HHS Public Access

Author manuscript

Biomacromolecules. Author manuscript; available in PMC 2019 November 12.

Published in final edited form as:

Biomacromolecules. 2018 November 12; 19(11): 4370–4378. doi:10.1021/acs.biomac.8b01256.

Decoupling the functional roles of cationic and hydrophobic groups in the antimicrobial and hemolytic activities of methacrylate random copolymers

Hamid Mortazavian[1], **Leanna L. Foster**[2], **Rajani Bhat**[1], **Shyrie Patel**[1], and **Kenichi Kuroda**[1,2,*]

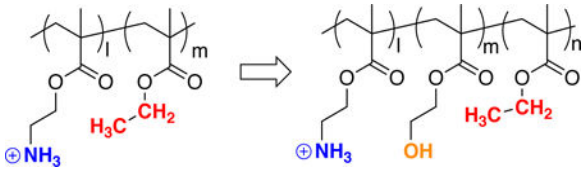
[1)]Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI 48176.

[2)]Macromolecular Science and Engineering Center, University of Michigan, Ann Arbor, MI 48176.

Abstract

In this study, we report that the antimicrobial and hemolytic activities of ternary statistical methacrylate copolymers consisting of cationic ammonium (amino-ethyl methacrylate: AEMA), hydrophobic alkyl (ethyl methacrylate: EMA), and neutral hydroxyl (hydroxyethyl methacrylate: HEMA) side chain monomers. The cationic and hydrophobic functionalities of copolymers mimic the cationic amphiphilicity of naturally occurring antimicrobial peptides (AMPs). The HEMA monomer units were used to separately modulate the compositions of cationic and hydrophobic monomers, and we investigated the effect of each component on the antimicrobial and hemolytic activities of copolymers. Our data indicated increasing the number of cationic groups of copolymers to be more than the 30 mole % did not increase their antimicrobial activity against *Escherichia coli*. The number of cationic side chains in a polymer chain at this threshold is 5.5 −7.7, which is comparable to those of natural antimicrobial peptides such as maginin (+6). On the other hand, the MIC values of copolymers with > 30 mole % of AEMA depend on only the mole % of EMA, indicating that the hydrophobic interactions of copolymers with *E. coli* cell membranes determine the antimicrobial activity of copolymers. These results suggest that the roles of cationic and hydrophobic groups can be controlled independently by design in the ternary copolymers studied here.

Ternary random copolymers as synthetic mimics of AMPs



*Corresponding Author(s): (K.K.), kkuroda@umich.edu.

Supporting Information

Supporting information (SI) includes ^{1}H NMR spectrum of AE34HE29E37, characterization of copolymers, MIC (*E. coli*). This material is available free of charge via the Internet at http://pubs.acs.org.

Keywords

antimicrobials; amphiphilic copolymers; E. coli; antimicrobial peptides

INTRODUCTION

Cationic amphiphilic random copolymers have been a molecular platform to mimic the mode of action of naturally occurring antimicrobial peptides.[1–11] AMP-mimetic polymers are designed to act by disrupting bacterial cell membranes for bactericidal activity.[7, 12] The cationic groups of polymers facilitate their binding to anionic bacterial cell membranes by electrostatic interactions. Because the bacterial cell membranes are highly negatively charged as compared to the human cell membranes, the polymers are expected to selectively bind to bacteria over human cells. Once the polymers bind to cell membranes, the hydrophobic groups of polymers are inserted to the hydrophobic domains of membranes, and the polymer chains form active amphiphilic conformations capable of membrane disruption.[13–15] Based on the expected mechanism of polymers, the electrostatic binding to bacterial membranes imparts the cell selectivity, and the hydrophobic insertion governs membrane disruption. However, highly hydrophobic polymers bind to human cell membranes due to non-specific hydrophobic interactions, causing undesired toxicity to human cells. Therefore, in the current optimization approach, many efforts have been made to find the optimal compositions of cationic and hydrophobic functionality of polymers such that they can maximize their antimicrobial activity and minimize their toxicity to human cells.

