The absorbance of the samples relative to the background control was measured at 475 nm using a microplate reader (BioTek Synergy H1) at a wavelength of 475 nm. In order to measure reference absorbance (to measure non-specific readings), a wavelength of 660 nm was used and subtracted from the 475 nm measurement.

The following formula was used to calculate the percentage of cell viability:

$$\text{cell viability (\%)} = \frac{[OD_{660} (\text{test sample}) - OD_{475} (\text{test sample})]}{[OD_{660} (\text{negative control}) - OD_{475} (\text{negative control})]} \times 100\%$$

where OD₄₇₅ = absorbance at 475 nm; OD₆₆₀ = absorbance at 660 nm (test sample) was the average of the observed results of 3 wells, (negative control) was the average of 9 control wells with medium.

Live/Dead Membrane Integrity Assay

Bacterial cell membrane integrity was studied using the LIVE/DEAD BacLight Bacterial Viability Kit^{58, 59} (bought from Thermo Fisher Scientific (Catalog number L7012)). The kit utilize mixtures of SYTO9 green fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. Bacteria cells with a compromised membrane that were dead or dying will stain red with propidium iodide, whereas cells with an intact membrane will stain green with SYTO9. The bacterial suspension of 10⁸ cells mL⁻¹ in PBS was incubated in the absence (as control) or presence of different concentration (MIC and 4 x MIC) of polymer for 4 h at 37°C. After that the bacterial were harvest by centrifugation and washed in PBS by 3 times. Then the bacteria were resuspended in 0.9% salt solution to reach a final density of 1 × 10⁶ cells mL⁻¹. 10 µL of the bacterial suspension was added to 3 µL of the dye mixture (SYTO9 with propidium iodide 1:1). Mix each sample solution well and incubate in the dark at room temperature for 15 min. Then they were examined by confocal laser scanning microscopy (CLSM), DM4000B, Leica.

Scanning Electron Microscopy (SEM)

Bacteria were collected by centrifugation, washed cells (after three times washed with PBS) were adhered to slides and fixed with 2% glutaraldehyde for 10 min at 4 °C. They were then washed with 0.1 M cacodylate buffer, incubated with 1% OsO₄ in 0.1 M cacodylate buffer for 1 h at room temperature, washed with water, and then dehydrated using a series of graded ethanol solutions (30, 50, 70%) for 15 min each at room temperature, and finally dehydrated three times with 100% ethanol for 30 min each,



and then dried. Dehydrated samples were gold-coated for 20 s and imaged under high vacuum using a Supra 55 Scanning Electron Microscope (Zeiss).

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DOI: 10.1039/D6PY00298F

Lipid vesicles preparation

A lipid mixture composed of *E. coli* polar lipids and egg PC in a 3:1 molar ratio was prepared for vesicle formation. *E. coli* polar lipids were obtained from a commercial total lipid extract (Avanti Polar Lipids) by acetone precipitation followed by diethyl ether extraction, as described in reference ⁶⁰. Both *E. coli* lipids and egg PC were dissolved in chloroform, mixed, and the solvent was evaporated using a rotary evaporator (Büchi Labortechnik AG). The resulting lipid film was washed with diethyl ether, then hydrated in 50 mM potassium phosphate (pH 7.0) to a final concentration of 20 mg mL⁻¹. The lipid suspension was sonicated using a tip sonicator (70% amplitude, 15 s on/45 s off, 16 cycles) while kept on ice, and subjected to three freeze–thaw cycles (liquid nitrogen and room-temperature water bath). The prepared large-unilamellar vesicles (LUVs) were aliquoted and stocked in liquid nitrogen to prevent oxidation.

Liposomes preparation for calcein leakage experiment

A 100 mM calcein stock solution was prepared in 50 mM potassium phosphate buffer (KPi) and adjusted to pH 7.0 using 2 M NaOH (Sigma-Aldrich). Liposomes were resuspended in a solution containing 70 mM calcein and 20 mM KPi (pH 7.0) to a final lipid concentration of 5 mg mL⁻¹. Calcein, at a self-quenching concentration, was encapsulated into the vesicles by subjecting the suspension to three freeze–thaw cycles at 30 °C. Following this, the vesicle suspension was extruded 13 times through a 200 nm polycarbonate membrane (Whatman, GE Healthcare) to obtain large-unilamellar vesicles (LUVs). Unencapsulated calcein was removed via size-exclusion chromatography using a 22-cm Sephadex G-75 column (Sigma-Aldrich) pre-equilibrated with an iso-osmotic buffer composed of 50 mM KPi (pH 7.0) plus 50 mM sucrose. Fractions (1 mL each) containing calcein-loaded liposomes were identified under ultraviolet illumination and pooled. The final vesicle suspension was diluted to a total volume of 12 mL in the same buffer (KPi 50 mM, pH 7.0, sucrose 50 mM).

Calcein Leakage Experiment

The ability of the polymer to induce membrane lysis or pore formation was assessed using a calcein leakage assay. Calcein-loaded vesicles were incubated with increasing concentrations of the polymer (0–1000 µg mL⁻¹), and the release of calcein was monitored as an increase in fluorescence intensity. Measurements were performed using a Spark Multimode Plate Reader (TECAN) at 25 °C, with an excitation wavelength of 495 nm and emission recorded at 517 nm. Fluorescence was measured every minute for a total of 30 minutes, until a fluorescence plateau was reached. Afterward, Triton X-100 (50% w/w) was added to fully disrupt the vesicles and determine the maximum fluorescence signal, F(max).

The percentage of calcein leakage was calculated according to the following equation:

$$\text{Leakage (\%)} = \frac{F(t) - F(0)}{F(\text{max}) - F(0)} 100$$



where:

- $F(0)$ is the fluorescence at time 0 (prior to polymer addition),
- $F(t)$ is the fluorescence at a given time point after polymer addition,
- $F(max)$ is the fluorescence after complete vesicle dissolve with Triton X-100.

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Each measurement was performed in triplicate.

4. Results and discussion

Polymer Synthesis and Characterization

In this study, the synthetic route shown in **Figure 1** was used to functionalize the bio-based starting material by introducing carbon-carbon double bonds into the Boc-protected amino acid moieties (Ala, methyl; Phe, aromatic; Lys, cation) and THG. The ^1H and ^{13}C NMR characterizations confirmed that the synthesized monomer was successfully synthesized (**Figure S1-S10**). Then, a library of amphiphilic copolymers was successfully synthesized using Reversible Addition–Fragmentation Chain-Transfer (RAFT) Polymerization (**Figure 1**)⁶¹. Specifically, to closely approximate the molar mass of antimicrobial peptides (several kDa) and avoid increased cytotoxicity associated with higher molar mass, all polymers were prepared with a target DP of 20, with the Chain Transfer Agent (CTA) constituting 5% of the total molar amount of monomers. The polymers include three components: (1) cationic groups, represented by three amino acids with different side chains: Ala (methyl side chain), Phe (aromatic phenyl ring), or Lys (positively charged amino side chain); (2) hydrophobic monomer, derived from bio-based THG and modified with a double bond to form THGA; and (3) end-groups from the CTA, specifically CDPA, which contains a long aliphatic chain.

