Synthesis of Lysine Mimicking Membrane Active Antimicrobial Polymers

Ankita Arora, Wan Zheng, Hongjun Liang and Abhijit Mishra

Abstract Antibiotic resistance in bacteria is a major health concern. Antimicrobial Peptides (AMPs) are efficient in killing most microbes and yet the development of resistance to AMPs is rare. Although AMPs show promising antimicrobial activities, commercializing them as antibiotics is difficult as in vitro extraction and purification of AMPs is complicated and expensive. AMP mimicking antimicrobial polymers can overcome such problems while maintaining the necessary features of AMPs. Here, we have developed meth-acrylamide based polymers to mimic AMPs which possess high antimicrobial activities with low cytotoxicity. Bactericidal and scanning electron microscopy studies show that the synthesized polymers are effective against Gram-positive and Gram-negative bacteria. We find that these polymers are lethal to bacteria and at the same time, they are also non-cytotoxic to mammalian cells, thereby increasing the potential of these polymers to be used as antibiotics.

Keywords Antimicrobial polymers · Lysine mimicking polymer
Antimicrobial peptides · Methacrylamide

1 Introduction

Acquisition of antibiotic-resistance genes by all the major disease-causing bacteria and their quick transmission is a major health concern worldwide [1]. Apart from using antibiotics in the past, it depicts a troublesome picture of antibiotic resistance emergence, development, propagation, and persistence, with various risky features [2–4]. If not treated this troublesome problem could result in 10 million deaths in next

A. Arora · A. Mishra (✉)
Department of Materials Science and Engineering, Indian Institute of Technology, Gandhinagar 382355, Gujarat, India
e-mail: amishra@iitgn.ac.in

W. Zheng · H. Liang
Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Science Center, Lubbock, TX 79430, USA

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30 years [5]. In this alarming situation we urgently need new antimicrobial agents that allow us to handle this problem. Antimicrobial peptides (AMPs), a broad and diverse range of peptides that kill a wide variety of microbes such as bacteria, protozoa, fungi, and virus are considered as suitable substitutes due to their broad spectrum of antibacterial activity, reduced bacterial resistance propensity and low immune response [6]. These are widely distributed throughout multicellular organisms as part of their defense mechanism and selectively fight back with microbes without host damage [7]. Antibiotic resistance against these naturally occurring AMPs is very limited [8], however, their transfer from bench to bedside is hindered because of their complex secondary and tertiary structures, tedious extraction and isolation, expensive production on large scale, pharmacokinetic properties and chemical instability have limited their use as antibiotics [9]. The challenge lies in developing AMP mimics which suppress such restrictions while maintaining the necessary features of an active antimicrobial agent i.e. high activity and low cytotoxicity.

In nature, AMPs are often cationic which enables them to interact with the negatively charged bacterial membranes due to electrostatic force, while remaining inactive to the zwitterionic mammalian cells [10, 11]. Another peculiar characteristic of AMPs is their net hydrophobicity which describes their membrane disrupting properties [12]. Cationicity and hydrophobicity are the two important components of AMPs. It has been reported (Fig. 1) that the arginine/lysine content and hydrophobicity of such AMPs are correlated, with lower arginine content necessitating increased lysine content and increased hydrophobicity.

In this study, we synthesize lysine mimicking homopolymer and lysine mimicking copolymer with hydrophobic group and study their antimicrobial activity against both Gram-positive and Gram-negative bacteria. The advantage of using such antibiotic polymers over conventional AMPs is their scalability, cost-effectiveness and chemical stability in in vivo environment [14].

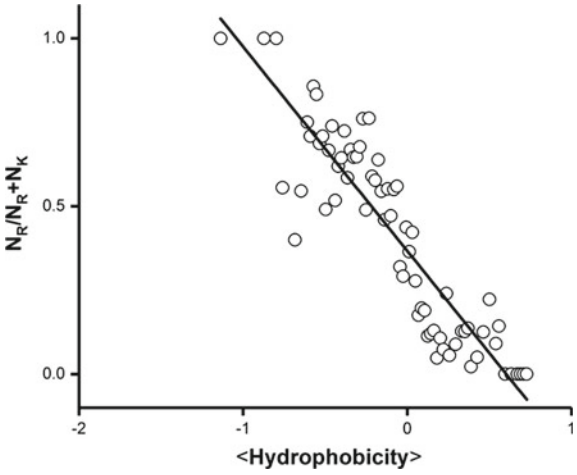

Fig. 1 Correlation between arginine/lysine content and hydrophobicity for AMPs [13]