Traditionally, many antimicrobial random copolymers have binary compositions of cationic and hydrophobic monomers because the cationic and hydrophobic functionalities are thought to be the minimum requirements for the antimicrobial activity and selectivity, as described above. The polymer design is simple; however, it is difficult to optimize monomer compositions for both potent antimicrobial activity and desired selectivity because the electrostatic binding and hydrophobic insertion of polymers to membranes cannot be independently controlled in the binary monomer system in which the cationic and hydrophobic monomer compositions are coupled. For example, increasing the hydrophobic monomer composition of polymers simultaneously decreases the cationic monomer composition. This compositional change would enhance the hydrophobic insertion of polymer chains into membranes and membrane disruption, which would increase the antimicrobial activity of polymers. However, at the same time, the electrostatic binding of polymer chains to bacteria would decrease because of the reduced number of cationic groups, which would reduce the antimicrobial activity and selectivity. Therefore, any compositional change of one monomer may compromise the benefits of the other monomer in the binary monomer system. For the same reason, it is also difficult to predict the activity of polymers from the binary monomer compositions, and thus, the compositional optimization of polymers often relies on screening large polymer libraries with a range of monomer compositions in a trial-and-error basis. These challenges led to the following question: *Can we independently control the cationic and hydrophobic effects of copolymers by design?*

On the other hand, we have previously demonstrated that the number of cationic groups of our antimicrobial methacrylate copolymers are significantly larger than AMPs. For example, the methacrylate polymers in our previous reports have typically 10–14 cationic ammonium groups in a polymer chain (the degree of polymerization = 15–20),[16] while natural AMP magainin II (23 amino acids) and LL-37 (37 amino acids) have net positive charges of +4 and +6, respectively[17]. In addition, the polymers also display higher hydrophobicity than AMPs.[16] These facts suggest that some cationic charges and hydrophobicity of polymers may be excess, not necessarily required for their activity. This led us to another question: *How many cationic and hydrophobic groups are minimally necessary for potent antimicrobial activity and desired selectivity?*

To answer these questions, we investigate in this report the antimicrobial and hemolytic activities of statistical methacrylate copolymers with ternary monomer compositions. Specifically, we designed new statistical copolymers consisting of the following monomers: cationic ammonium (amino-ethyl methacrylate: AEMA), hydrophobic alkyl (ethyl methacrylate: EMA), and neutral hydroxyl (hydroxyethyl methacrylate: HEMA) side chain monomers. The hydroxyl group is polar, but not charged, so that it would not contribute to the electrostatic binding and hydrophobic insertion of polymers to membranes. Therefore, we postulate that HEMA would be an inactive spacer monomer enabling us to separately control the compositions of cationic and hydrophobic monomers in a polymer chain. Yang and coworkers previously reported the antimicrobial and hemolytic activities of methacrylate random copolymers with cationic and hydroxyl groups in the side chains.[18] Their study demonstrated that the hemolytic activity of the polymers was significantly lower than the counterpart polymers with hydrophobic side chains instead of hydroxyl side chains. On the other hand, Gellman and coworkers also synthesized ternary nylon-3 copolymers with cationic, hydrophobic, and hydroxyl subunits and demonstrated partial replacement of hydrophobic subunits, cationic subunits, or both by hydroxyl subunits can reduce their hemolytic activity while their antimicrobial activity was minimally changed.[19] We here used the ternary copolymer system to systematically evaluate the effects of the cationic, neutral, and hydrophobic groups on their antimicrobial and hemolytic activities to examine if the functional roles of these groups in their antimicrobial activity and selectivity can be controlled independently.

EXPERIMENTAL

Materials.