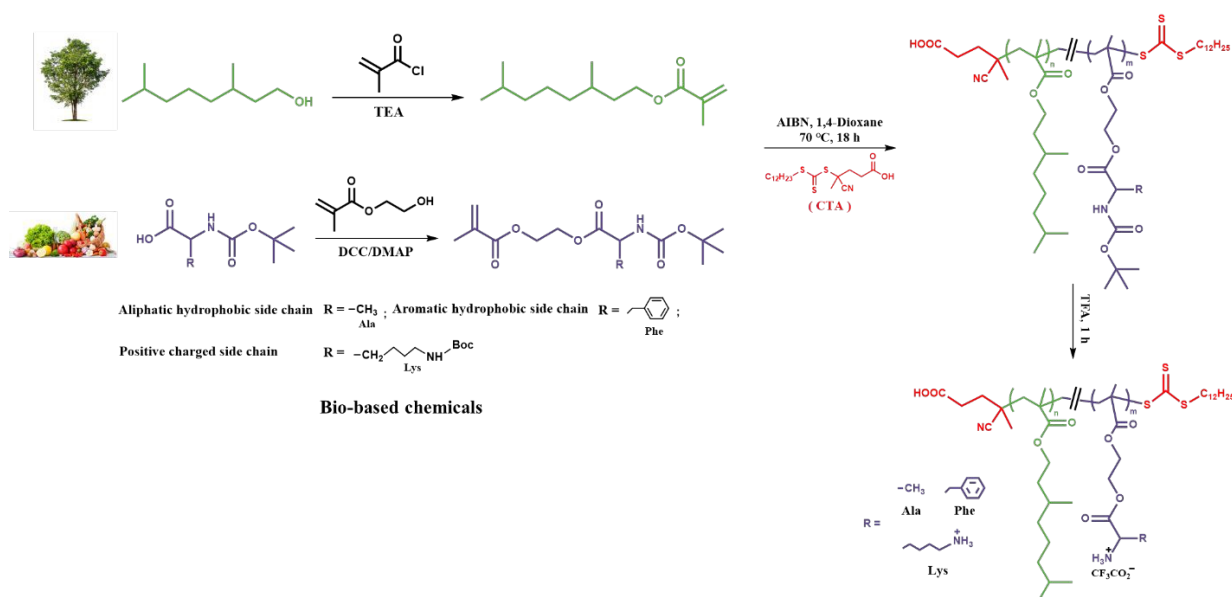


Figure 1. Schematic diagram the synthesis of bio-based monomer and amphiphilic random copolymers by RAFT polymerization.



By adjusting the ratio of cationic monomers to hydrophobic monomers, a series of amphiphilic random copolymers with varying molar ratios between the two components were synthesized to study their structure-activity relationships. Based on the cationic component, the polymers were classified into three major families: alanine group (A family), phenylalanine group (P family), and lysine group (L family). The hydrophobic monomer was THGA for all polymers. The polymers were named P(x -r-T) y , where x indicates the type of amino acids (A, P or L), and y is a number from 1 to 9, corresponding to the relative amount of cationic monomer (from 10 to 90%). For example, in P(A-r-T)1, A corresponds to the cationic monomer with Ala, r stands for random, T corresponds to the hydrophobic monomer THGA, and the number 1 indicates 10 mol% of cationic monomer (and 90 mol% of the hydrophobic one).

All the copolymers (with and without Boc deprotection) prepared by RAFT polymerizations were analyzed by ^1H NMR and SEC analysis to determine the monomer conversion (% conv^a), (M_n & M_w), copolymer composition (MP_{THGA}^a (%)), and \bar{D}^b (Table 1). The ^1H NMR analysis of the reaction solutions before and after the reaction was compared, and the monomer conversion was calculated by comparing the vinyl proton signals at 5.5 and 6.3 ppm with the solvent peak (Figure S11), indicating that the monomer conversion of all prepared polymers exceeded 80 %, with only the reaction monomer conversion of P(A-r-T)3 being 75.2 %. The M_n was estimated by SEC and ^1H NMR with good agreement between theoretical M_n and experimental values. The average DP was calculated by comparing the integral peak areas of the side chain terminal groups and main chain end groups (Figure S13, S15, S17). The MP_{THGA} value was determined by comparing the signals generated by the two different side chains in the ^1H NMR spectra. For each copolymer series, the fraction of hydrophobic repeating units (THGA) in the copolymer, MP_T , ranged from 10% to 90%, consistent with the monomer feed ratios. The SEC results (Figure S21) showed that the molecular weight (3 to 4 kDa) and \bar{D} values (1.2 to 1.4) indicated that the polymerization reaction was well controlled. After deprotection of the Boc group with TFA, the signal at about 1.4 ppm (attributed to the protons of the tert-butyl group) disappeared in the ^1H NMR spectrum of the polymer, confirming successful removal of the Boc protecting group.



Table 1. Polymer Characterization by ¹H NMR, SEC, DLS and Zeta potential analyses

Random copolymers	Theoretical composition (%)	% conv ^a	Boc-protected						Deprotected								
			MP _{THGA} ^a (%)	M _n ^a	DP ^a	M _n ^b	M _w ^b	Đ ^b	MP _{THGA} ^a (%)	M _n ^a	DP ^a	M _n ^b	M _w ^b	Đ ^b	H ₂ O sol	D _n ^c (nm)	ζ ^d (mV)
P(A-r-T)1	10 (Ala) : 90 (THGA)	90.2	85	3750	14	3400	4080	1.20	93	3570	14	2800	3900	1.41	×	nd	nd
P(A-r-T)3	30 (Ala) : 70 (THGA)	75.2	72	3660	13	3900	5000	1.28	75	3320	13	3300	4400	1.35	×	nd	nd
P(A-r-T)5	50 (Ala) : 50 (THGA)	90.9	49	4180	14	5000	6200	1.25	50	3350	14	4000	5800	1.45	×	nd	nd
P(A-r-T)7	70 (Ala) : 30 (THGA)	83.9	30	5290	17	5000	6200	1.25	34	3960	17	3600	5000	1.38	√	25.6	nd
P(A-r-T)9	90 (Ala) : 10 (THGA)	89.6	13	5709	18	5000	6200	1.23	26	3420	16	3500	4900	1.41	√	53.9	nd
P(P-r-T)1	10 (Phe) : 90 (THGA)	87.4	91	3620	15	3500	4400	1.25	93	nd	nd	3000	4000	1.35	×	nd	nd
P(P-r-T)3	30 (Phe) : 70 (THGA)	91.8	66	4180	14	4500	5500	1.22	76	4140	15	3400	4600	1.37	×	nd	nd
P(P-r-T)5	50 (Phe) : 50 (THGA)	92.7	47	5190	15	4800	6100	1.27	54	4350	15	3700	4900	1.31	×	nd	nd
P(P-r-T)7	70 (Phe) : 30 (THGA)	93.5	27	5170	14	5200	6400	1.25	34	nd	nd	3700	5000	1.35	×	nd	nd
P(P-r-T)9	90 (Phe) : 10 (THGA)	94.3	11	5520	14	5500	6600	1.18	11	nd	nd	3400	4400	1.28	×	nd	nd
P(L-r-T)1	10 (Lys) : 90 (THGA)	91.6	84	5480	19	5100	5600	1.10	91	4980	20	nd	nd	nd	×	nd	nd
P(L-r-T)3	30 (Lys) : 70 (THGA)	94.2	71	6820	21	5200	5800	1.12	81	4560	18	nd	nd	nd	×	nd	nd
P(L-r-T)5	50 (Lys) : 50 (THGA)	96.8	51	5760	16	5900	7100	1.09	53	nd	nd	nd	nd	nd	√	48.1	nd
P(L-r-T)7	70 (Lys) : 30 (THGA)	93.5	34	7070	18	6600	7200	1.08	29	nd	nd	nd	nd	nd	√	59.0	nd
P(L-r-T)9	90 (Lys) : 10 (THGA)	93.3	19	nd	nd	6300	7300	1.15	13	nd	nd	nd	nd	nd	√	64.6	nd

% conv^a (% monomer conversion during polymerization); MP_{THGA}^a (mole percentage of hydrophobic side chains); M_n^a (number average molecular weight); and DP^a (degree of polymerization) values were determined by ¹H NMR peak integration analysis. M_n^b, M_w^b, Dispersity^b (Đ^b) was determined by SEC analysis in DMF against PMMA standards; Hydrodynamic number average diameter size (D_n^c) and zeta potential (ζ^d) determined by Malvern Zetasizer Nano ZS at a concentration of 0.1 mg mL⁻¹ in PBS; (nd)-can not determined, indicating one of the following: (i) The NMR peak assignment is unclear because the polymer aggregates in the deuterated reagent after deprotection; (ii) insolubility of the polymer in THF preventing SEC measurement; or (iii) insolubility of the polymer in PBS preventing DLS measurement.