2 Materials and Methods

2.1 Materials

N-(3-aminopropyl)methacrylamide hydrochloride (APMA, 98%), α,α,′-azoisobutyronitrile (AIBN), 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDSP), methacrylic acid (MAA), *N*,*N*'- dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), trimethylamine (TEA, ≥99%), 1-ethylpiperidine hypophosphite (EPHP, 95%), dimethyl sulfoxide (DMSO, ≥99.5%), potassium phosphate monobasic (KH_2PO_4, ≥99%), agar and glutaraldehyde solution (50%) are purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals, unless otherwise discussed, are reagent grade and used as received from Sigma-Aldrich. The Gram-negative and Gram-positive bacterial strains *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) respectively, are purchased from American Type Culture Collection (ATCC) (Manassas, VA) and subcultured according to the instructions from ATCC. Fresh human red blood cell (HRBC) is purchased from Innovative Research Inc. (Novi, MI), stored at 4 °C and used within 2 weeks. Mueller Hinton (MH) broth is purchased from Becton, Dickinson and Company (BD) (Franklin Lakes, NJ) and used as received.

2.2 Methods

2.2.1 Polymer Synthesis

Polymerization of aminopropyl methacrylamide P(APMA). The schematic diagram as shown in Fig. 2, we synthesized P(APMA) by RAFT polymerization where RAFT agent CDSP (0.075 mmol), initiator AIBN (0.015 mmol) and monomer APMA (3.75 mmol) are dissolved in DMSO in a round-bottomed schlenk flask under nitrogen. After degassed by three freeze–pump–thaw cycles, the flask is vacuum-packed and dipped in a constant temperature bath. For all polymerizations, Raft agent/Initiator = 5. The polymerization is conducted at 70 °C for 24 h [15]. The solution is precipitated in acetone and dried overnight in vacuum oven. After the product is collected, a reduction step is performed using EPHP to remove the trithiocarbonate moieties to obtain final product.

Synthesis of butyl methacrylamide (BMA). For the synthesis of BMA, methacrylic acid (0.15 mol) is mixed with NHS (0.16 mol), DCC (0.15 mol) and Dioxane (with some THF), stirred overnight at 0 °C, the solution is filtered resulting in the formation of an intermediate product. After recrystallization in hexanes, undissolved impurities are removed and the filtrate is dissolved in anhydrous THF. The solution is mixed with butylamine and triethylamine, stirred at 0 °C the impurities are removed and the solvent is removed with rotavapor. Light yellow solution appears which is run through the column (Si gel), the solvent used is ethyl acetate

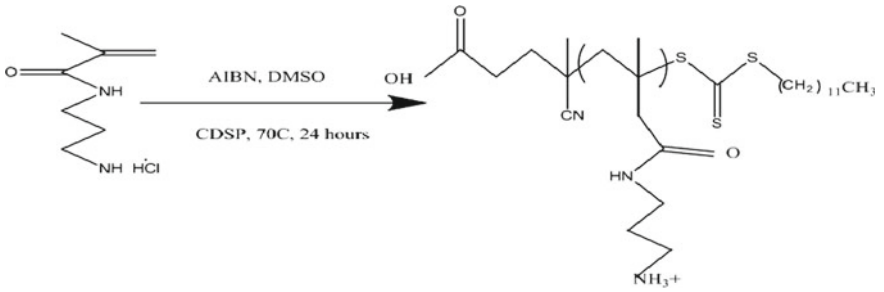

Fig. 2 Schematic synthesis of P(APMA)

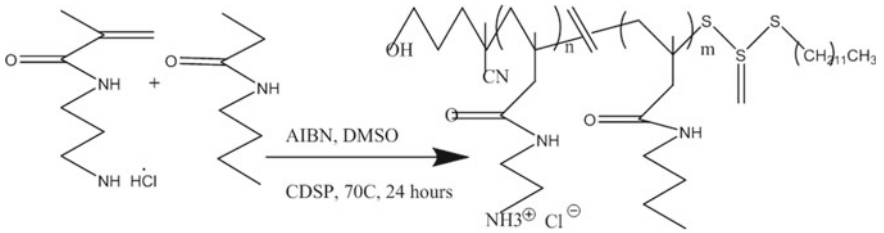

Fig. 3 Schematic synthesis of P(APMA-BMA)

and hexane with 5/4 ratio on the basis of their polarity. The solvent is finally pumped out and butyl meth-acrylamide is obtained.