Ethanolamine, 4-amino-1-butanol, di-*tert*-butyl dicarbonate, triethylamine, methyl 3-mercaptopropionate, and ethyl methacrylate were purchased from Acros Organics. 2,2'-azobisisobutyronitrile (AIBN) and 2-(trimethylsiloxy)ethyl methacrylate were purchased from Sigma-Aldrich Co. LLC. Trifluoroacetic acid (TFA) and solvents were purchased from Thermo Fisher Scientific, Inc. The chemicals were used without further purification, with the exception of methacryloyl chloride, which were distilled before use. ^{1}H NMR was performed using a Varian MR400 (400 MHz) and analyzed using VNMRJ 3.2 and MestReNova. Gel permeation chromatography (GPC) analysis was performed using a Waters 1515 HPLC instrument equipped with Waters Styragel (7.8 × 300 mm) HR 0.5, HR

1, and HR 4 columns in sequence and detected by a differential refractometer (RI). Mueller Hinton broth (MHB, BD and Company ©) and phosphate buffered saline (PBS, pH=7.4, Gibco®) were prepared according to manufacturer instructions and sterilized prior to use. Human red blood cells (RBCs) (leukocytes reduced adenine saline added) were obtained from the American Red Cross Blood Services Southeastern Michigan Region and used prior to the out date indicated on each unit.

Polymer synthesis and characterization

The synthesis of copolymer with a monomer composition of AEMA 34 mole % HEMA 29 mole %, ETA 37 mole %(AE34HE29E37) is described here as a representative sample. 4-((*tert*butoxycarbonyl)amino)butyl methacrylate (Boc-AEMA) (0.36 mmol, 82.5 mg), 2-(trimethylsiloxy)ethyl methacrylate (HEMA-TMS) (0.36 mmol, 72.8 mg), ethyl methacrylate (EMA) (0.48 mmol, 54.8 mg), AIBN 0.012 mmol, 2 mg), and methyl 3-mercaptopropionate (0.12 mmol, 14.4 mg) in acetonitrile (0.8 mL)/DMF (0.2 mL) were mixed in a flask. The oxygen in the reaction mixture was removed by nitrogen gas bubbling for 10 minutes, and the reaction solution was stirred at 60°C for 16 hours. The reaction mixture was cooled down to room temperature. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in dichloromethane and precipitated in water/methanol (50/50). To simultaneously remove the Boc and TMS groups of copolymer, the protected polymers were dissolved in TFA and stirred for 10 minutes. TFA was removed by blowing with nitrogen gas in a closed container, and the gas was passed through a base (NaOH) aqueous solution to trap TFA. The residues were dissolved in methanol and precipitated in diethyl ether. Subsequently, the precipitate was dissolved in distilled water and lyophilized. It should be noted that polymer samples with high percentages of HEMATMS (> 50 mole %) were dissolved in dichloromethane and precipitated in hexanes. The degree of polymerization and mole percentages of monomers (Table 1) were determined by comparing the integrated areas of peaks from the side chains to that of the end group (methyl 3-mercaptopropionate) of polymer chain (See Supporting Information for the NMR spectrum, assignments, and calculation).

Antimicrobial assay

The minimum inhibitory concentration (MIC) of copolymers against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *S. mutans* (ATCC 25175) was determined in a standard microbroth dilution assay according to the Clinical and Laboratory Standards Institute guidelines with suggested modifications by R. E.W Hancock Laboratory (University of British Columbia, British Columbia, Canada)[20] and Giacometti et al.[21] *E. coli* and *S. aureus* were grown overnight (~18 hours) at 37 °C with stirring in MHB and regrown to the exponential phase (OD_{600}= 0.5–0.7, 2 hours). *S. mutans* were grown overnight at 37 °C with 5% CO_2 in the absence of stirring, and regrown to exponential phase (OD_{600}= 0.2, 3 hours). The bacteria culture was diluted with MHB to give the bacterial suspension with a concentration of OD_{600}= 0.001, corresponding to approximately 4×10^5 cfu/mL. Polymers were dissolved in 0.01% acetic acid to achieve stock concentrations of 20 mg/mL, with the exception of the copolymers with high percentages of HEMA (> 80 mole %) (Polymers 5, 12, and 21 in Tables S1–S3) which were dissolved in DMSO due to low solubility in water. Serial dilutions of polymers were prepared from stock solutions in phosphate buffer saline

(PBS) (GIBCO®, pH 7.4). After serial dilutions, polymers (10 μL) were transferred to a 96-well sterile round-bottom polypropylene plate, followed by the bacterial suspension (90 μL). PBS or DMSO diluted in PBS was used as a control. Plates were incubated for 18 hours at 37 °C. *S. mutans* plates were incubated with 5% CO_2. MIC was defined as the lowest concentration of polymers to completely inhibit bacterial growth. Assays were repeated a minimum of three times in triplicate on different days.