The water solubility of the random copolymers also correlates with the increasing content of cationic components. In the Ala series random copolymers, which have methyl side chains, the polymers become water-soluble when the cationic content reaches approximately 70%. In contrast, the Phe series polymers, bearing more hydrophobic phenyl side chains, remain insoluble regardless of cationic content. For the Lys series, which contains 2 cationic side chains per monomer, water solubility is achieved when the cationic component reaches around 50%. Subsequently, the amphiphilic polymers that could be dissolved in water were also characterized in aqueous media. All characterized polymers have positive ζ (8-21 mV) due to the cationic charge of the amino groups (**Table 1**). This low ζ value may be due to the difficulty of random copolymers in forming uniform and stable micelles, and the partial shielding of cationic charges by polymer intra- and inter-chain interactions. It can be pointed out here that lower stability of the aggregates should be beneficial for the antimicrobial activity, as the polymers most likely interact with the membrane as single chains. Random copolymers with Ala and Lys as cationic components all showed a clear trend of increasing self-assembled aggregate size with the increase of cationic components (Ala or Lys) (**Figure 2a and 2b**), suggesting that the more cationic polymers have larger aggregation numbers. Despite the lower ζ , TEM images confirmed the formation of sufficiently stable nanoscale aggregates (**Figure 2c**). The aggregates seem irregular in shape, and have sizes below 10 nm. This is obviously smaller than the hydrodynamic sizes obtained by DLS. This is because TEM is performed on dry samples under vacuum, and dehydration can also cause the nanoscale aggregates to shrink or disintegrate. Also, DLS measures the hydrodynamic diameter of the hydrated shell, so the measured diameter is larger than that observed by TEM⁶². Furthermore, because the TEM-observed aggregates are only a few nanometers in size, this approaches the resolution limit of TEM, making it difficult to reliably compare aggregate sizes between samples.

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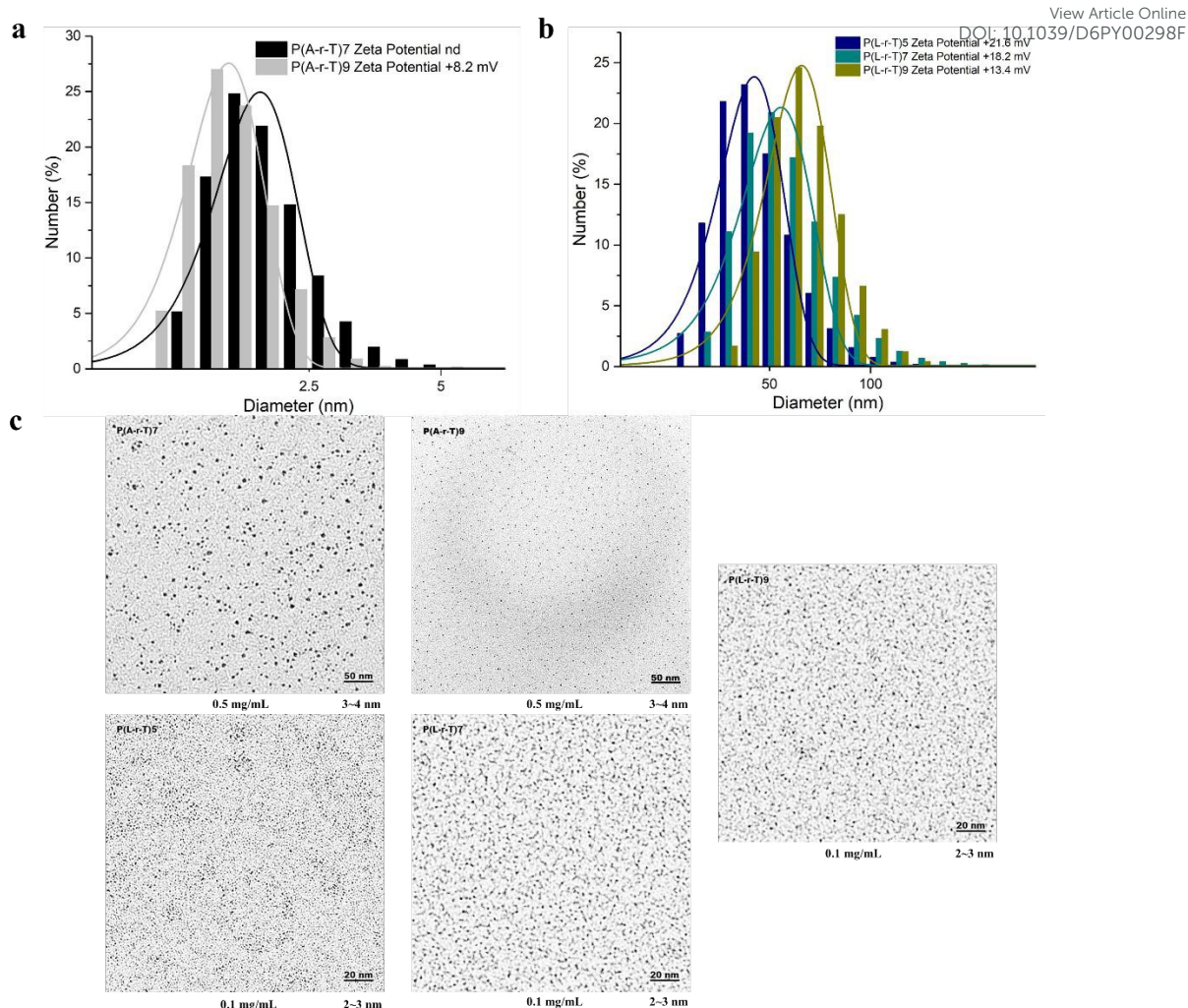


Figure 2. (a) DLS number size distributions of Ala series water-soluble copolymers in PBS solution (0.1 mg mL^{-1}). (b) DLS number size distributions of Lys series water-soluble copolymers in PBS solution (0.1 mg mL^{-1}). (c) TEM images of nanoscale aggregates formed by water-soluble antibacterial copolymers in PBS

Antimicrobial Activity

The antimicrobial activity of the polymers was evaluated by determining the MIC (the lowest concentration of the polymer that inhibited visible bacterial growth) and the MBC (the lowest concentration of the polymer at which the bacteria could not survive): Gram-negative bacterium *E. coli* (strain ATCC 25922); and Gram-positive bacterium *S. aureus* (strain ATCC 6538) (**Table 2**) were tested. Due to the limited water solubility of the polymers, DMSO was used as cosolvent.

As previously mentioned, these kind of antimicrobial polymers are believed to kill bacteria mainly via membrane disruption^{63, 64}. These amphiphilic polymers contain cationic and hydrophobic groups, each serving a distinct function. The cationic groups attract anionic components on the surface of bacterial cell membranes through electrostatic interactions, promoting the adsorption of the polymer onto the membrane while compromising membrane integrity and disrupting the transport of compounds across it⁶⁵⁻⁶⁷. Simultaneously, the interaction between the cationic groups and the cell membrane induces the polymer to adopt an overall amphiphilic conformation, allowing the hydrophobic groups to insert into the membrane, leading to membrane disruption and cell death^{68, 69}.