Polymerization of copolymer P(APMA-BMA). The schematic diagram as shown in Fig. 3, we synthesized P(APMA-BMA) by RAFT polymerization where RAFT agent CDSP (0.067 mmol), initiator AIBN (0.0134 mmol), monomers APMA (2.4 mmol) and BMA (0.27 mmol) are dissolved in DMSO and methanol in a round-bottomed schlenk flask under nitrogen. After degassed by three freeze–pump–thaw cycles, the flask is vacuum-packed and dipped in a constant temperature bath. The reaction mixture is stirred at 70 °C for 24 h. The solution is precipitated in petroleum ether and acetone and dried overnight in vacuum oven. After the product is collected, a reduction step is performed using EPHP to remove the trithiocarbonate moieties to obtain product. For characterizing the successful synthesis of products, we use ^{1}H NMR and UV–Vis Spectrophotometer.

2.2.2 NMR

^{1}H NMR is performed with a JEOL ECS 400 MHz NMR Spectrometer in D_2O to determine monomer purity. The NMR is used to identify the structures of P(APMA) and P(APMA-BMA) homopolymers and copolymer, respectively. For every experiment, 64 scans are taken. For each of the homopolymers, a characteristic peak is

assigned and the copolymer constituents are analyzed via peak integration of the APMA and BMA monomer residues, respectively.

2.2.3 Bacterial Killing Assay

The bactericidal activity is determined based on the protocol suggested by Clinical and Laboratory Standards Institute (CLSI) and literature [16, 17]. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) are selected as prototypical Gram-negative and Gram-positive bacteria, respectively. Bacteria are grown in MH broth at 37 °C for 18 h, and then diluted into fresh MH broth for re-growth. Growth in bacterial cells is observed by optical density at 600 nm (OD_{600}) using a UV–Vis spectrometer (Hewlett Packard 8435, Palo Alto, CA). After the mid-log phase ($OD_{600} = 0.5–0.6$) is reached, the bacteria are washed twice with sterile PBS buffer (10 mM KH_2PO_4, 150 mM NaCl, pH 7.0) and centrifuged for 5 min at 10,000 rpm. The harvested bacteria are re-suspended and diluted by PBS. To determine the minimum bactericidal concentration (MBC), polymers are diluted in microplate and different range of concentrations is tested. To each well of polymer, diluted bacterial suspension with final concentration ~5×10^5 CFU/ml is added. The plates are incubated at 37 °C for 3 h. Serial 10-fold dilution is subsequently made with PBS buffer. For each dilution, 20 μl of the solution is taken and plated onto MH agar plates, which are then incubated at 37 °C overnight to yield visible colonies. The minimum bactericidal concentration 99 (MBC_{99}) is defined as the minimum antimicrobial concentration that resulted in $\leq 1\%$ bacterial survival rate. All experiments are performed two times, each in triplicates on different days.

2.2.4 Scanning Electron Microscopy (SEM) Characterization

The bacterial suspension is first grown to mid-log phase ($OD_{600} = 0.5–0.6$) and the cells are collected by centrifugation at 5000*g* for 10 min and washed with sterile PBS buffer for 2 times. The bacterial cells are then resuspended in PBS buffer and incubated with polymer solutions for 3 h. Bacteria incubated without polymer solutions are used as a control. After the incubation, bacteria suspensions are washed by PBS buffer for 2 times and then fixed by the PBS buffer consisting of glutaraldehyde (2.5%) solution for 24 h [18]. Finally, the bacterial cells are further washed with sterile millipore water for three times, then dehydrated using a cycle of ethanol wash and dried in a lyophilizer. The dried bacterial cell sample is placed on a carbon tape, which is mounted onto an aluminum stud and coated with a thin layer of gold prior to analysis using a JEOL JSM7000F Field Emission SEM (Peabody, MA) with an accelerating voltage of 20 kV and a medium probe current.