Hemolysis assay

Hemolysis, the lysis of human red blood cells (RBCs), was used to assess the toxicity of polymers to human cells. A 10% solution of human RBCs in PBS was centrifuged at 2000 rpm for 5 minutes and washed with PBS x2 to remove initial hemoglobin. The number of RBCs in the resulting solution was determined by counting chamber, and the solution diluted in PBS to give a final concentration of 3.33×10^8 cells/mL. Polymers were dissolved in 0.01% acetic acid to achieve stock concentrations of 20 mg/mL, with the exception of the copolymers with high percentages of HEMA (> 80 mole %) (Polymers 5, 12, and 21 in Tables S1–S3) which were dissolved in DMSO due to low solubility in water. Serial dilutions of polymers were prepared from stock solutions in phosphate buffer saline. After serial dilutions, polymers (10 μL) were transferred to a 96-well sterile round-bottom polypropylene plate, followed by the RBC suspension (90 μL). Plates were incubated at 37 °C with orbital shaking (180 rpm) for 1 hour. Triton X-100 (0.1% v/v in water) was used as the positive lysis control and PBS used as a negative control. Following incubation, the plate was centrifuged at 2000 rpm for 5 minutes. The supernatant (5 μL) from each well diluted in PBS (100 μL) with thorough mixing in a 96-well flat bottomed polystyrene plate. The absorbance of released hemoglobin (415 nm) was measured using a Varioskan Flash microplate reader (Thermo Fisher). The percentage hemolysis was determined relative to Triton X-100 (100%) and PBS negative control (0%). The polymer concentration causing 50% hemoglobin release (HC_{50}) was determined. Assays were repeated a minimum of three times in triplicate.

RESULTS

Polymer design and synthesis.

In our polymer design, we incorporated HEMA to our previous antimicrobial methacrylate copolymers consisting of binary cationic-hydrophobic monomers. The ternary copolymers were synthesized by free-radical polymerization in the presence of methyl 3-mercaptopropionate as a chain transfer agent. We used boc-protected AEMA and TMS-protected HEMA to facilitate the polymer synthesis in a non-polar organic solvents (acetonitrile/DMF) and characterization using GPC with an eluent of THF. We initially attempted to use non-protected HEMA for the polymerization; however, the precipitation of resultant polymers from the reaction mixture was not quantitative, and the precipitated polymers showed monomer compositions largely different from the feed compositions. This is likely because the polymers have mixed polar (hydroxyl) and non-polar (t-boc and ethyl) properties, resulting in selective precipitation. The boc and TMS-protected polymers were treated with TFA to produce polymers with primary ammonium, ethyl, and hydroxyl side chains. The degree of polymerization (DP) of polymers based on ^{1}H NMR spectra was 15–

20. The monomer compositions of copolymers were also determined by ^{1}H NMR (See Supporting Information for DP and monomer compositions). The monomer compositions of resultant polymers were found close to the feed monomer compositions (Tables S1–S5 in Supporting Information). We did not prepare copolymers with EMA more than 50% in this study because we were concerned that theses copolymers would be poorly soluble to water due to high hydrophobicity. The molecular weight distribution was relatively broad, determined for selected protected polymers using GPC (M_w/M_n = 1.4–1.9) (Tables 1 and 2).

Antimicrobial activity.

Next, we investigated the relationship between the monomer composition and the antimicrobial activity of copolymers. The activity of copolymers was evaluated as the minimum inhibitory concentration (MIC) of copolymers against Gram-negative *Escherichia coli* as a model bacterium. To facilitate data analysis and discussion, the same MIC data were shown in two different presentations (Fig. 2). The MIC values of a series of copolymers with different EMA compositions were plotted as a function of the average composition of HEMA (hydroxyl) (Fig. 2A) or AEMA (ammonium) (Fig. 2B) in a polymer chain.