For the Gram-negative bacteria *E. coli*, among the random copolymers composed of Ala and Phe only, the samples with the highest cationic content (up to 90%) showed measurable antibacterial activity; which may be strictly correlated with their water solubility; however, their MICs were very high (2500 and 5000 $\mu\text{g mL}^{-1}$, respectively), indicating that these polymers are unlikely to be suitable for prospective in vivo applications. In contrast, for the Lys-based copolymers, increasing the cationic monomer content gradually enhanced the polymer antibacterial activity, with MICs decreasing from 1000 $\mu\text{g mL}^{-1}$ for P(L-r-T)5 to 125 $\mu\text{g mL}^{-1}$ for P(L-r-T)9. For the Gram-positive bacteria *S. aureus*, the balance between the cationic part and the hydrophobic chain with Ala, Phe and Lys has a more obvious effect on the MIC. As the content of the hydrophobic chain decreases, the antibacterial activity first weakens and then increases, with the exception of the P(A-r-T)9. Among them, the most hydrophobic one, P(P-r-T)1, showed the lowest MIC (250 $\mu\text{g mL}^{-1}$), followed by P(A-r-T)1, P(A-r-T)7, P(L-r-T)1, and P(L-r-T)9, with MICs of 500 $\mu\text{g mL}^{-1}$. The MBCs of the random copolymers tested were all higher than the MIC. Only the P(L-r-T)9 with the strongest antibacterial activity had the same MBC and MIC against *S. aureus*, which was 500 $\mu\text{g mL}^{-1}$. As reported in the previous section, the studied polymers are either insoluble in water, or they form relatively stable aggregates. The low value of ζ suggests that the aggregates are labile enough to allow the polymers to interact with the bacterial membranes as single chains.

The results of zone of inhibition experiments are shown in **Figure S22&S23**. For the *E. coli*, it can be clearly seen that there is no growth of *E. coli* in the areas where P(L-r-T)7 and P(L-r-T)9 were dropped, while *E. coli* grew in the areas where other samples. For the *S. aureus*, the inhibition zone of P(L-r-T)9 is very obvious, and the inhibition zone of P(P-r-T)1 can also be observed, while the inhibition zones of P(A-r-T)1, P(A-r-T)7 and P(L-r-T)1 need to be observed very carefully, which may be related to the diffusion intensity of the samples on the agar plate. This also illustrates the limitation of this method, which is only suitable for samples with strong antibacterial effect and easy diffusion on the agar plate. Notably, increased hydrophobicity does not universally enhance antimicrobial activity in this system. While moderate amphiphilicity is sufficient for effective activity against *S. aureus*, overcoming the outer membrane barrier of *E. coli* requires a substantially higher hydrophilic and cationic content. This observation highlights the crucial role that the fundamental differences in the cell membrane structure of Gram-positive and Gram-negative bacteria play in determining the antimicrobial properties of polymers. For *S. aureus*, the absence of an outer membrane allows amphiphilic polymers with moderate hydrophobicity and cationic content to access and disrupt the cytoplasmic membrane relatively efficiently, leading to higher overall activity across much of the polymer library. In contrast, *E. coli* possesses a lipopolysaccharide-rich outer membrane that constitutes a significant permeability barrier. In this case, initial electrostatic interactions and charge accessibility dominate antimicrobial efficacy. Polymers with higher hydrophobic content may experience reduced aqueous dispersibility or partial self-association, limiting effective interaction with the negatively charged outer membrane. Only



polymers with sufficiently high hydrophilic and cationic content remain well dispersed and exhibit enhanced activity against *E. coli*. Article Online
DOI: 10.1039/D6PY00298F

Table 2. Antimicrobial Activity (MIC&MBC) of random copolymers with varying cationic monomers and monomer feed ratios against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 6538)

Random copolymers	MIC ($\mu\text{g mL}^{-1}$)		MBC ($\mu\text{g mL}^{-1}$)	
	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 6538)	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 6538)
P(A-r-T)1	>5000	500	>10000	>2000
P(A-r-T)3	>5000	2000	>10000	>2000
P(A-r-T)5	>5000	2000	>10000	>2000
P(A-r-T)7	>5000	500	>5000	1000
P(A-r-T)9	2500	>2000	5000	>2000
P(P-r-T)1	>5000	250	>10000	>2000
P(P-r-T)3	>5000	2000	>10000	>2000
P(P-r-T)5	>5000	2000	>10000	>2000
P(P-r-T)7	>5000	2000	>10000	>2000
P(P-r-T)9	5000	1000	5000	>2000
P(L-r-T)1	>5000	500	10000	>2000
P(L-r-T)3	>5000	1000	10000	>2000
P(L-r-T)5	1000	1000	5000	>2000
P(L-r-T)7	250	2000	2000	>2000
P(L-r-T)9	125	500	2000	500

Cytotoxicity

Since there are many reports that cationic polymers can cause cytotoxicity by increasing the permeability of mammalian cell membranes, and the change in membrane permeability depends largely on the number of cationic charges on the polymer surface^{70, 71}, in order to evaluate the toxicity characteristics of such polymers in future applications, the cell viability of mouse fibroblasts (*L929*) after 24 h of exposure to the random copolymers was tested, and the results are shown in **Figure 3**. The cell viability after 24 hours showed the same pattern for random copolymers composed of all cationic species in this study (Ala, Phe and Lys). In random polymers with lower cationic component content, no cytotoxicity was shown at a concentration of up to 5000 $\mu\text{g mL}^{-1}$, while the cytotoxicity gradually increased with the increase of cationic component content. Similar results have been reported in the literature previously^{40, 47}. Polymers with higher cationic content contain more protonated units, which catalyzes the acidic hydrolysis of ester bonds in the phospholipid bilayer of the cell membrane to form single-chain lipids and fatty acids. Then, the single-chain lipids will quickly destroy the stability of the membrane and cause cytotoxicity^{72, 73}. For random copolymers with Ala and Phe as cations, among the tested polymers, only the polymer with a cationic content of 90% showed certain cytotoxicity at concentrations of 5000 and 2500 $\mu\text{g mL}^{-1}$ respectively. It is worth mentioning that for the P(P-r-T)9 sample, the cell viability at



a concentration of 2500 $\mu\text{g mL}^{-1}$ was just 50%. For random copolymers with Lys as cations, copolymers with more than 50% cationic components began to show cytotoxicity at a concentration of 5000 $\mu\text{g mL}^{-1}$, while cytotoxicity was reduced at 2500 $\mu\text{g mL}^{-1}$, and cell viability remained above 50 %.