2.2.5 Hemagglutination

Hemagglutination is formation of blood aggregates or clots due to toxicity. Fresh HRBC suspension (600 μl) is washed twice with PBS buffer (12 ml) and harvested by centrifugation at 3000 rpm (1000*g*), then re-dispersed in PBS buffer (15 ml) to reach a HRBC concentration of ~1.0%. Aliquots of this HRBC suspension (800 μl) are then mixed with polymer solutions of different concentration (200 μl) in 24 well microplate. PBS buffer (200 μl) mixed with HRBC suspension (800 μl) is used as negative controls. The microplate is kept in shaking incubator at 37 °C at 250 rpm for 60 min and settled for another 60 min before photo recording [19]. The relative hemagglutination is determined as ++++, +++, ++, +, 0, according to the size of blood clot in each well.

3 Results and Discussion

The NMR data of lysine mimicking homopolymer is shown in Fig. 4a, in order to check the purity of the polymer, the peaks are identified corresponding to each proton. For analyzing the degree of polymerization, the monomer peaks *e* and *d* are integrated and compared with that of the peak *a* at bulky hydrocarbon end group by using the following formula:

$$\frac{3n+23}{4n} = \frac{I_a}{I_{e,d}}$$

where *n* is the degree of polymerization and *Ia* is the integrated chemical shift value of peak *a*. The DP for lysine mimicking homopolymer P(APMA) is 32 units after solving the above equation.

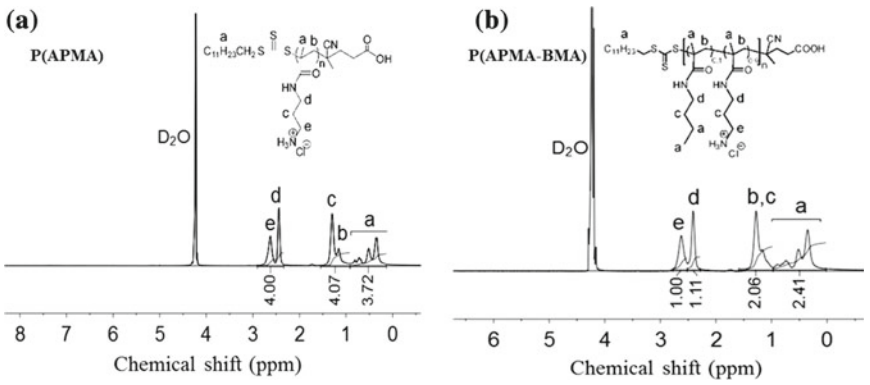

Fig. 4 NMR for **a** P(APMA) and **b** P(APMA-BMA) with D_2O

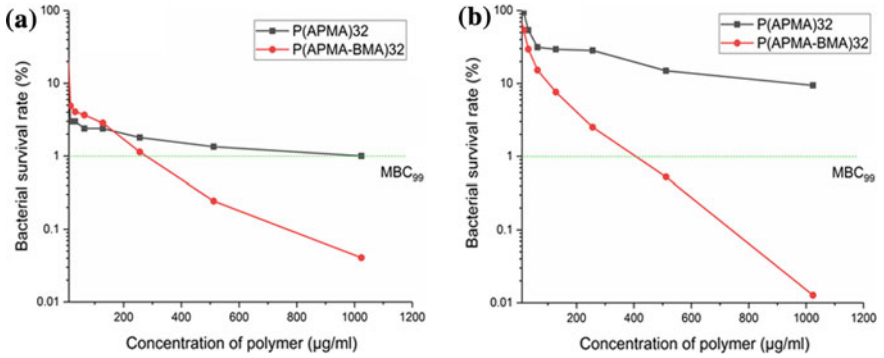

Fig. 5 MBC_{99} of P(APMA) and P(APMA-BMA) with **a** *E. coli* and **b** *S. aureus*

The NMR data of lysine mimic with hydrophobic moiety is shown in Fig. 4b, in case of copolymers the composition of each polymer is characterized by NMR where the characteristic peak in either of the polymer is integrated and given a fixed value, it is then compared with the integrated peaks common to both the polymers, according to the following formula:

$$\frac{I_d}{I_e} = \frac{2_x + 2_y}{2_x} = \frac{1.11}{1} \rightarrow x/y = 9.1$$

where I_d is the integrated chemical shift value of peak *d,* I_d is the integrated chemical shift value of peak *d* and *x, y* is the proportion of APMA and BMA, respectively. Therefore the ratio of lysine mimicking polymer to the hydrophobic moiety observed to be 9:1.