The copolymers with 0 and 10 mole % EMA did not show any activity against *E. coli* (MIC > 1000 μg/mL) (Supporting Information). The MIC values increased as the mole percentage of HEMA increased (Fig. 2A) or the mole percentage of AEMA decreased (Fig. 2B), suggesting that increasing the cationic groups in a polymer chains increased the antimicrobial activity against *E. coli*. However, as the AEMA increased, the MIC values of all series of copolymers started to level off at ~30 mole % of AEMA, despite different EMA compositions. This result indicates that the antimicrobial activity of copolymers cannot be improved by increasing the cationic charges more than 30 mole %. In addition, because the degree of polymerization was 15–20 for these copolymers, each polymer chain has 4.5 – 6 cationic groups in average for 30 mole % AEMA, which are comparable to the net positive charge of natural AMPs including, for example, magainin II (+4) and LL-37 (+6)[17]. Because the net charge of AMPs is likely to be evolutionarily optimized for the antimicrobial mechanism of AMPs, the number of cationic groups of copolymers may be reasonable and optimal for the binding to anionic lipids of *E. coli* cell membranes and/or cause membrane disruption. On the other hand, the MIC values of copolymers in saturation at the high mole percentages of AEMA decreased as the mole percentage of EMA increased, indicating that increasing the hydrophobicity of copolymers increased their maximum antimicrobial activity of each polymer series against *E. coli*. In our previous reports,[13–14, 22] molecular dynamic simulations demonstrated that the hydrophobic groups of copolymers are inserted into the hydrophobic domains of bacterial cell membranes to form bioactive amphiphilic conformations capable of membrane disruption. Therefore, the result suggests that the antimicrobial activity of copolymers (MIC) might be determined by the hydrophobic insertion of copolymers into the bacterial membranes and subsequent membrane disruption, once the electrostatic binding of polymers to membranes are sufficient (AEMA > 30 mole %).

Hemolytic activity.

We also investigated the effect of monomer compositions on the hemolytic activity of copolymers. In general, the hemolytic activity of copolymers was increased as the hydrophobic EMA monomer composition was increased, which is in good agreement with our previous reports[23–24] and others[25–26]. The series of copolymers with 30 mole % EMA did not show any substantial hemolytic activity, with < 15% hemolysis at a polymer centration of 1000 μg/mL. The copolymers with 40 mole % EMA showed ~50% hemolysis at a polymer concentration of 1000 μg/mL. The copolymers with 50 mole % EMA showed high hemolytic activity to give HC_{50} values (Fig. 3). The HC_{50} value of copolymers was increased as the mole % of HEMA was increased (Fig. 3A) or the HC_{50} value was decreased as the mole % of AEMA was increased (Fig. 3B). This result suggests that replacing cationic ammonium groups by HEMA reduces the hemolytic activity of copolymers, or increasing the number of cationic groups increased the hemolytic activity of copolymers. One may think that this result is counterintuitive; the copolymers with more cationic groups (AEMA) would be more hydrophilic so that more copolymer chains should remain in solution rather than binding to the surface of red blood cells. Therefore, their hemolytic activity should be reduced by increasing the number of cationic groups (AEMA monomer). However, the result suggests that the copolymers with more cationic groups are more hemolytic. This can be explained by the fact that the surface of red blood cells has net negative charge due to anionic components such as sialic acids, and the cell membrane expresses anionic lipids such as phosphatidylserine (PS) lipids while the majority of PS is located in the inner leaflet (cytoplasmic side) of membrane.[27] In addition, we previously demonstrated that the primary ammonium groups of polymers facilitate the binding of polymers to the anionic phosphate groups of lipids possibly by a combination of electrostatic interaction and hydrogen bond.[28] Therefore, the cationic net charges and primary ammonium groups of copolymers are likely to enhance the binding of copolymers to the cell membrane of RBCs, increasing their hemolytic activity. This mechanism is analogous to the electrostatic binding of copolymers to anionic bacterial membranes for their antimicrobial activity. In similar to the MIC values (Fig. 2), the HC_{50} value of copolymers appears to level off at ~40 mole % of AEMA. This result suggests that some cationic charges are also excess for their hemolytic activity. Since the copolymers showed the threshold AEMA content of 30 mole % for the saturation of MIC values (Fig. 2), one may wonder how different in antimicrobial and hemolytic activities the copolymers with 30 mole % AEMA but different EMA compositions would be. We will discuss in the following section the antimicrobial and hemolytic activities of those copolymers in our effort to identify the optimal monomer compositions for potent and selective antimicrobial copolymers.