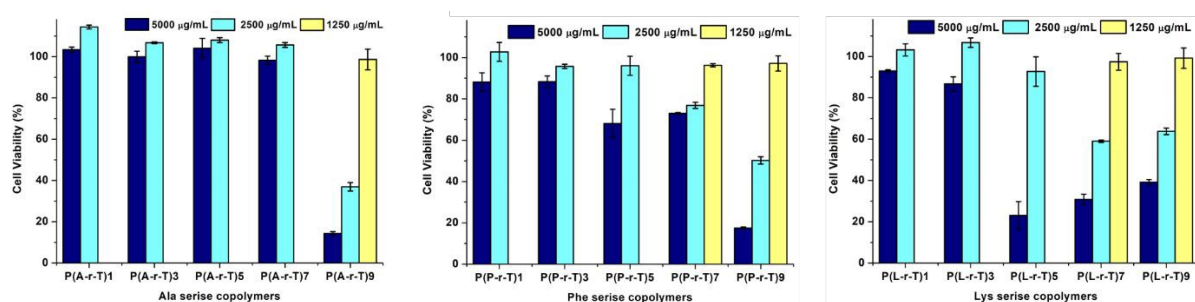


Figure 3. The cytotoxicity of random copolymers with different cationic monomers and monomer feed ratios on cultured mouse fibroblasts (*L929*) after 24 h of incubation

Selectivity to bacteria over mammalian cells

To examine the selective activity of random copolymers to bacterial cells over mammalian cells, we determined the IC₅₀/MIC as a selectivity measure (**Table 3**). In general, copolymers with Ala and Phe as cationic components have weak antibacterial activity against Gram-negative bacteria *E. coli* (MIC \geq 2500 $\mu\text{g mL}^{-1}$), and their selectivity indexes for *E. coli* are very small (\leq 0.5), indicating that these polymers have no selectivity for *E. coli* compared to animal cells. However, copolymers with Lys as cationic components have obvious selectivity for *E. coli* over animal cells, that becomes higher with the increase of the content of the cationic component. The selectivity coefficient increases from 2.5 for P(L-r-T)5 to 20 for P(L-r-T)9. For Gram-positive bacteria *S. aureus*, only P(A-r-T)9 and P(L-r-T)7 showed low or no selectivity among the tested polymers. The other samples showed certain selectivity, especially when the content of cationic components is small (10 %); their selectivity is larger than 10, and the selectivity of Phe (with a hydrophobic benzene ring on the side chain) series polymers of the cationic component is larger than 20. It is worth mentioning that P(L-r-T)9 shows a certain selectivity for both *E. coli* and *S. aureus* over *L929* cell, with values of 20 and 5, respectively, indicating the possibility that it can be used to treat bacterial infections. Therefore, the subsequent antibacterial mechanism research was carried out using P(L-r-T)9.

Table 3. Antimicrobial activity (MIC) and cytotoxicity (IC₅₀) of random copolymers with varying cationic monomers and monomer feed ratios

Random copolymers	MIC ($\mu\text{g mL}^{-1}$)		IC ₅₀ ^a ($\mu\text{g mL}^{-1}$)	Selectivity ^b	
	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 6538)	<i>L929</i>	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 6538)
P(A-r-T)1	>5000	500	>5000	/	>10
P(A-r-T)3	>5000	2000	>5000	/	>2.5
P(A-r-T)5	>5000	2000	>5000	/	>2.5
P(A-r-T)7	>5000	500	>5000	/	>10
P(A-r-T)9	2500	>2000	1250	0.5	<0.625
P(P-r-T)1	>5000	250	>5000	/	>20
P(P-r-T)3	>5000	2000	>5000	/	>2.5
P(P-r-T)5	>5000	2000	>5000	/	>2.5



P(P-r-T)7	>5000	2000	>5000	/	>2.5
P(P-r-T)9	5000	1000	2500	0.5	2.5
P(L-r-T)1	>5000	500	>5000	/	>10
P(L-r-T)3	>5000	1000	>5000	/	>5
P(L-r-T)5	1000	1000	2500	2.5	2.5
P(L-r-T)7	250	2000	2500	10	1.25
P(L-r-T)9	125	500	2500	20	5

^a Concentration for a 50 % reduction in cell viability; ^b Selectivity is calculated by IC_{50}/MIC

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DOI: 10.1039/D6PY00298F

Bacterial Membrane Integrity Assay

Several researchers have proposed that the bactericidal mechanism of these amphiphilic antimicrobial agents involves an initial electrostatic interaction between the cationic components and the microbial cell membrane. This is followed by the insertion of hydrophobic groups, which disrupts membrane permeability, leading to a breakdown of transmembrane potential and leakage of intracellular contents, ultimately resulting in cell death⁶⁵⁻⁶⁷. To further investigate the said mechanism, bacterial viability was assessed using the Thermo Fisher Scientific LIVE/DEAD BacLight kit to evaluate the integrity of bacterial cell membranes following treatment with polymer P(L-r-T)9. This kit includes two dyes: green-fluorescent SYTO 9, which labels all bacterial cells in the sample, including those with intact and damaged membranes, and red-fluorescent Propidium Iodide (PI), which selectively penetrates only bacteria with compromised membranes.

In the control group, *E. coli* and *S. aureus* cells not treated with the polymer P(L-r-T)9 exhibited strong green fluorescence with no red fluorescence, indicating that all bacteria were live with intact cell membranes (**Figure 4a- I , a- II , a- III, d- I , d- II , d- III**). In contrast, cells exposed to a 1 MIC concentration of the polymer solution for 4 hours showed reduced green fluorescence along with some red fluorescence signals, suggesting partial membrane damage; however, a substantial number of bacteria remained viable at this concentration (**Figure 4b- I , b- II , b- III, e- I , e- II , e- III**). At the higher concentration of 4 MIC, red fluorescence became more prominent, indicating extensive membrane damage and bacterial cell death, although some cells still survived (**Figure 4c- I , c- II , c- III, f- I , f- II , f- III**). These observations suggest that the polymer exerts bactericidal activity by disrupting bacterial cell membranes, that was further confirmed with SEM.



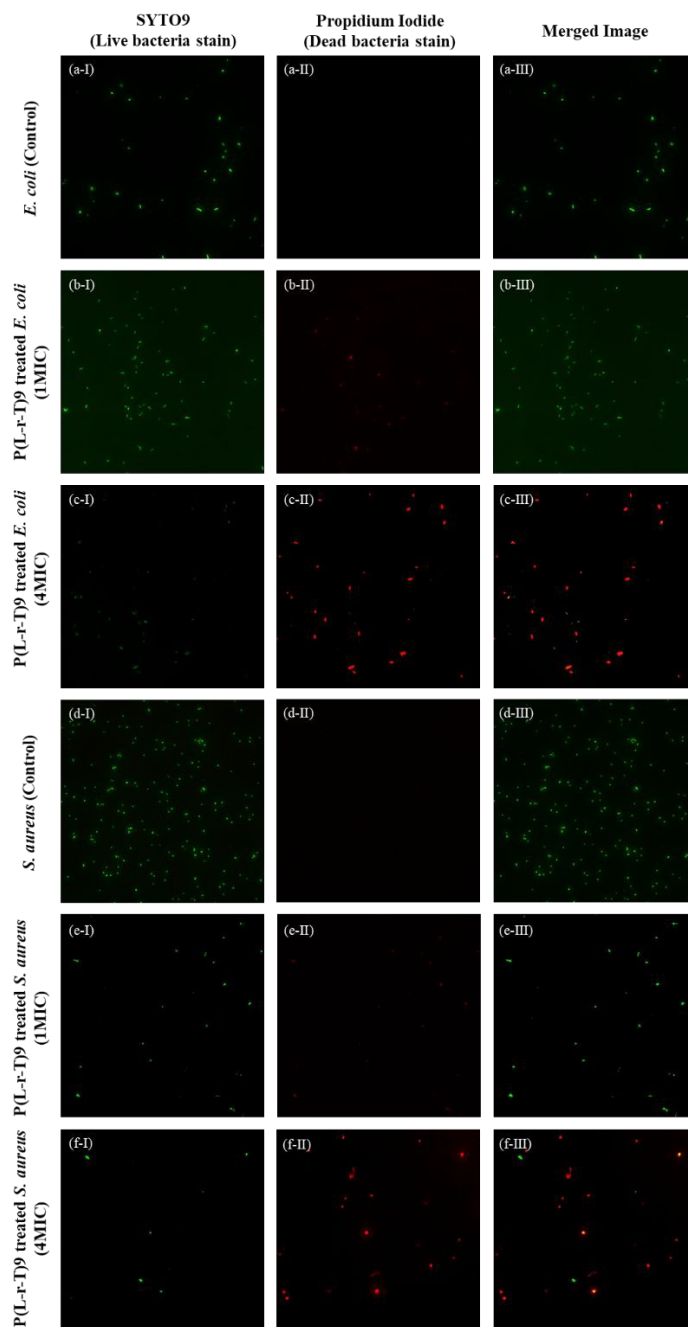


Figure 4. Confocal laser scanning microscopy (CLSM) images of *E. coli* (from a- I to c-III) and *S. aureus* (from d- I to f-III) treated with P(L-r-T)9 at different concentration (MIC and 4 x MIC) for 4 h. (Green dots indicate live cells with an intact membrane, while red dots indicate dead cells with a compromised membrane, all images were taken at 40x magnification)