The synthesized polymers are studied against Gram-negative *E. coli* and Gram-positive *S. aureus.* The $MBC_{99,}$ i.e., bactericidal concentration at which 99% of bacteria are killed, Fig. 5 shows that P(APMA-BMA) is more effective as compared to the P(APMA). The copolymer has a slightly lower MBC_{99} against *E.coli* (275 μg/ml) as compared to *S. aureus* (420 μg/ml). These results show that hydrophobicity in addition to cationicity is required for an effective antibacterial polymer.

Mode of action of killing by polymers is evaluated using SEM. Surface morphology of bacteria incubated with both the polymers shows multiple blisters like disruptions on the surface of *E. coli* (Fig. 6b, c) as compared to the control *E. coli* shown in Fig. 6a. This indicates that both the polymers have likely punctured the bacterial membrane leading to discharge of intracellular content into the periplasmic space. The SEM results, therefore, indicate a pore-forming mode of action for these polymers.

Increase in hydrophobicity in antimicrobial polymers may stimulate the antibacterial activity but may also show cytotoxicity towards mammalian cells. To test the cytotoxicity of the designed polymers in mammalian cells, hemagglutination assay is carried out where the human red blood cells are treated with P(APMA) and P(APMA-

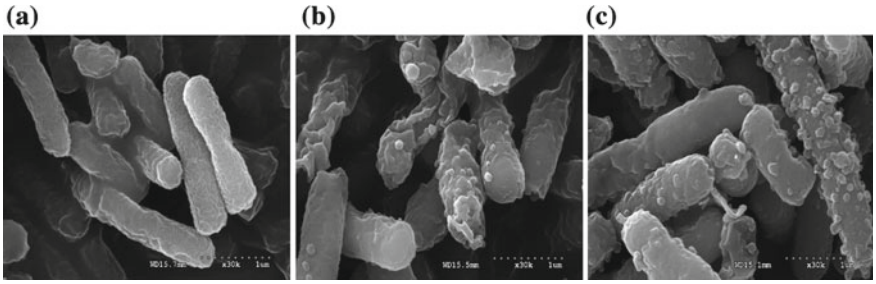

Fig. 6 SEM images for **a** *E. coli* **b** *E. coli* with P(APMA) and **c** *E. coli* with P(APMA-BMA), the scale bar is 1 μm

Table 1 Hemagglutination with P(APMA) and P(APMA-BMA)

Sample	P(APMA)32	P(APMA-BMA)32	Negative control	Positive control
Hemagglutination at 512 μg/ml	0	0	0	++++
Hemagglutination at MBC	0	0	0	+++

BMA) at different concentrations. The results in Table 1 indicate that no aggregates are formed with both polymers even at 512 μg/ml, therefore these are nontoxic to human blood cells.

4 Conclusion

Methacrylamide-based polymers are synthesized to mimic features of AMPs. Here, lysine mimicking homopolymer and 90% lysine mimicking co-polymer with 10% hydrophobic moiety are synthesized and characterized with some biochemical tests against *E. coli* and *S. aureus*. These polymers are found to be effective against both the bacteria at low concentrations with no toxicity to human blood. However, P(APMA-BMA) is observed to be more active as compared to the P(APMA) as cationicity and hydrophobicity are the two critical pre-requisite for an effective antimicrobial polymer. The mode of action of these antimicrobial polymers is pore forming as shown in SEM image, therefore antibiotic resistance is likely to be difficult to achieve. The advantages associated with these polymers are that they are cost-effective, can be commercialized at large scale and are efficient in killing bacteria.

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Conflict of Interest The authors declare that they have no conflict of interest.

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