SEM can be employed to analyze the antimicrobial effects of synthetic polymers by observing characteristic morphological changes induced on bacterial membrane surfaces (**Figure 5**). Untreated *E. coli* and *S. aureus* control cells exhibited bright, smooth, and undamaged membrane surfaces, as shown in the SEM micrographs (**Figure 5a-i, b-i**). However, after treatment with P(L-r-T)9 at a concentration equal to MIC for 4 hours, noticeable alterations were observed. The surface of *E. coli* showed distinct depressions and the presence of particulate structures, while the membrane of *S. aureus* appeared rough, wrinkled, and featured some vesicle formation, with even visible membrane disruptions in some cases.



At a higher concentration of 4MIC for 4 hours, the membranes of both *E. coli* and *S. aureus* exhibited widespread damage and rupturing, resulting in collapsed cells.

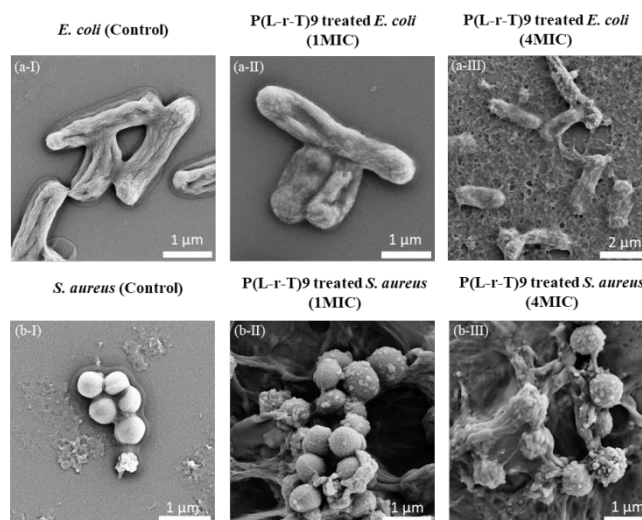


Figure 5. SEM images of *E. coli* (from a- I to a-III) and *S. aureus* (from b- I to b-III) treated with P(L-r-T)9 at different concentration (MIC and 4 x MIC) for 4 h.

In summary, these observations indicate that P(L-r-T)9 induces significant characteristic changes in the bacterial membrane surfaces, as evident from the SEM and Live/Dead staining. This leads to membrane rupture and the leakage of intracellular contents, ultimately causing bacterial cell death. To further support the cell membrane breaking mechanism, a study of the interaction of the most active polymers, P(L-r-T)7 and P(L-r-T)9, with *E. coli*: PC vesicles was performed using a calcein leakage experiment as described in the Material & Methods. Both polymers, P(L-r-T)7 and P(L-r-T)9, induced a concentration-dependent increase in calcein leakage (**Figure 6**), indicating their ability to permeabilize the lipid bilayer and promote pore formation or membrane destabilization. However, their leakage profiles differed in magnitude and threshold of activity.



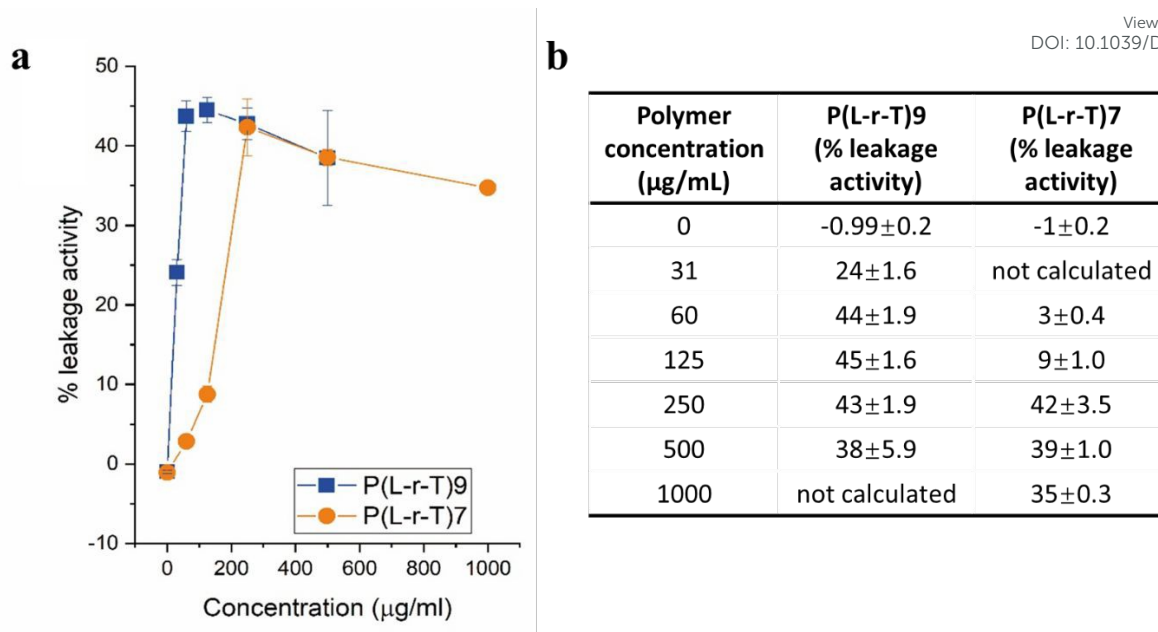
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Figure 6. (a) Calcein leakage activity of P(L-r-T)7 and P(L-r-T)9 as a function of polymer concentration. Large-unilamellar vesicles (LUVs) composed of *E. coli* polar lipids and egg PC (3:1 molar ratio) were incubated with increasing concentrations of polymer (0–1000 µg mL⁻¹). Leakage was quantified as a percentage of total calcein release induced by Triton X-100. Data represent mean ± SD (n = 3). (b) Summary table of leakage values at different polymer concentrations for P(L-r-T)7 and P(L-r-T)9.

For P(L-r-T)7, leakage activity progressively increased with concentration, reaching a maximum of 42% at 250 µg mL⁻¹. Notably, this concentration corresponds to the previously determined minimum inhibitory concentration (MIC) for this polymer, suggesting that membrane permeabilization may be directly linked to its antibacterial efficacy.

In contrast, P(L-r-T)9 showed significant leakage (44%) already at 60 µg mL⁻¹, below its MIC of 250 µg mL⁻¹. At 125 and 250 µg mL⁻¹, leakage values remained similar (45% and 43%, respectively), indicating an early saturation of membrane-disruptive activity. This earlier onset of leakage may reflect a more specific or efficient interaction of P(L-r-T)9 with the lipid membrane compared to P(L-r-T)7. Interestingly, at concentrations above 250 µg mL⁻¹, both polymers showed a plateau or slight decrease in leakage activity. This non-linear trend suggests that optimal membrane interaction occurs at intermediate concentrations. Several mechanisms may underlie the reduced leakage at higher polymer doses, including polymer aggregation, limiting effective interaction with the bilayer; membrane rigidification, leading to stabilization rather than disruption; direct interaction with calcein, potentially quenching fluorescence without additional leakage. Further investigation of this phenomenon is outside the scope of this work, but may represent a follow-up study.

Overall, these results confirm that both polymers can disrupt lipid membranes in a concentration-dependent manner, with P(L-r-T)9 displaying earlier and more potent activity, possibly due to enhanced membrane targeting properties. In contrast, P(L-r-T)7 exhibits its maximum leakage activity at 250 µg



mL⁻¹, which coincides with its MIC, suggesting a direct correlation between membrane permeabilization and antimicrobial efficacy for this polymer.

5. Conclusions

In summary, we successfully synthesized a library of 15 statistical amphiphilic random copolymers by using bio-based monomers, utilizing amino acids with different side chains as cationic components and tetrahydro geraniol as hydrophobic component as starting materials, turning them into methacrylates, and employing RAFT polymerization. These copolymers were screened against Gram-negative *E. coli* and Gram-positive *S. aureus*, as well as mouse fibroblast cells (*L929*), to evaluate their antimicrobial activity and cytotoxicity. We systematically examined the impact of monomer ratio and the type of amino acid side chains (Ala, methyl; Phe, aromatic; Lys, cationic) on both antimicrobial activity and cytotoxicity. The results demonstrated that for *E. coli*, only the copolymers with the highest cationic content (up to 90%) from Ala and Phe exhibited antimicrobial activity. In contrast, the random copolymers composed of Lys showed gradually enhanced antimicrobial activity as the cationic content increased. For *E. coli* there seems to be a direct correlation between water solubility and antimicrobial activity, but not for *S. aureus*. In this case, the balance between cationic components and hydrophobic chains influenced the MIC, with antimicrobial activity first decreasing and then increasing as the hydrophobic chain content decreased, with P(A-r-T)9 being the only outlier. In general, low solubility and polymer aggregation may be limiting factors in antimicrobial activity. Cytotoxicity also increased with higher cationic content. Among all the copolymers, P(L-r-T)9 exhibited notable antimicrobial activity against both *E. coli* and *S. aureus*, while demonstrating optimal selectivity for mammalian cells, and it is therefore the best candidate for practical applications. Live/Dead staining and SEM results confirmed that its antimicrobial effect was mediated by disruption of the bacterial cell membrane. Consistently, calcein leakage assays in model liposomes composed of *E. coli* polar lipids and phosphatidylcholine (3:1) revealed that P(L-r-T)9 induces significant membrane permeabilization already at concentrations below its MIC, supporting a mechanism of action based on membrane destabilization and subsequent bacterial cell lysis. These findings provide valuable insights for the design and synthesis of more sustainable and effective antimicrobial amphiphilic polymers with strong biocompatibility.

6. Supporting Information

Experimental section and characterization details; supplementary Synthetic routes of amphiphilic antimicrobial random copolymers; ¹H and ¹³C NMR spectra and analyses, SEC and inhibition zone test results of polymers.



7. Data Availability Statement

Data for this article, including SEC, NMR, SEM, cytotoxicity, and dead cell stain, are available at Figshare at <https://doi.org/10.6084/m9.figshare.31852735>.

8. Acknowledgments

The first author of this work was financially supported by the China Scholarship Council (CSC) under Grant Number 202204910075. We thank the European Union's Horizon 2020 research and innovation program, under the Marie Skłodowska-Curie grant agreement BioInspireSensing No. 955643, for supporting the research of Caterina Presutti.

9. References

1. P. Sarmah, M. M. Dan, D. Adapa and T. Sarangi, *Electronic Journal of Biology*, 2018, **14**, 50–58.
2. D. M. Brogan and E. Mossialos, *Global Health*, 2016, **12**, 8.
3. M. Kumar, D. K. Sarma, S. Shubham, M. Kumawat, V. Verma, P. B. Nina, D. Jp, S. Kumar, B. Singh and R. R. Tiwari, *Front Microbiol*, 2021, **12**, 609459.
4. S. Shahzadi, N. Zafar and R. Sharif, *Bact. Pathog. Antibact. Control*, 2018, **51**.
5. R. Rawashdeh and Y. Haik, *Dynamic Biochemistry, Process Biotechnology and Molecular Biology*, 2009, **3**, 12–20.
6. Y. Yan, Y. Li, Z. Zhang, X. Wang, Y. Niu, S. Zhang, W. Xu and C. Ren, *Colloids and Surfaces B: Biointerfaces*, 2021, **202**, 111682.
7. J. K. Boparai and P. K. Sharma, *Protein and peptide letters*, 2020, **27**, 4–16.
8. S. Duan, R. Wu, Y. Xiong, H. Ren, C. Lei, Y. Zhao, X. Zhang and F. Xu, *Progress in Materials Science*, 2022, **125**.
9. K. Huang, C. Yang, S. Huang, C. Chen, Y. Lu and Y. Lin, *International journal of molecular sciences*, 2016, **17**, 1578.
10. L. Timofeeva and N. Kleshcheva, *Applied microbiology and biotechnology*, 2011, **89**, 475–492.
11. J. Chen, F. Wang, Q. Liu and J. Du, *Chemical communications*, 2014, **50**, 14482–14493.
12. T. I. Shabatina, O. I. Vernaya and M. Y. Melnikov, *Molecules*, 2023, **28**, 1603.
13. U. Patel and E. C. Hunt, *Journal of Nanotheranostics*, 2023, **4**, 429–462.
14. M. Manoswini, D. Bhattacharya, P. Sen, N. Ganguly and P. S. Mohanty, *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 2021, **12**.
15. L. J. Zhang and R. L. Gallo, *Curr Biol*, 2016, **26**, R14–19.
16. W. C. Wimley, *ACS Chem Biol*, 2010, **5**, 905–917.
17. K. Matsuzaki, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1999, **1462**, 1–10.
18. Y. Q. Xiong, K. Mukhopadhyay, M. R. Yeaman, J. Adler-Moore and A. S. Bayer, *Antimicrob Agents Chemother*, 2005, **49**, 3114–3121.
19. N. Malanovic and K. Lohner, *Pharmaceuticals*, 2016, **9**, 59.
20. V. I. Band and D. S. Weiss, *Antibiotics*, 2015, **4**, 18–41.
21. A. H. Benfield and S. T. Henriques, *Frontiers in Medical Technology*, 2020, **Volume 2 - 2020**.
22. G. Moad, E. Rizzardo and S. H. Thang, *Chem Asian J*, 2013, **8**, 1634–1644.
23. F. L. Hatton, *Polymer Chemistry*, 2020, **11**, 220–229.
24. N. P. Truong, G. R. Jones, K. G. Bradford, D. Konkolewicz and A. Anastasaki, *Nature Reviews Chemistry*, 2021, **5**, 859–869.
25. N. Corrigan, K. Jung, G. Moad, C. J. Hawker, K. Matyjaszewski and C. Boyer, *Progress in Polymer Science*, 2020, **111**, 101311.
26. D. S. Uppu, S. Samaddar, C. Ghosh, K. Paramanandham, B. R. Shome and J. Haldar, *Biomaterials*, 2016, **74**, 131–143.



27. K. E. Locock, T. D. Michl, J. D. Valentin, K. Vasilev, J. D. Hayball, Y. Qu, A. Travençolo, H. J. Griesser, L. Meagher and M. Haeussler, *Biomacromolecules*, 2013, **14**, 4021–4031. View Article Online
DOI: 10.1039/D3PY00298F
28. K. Kuroda and W. F. DeGrado, *Journal of the American Chemical Society*, 2005, **127**, 4128–4129.
29. K. Kuroda, G. A. Caputo and W. F. DeGrado, *Chemistry—A European Journal*, 2009, **15**, 1123–1133.
30. K. Lienkamp and G. N. Tew, *Chemistry—A European Journal*, 2009, **15**, 11784–11800.
31. K. Lienkamp, A. E. Madkour, A. Musante, C. F. Nelson, K. Nusslein and G. N. Tew, *Journal of the American Chemical Society*, 2008, **130**, 9836–9843.
32. G. J. Gabriel, J. A. Maegerlein, C. F. Nelson, J. M. Dabkowski, T. Eren, K. Nüsslein and G. N. Tew, *Chemistry—A European Journal*, 2009, **15**, 433–439.
33. H. Takahashi, G. A. Caputo, S. Vemparala and K. Kuroda, *Bioconjugate Chemistry*, 2017, **28**, 1340–1350.
34. K. Lienkamp, K. N. Kumar, A. Som, K. Nüsslein and G. N. Tew, *Chemistry—A European Journal*, 2009, **15**, 11710–11714.
35. J. Pachla, R. J. Kopiasz, G. Marek, W. Tomaszewski, A. Głogowska, K. Drężek, S. Kowalczyk, R. Podgórski, B. Butruk-Raszeja, T. Ciach, J. Mierzejewska, A. Plichta, E. Augustynowicz-Kopeć and D. Jańczewski, *Biomacromolecules*, 2023, **24**, 2237–2249.
36. Z. Cheng and P. Raffa, *Materials Advances*, 2025, **6**, 4939–4968.
37. P. Pham, S. Oliver, E. H. Wong and C. Boyer, *Polymer Chemistry*, 2021, **12**, 5689–5703.
38. R. Namivandi-Zangeneh, R. J. Kwan, T.-K. Nguyen, J. Yeow, F. L. Byrne, S. H. Oehlers, E. H. Wong and C. Boyer, *Polymer Chemistry*, 2018, **9**, 1735–1744.
39. R. Bhat, L. L. Foster, G. Rani, S. Vemparala and K. Kuroda, *RSC advances*, 2021, **11**, 22044–22056.
40. S. Schaefer, D. Melodia, C. Pracey, N. Corrigan, M. D. Lenardon and C. Boyer, *Biomacromolecules*, 2024, **25**, 871–889.
41. Y. Hu, J. Zhao, J. Zhang, Z. Zhu and J. Rao, *ACS Macro Letters*, 2021, **10**, 990–995.
42. A. Tyagi and A. Mishra, *ACS omega*, 2021, **6**, 34724–34735.
43. S. Hong, H. Takahashi, E. T. Nadres, H. Mortazavian, G. A. Caputo, J. G. Younger and K. Kuroda, *PLoS one*, 2017, **12**, e0169262.
44. P. T. Phuong, S. Oliver, J. He, E. H. H. Wong, R. T. Mathers and C. Boyer, *Biomacromolecules*, 2020, **21**, 5241–5255.
45. V. Sambhy, B. R. Peterson and A. Sen, *Angew Chem Int Ed Engl*, 2008, **47**, 1250–1254.
46. I. Sovadinova, E. F. Palermo, M. Urban, P. Mpiğa, G. A. Caputo and K. Kuroda, *Polymers*, 2011, **3**, 1512–1532.
47. E. F. Palermo and K. Kuroda, *Biomacromolecules*, 2009, **10**, 1416–1428.
48. Y. Oda, S. Kanaoka, T. Sato, S. Aoshima and K. J. B. Kuroda, *Biomacromolecules*, 2011, **12**, 3581–3591.
49. T. Maity, S. Paul and P. Y. De, *Journal of Macromolecular Science, Part A*, 2023, **60**, 2–17.
50. I. Mukherjee, A. Ghosh, P. Bhadury and P. De, *ACS omega*, 2017, **2**, 1633–1644.
51. J. Zhu, H. Han, F. Li, X. Wang, J. Yu, C.-C. Chu and D. Wu, *Journal of Colloid and Interface Science*, 2019, **540**, 634–646.
52. J. Koehbach and D. J. Craik, *Trends in Pharmacological Sciences*, 2019, **40**, 517–528.
53. S. Noppalit, A. Simula, N. Ballard, X. Callies, J. M. Asua and L. Billon, *Biomacromolecules*, 2019, **20**, 2241–2251.
54. J. Tan, Y. Zhao, J. L. Hedrick and Y. Yang, *Advanced Healthcare Materials*, 2022, **11**, 2100482.
55. S. G. Roy and P. De, *Polymer*, 2014, **55**, 5425–5434.
56. L. M. Jost, J. M. Kirkwood and T. L. Whiteside, *Journal of Immunological Methods*, 1992, **147**, 153–165.
57. D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff and M. R. Boyd, *Cancer Research*, 1988, **48**, 4827–4833.
58. M. A. Auty, G. E. Gardiner, S. J. McBrearty, E. O. O'Sullivan, D. M. Mulvihill, J. K. Collins, G. F. Fitzgerald, C. Stanton and R. P. Ross, *Appl Environ Microbiol*, 2001, **67**, 420–425.
59. L. Boulos, M. Prévost, B. Barbeau, J. Coallier and R. Desjardins, *J Microbiol Methods*, 1999, **37**, 77–86.



60. M. J. Newman and T. H. Wilson, *Journal of Biological Chemistry*, 1980, **255**, 10583–10586. Article Online
61. E. F. Palermo, S. Vemparala and K. Kuroda, *Biomacromolecules*, 2012, **13**, 1632–1641. DOI: 10.1039/D6PY00298F
62. S. C. Owen, D. P. Y. Chan and M. S. Shoichet, *Nano Today*, 2012, **7**, 53–65.
63. A. Jain, L. S. Duvvuri, S. Farah, N. Beyth, A. J. Domb and W. Khan, *Advanced Healthcare Materials*, 2014, **3**, 1969–1985.
64. H. Takahashi, G. A. Caputo and K. Kuroda, *Biomaterials Science*, 2021, **9**, 2758–2767.
65. R. F. Epand, B. P. Mowery, S. E. Lee, S. S. Stahl, R. I. Lehrer, S. H. Gellman and R. M. Epand, *Journal of Molecular Biology*, 2008, **379**, 38–50.
66. H. Murata, R. R. Koepsel, K. Matyjaszewski and A. J. Russell, *Biomaterials*, 2007, **28**, 4870–4879.
67. J. Y. Huang, R. R. Koepsel, H. Murata, W. Wu, S. B. Lee, T. Kowalewski, A. J. Russell and K. Matyjaszewski, *Langmuir*, 2008, **24**, 6785–6795.
68. N. Kawabata and M. Nishiguchi, *Appl. Environ. Microbiol.*, 1988, **54**, 2532–2535.
69. P. Pham, S. Oliver and C. Boyer, *Macromolecular Chemistry and Physics*, 2022, **224**.
70. S. Hong, P. R. Leroueil, E. K. Janus, J. L. Peters, M. M. Kober, M. T. Islam, B. G. Orr, J. R. Baker, Jr. and M. M. Banaszak Holl, *Bioconj Chem*, 2006, **17**, 728–734.
71. F. Unger, M. Wittmar and T. Kissel, *Biomaterials*, 2007, **28**, 1610–1619.
72. D. M. L. Morgan, V. L. Larvin and J. D. Pearson, *Journal of Cell Science*, 1989, **94**, 553–559.
73. D. M. L. Morgan, J. Clover and J. D. Pearson, *Journal of Cell Science*, 1988, **91**, 231–238.



Data for this article, including GPC, NMR, SEM, cytotoxicity, and dead cell stain, are available at <https://doi.org/10.1039/D6PY00298F> Figshare at <https://doi.org/10.6084/m9.figshare.31852735>.

View Article Online
DOI: 10.1039/D6PY00298F