Identifying the optimal monomer compositions.

The hemolysis data described above suggest that the cationic AEMA composition of copolymers should be minimized to reduce their electrostatic binding to RBCs, thus reducing their hemolytic activity. On the other hand, the MIC data suggest that approximately 30 mole % of cationic groups or 5.5 – 7.7 net cationic charges are sufficient to exert their antimicrobial effect against *E. coli*, which present the minimal number of cationic groups for potent antimicrobial activity against *E. coli*. Therefore, the series of copolymers with 30 mole % of AEMA would be candidates for potent antimicrobial

polymers with selective activity. The copolymers with approximately 30 mole % of AEMA were listed in Table 1. Regarding the hydrophobic EMA composition, the MIC data indicated that the copolymers with higher mole percentage of EMA are more active against *E. coli*. Therefore, the EMA composition should be maximized for their potent antimicrobial activity. On the other hand, the hemolytic activity of copolymers increased as the EMA composition increased, and the hemolytic activity of copolymers significantly increased as the EMA composition increased from 40 mole % to 50 mole %, which appears to be the threshold EMA composition for high hemolytic activity. Indeed, AE32HE22E46 showed the highest antimicrobial activity (MIC = 13 μg/mL) among the copolymers in this series and high hemolytic activity (HC_{50} = 42 μg/mL) (Fig. 2 and Table 1). By reducing the EMA composition to 37 mole %, AE34HE29E37 showed the slightly reduced antimicrobial activity (MIC = 31 μg/mL) and significantly reduced hemolytic activity (HC_{50} = 1000 μg/mL) (Fig. 2 and Table 1). Further reducing the EMA composition of copolymers such as AE29HE39E33 significantly reduced both their antimicrobial and hemolytic activities (Table 1). Taken together, we identified AE34HE29E37 as the optimal monomer composition for best antimicrobial activity and selectivity among the series of polymers studied here. From the viewpoint of traditional binary copolymers, it can be stated that replacing the cationic and hydrophobic monomers with neutral hydroxyl monomers retained the antimicrobial activity, but reduced the hemolytic activity in the copolymers, which is in good agreement with the conclusion of previous study on ternary nylon-3 copolymers from Gellman's group[19].

Binary vs. ternary monomer compositions.

In the previous section, we determined the ternary monomer compositions of copolymers for best antimicrobial activity and selectivity as we used HEMA presumably as an inert spacer. However, if the cationic and hydrophobic groups of copolymers were only the structural determinants of copolymers responsible for their antimicrobial mechanism, and their compositions were already optimal, we wondered if the HEMA monomers may not be needed anymore to exhibit the same antimicrobial and hemolytic activities. To answer this question, we synthesized binary copolymer derivatives with the similar numbers of cationic (AEMA) and hydrophobic (EMA) groups in a polymer chain to the parent ternary copolymer, but without the hydroxyl groups (HEMA), and we examined their antimicrobial and hemolytic activities. If only the cationic and hydrophobic groups determine the activities of copolymers, this binary copolymer should show the same activities with the parent ternary copolymer with HEMA.

We prepared the binary copolymers AE45HE0E55, AE46HE0E56, and AE47HE0E53, which have the similar numbers of cationic and ethyl groups in a polymer chain to parent ternary copolymer AE34HE29E37 (Table 2). The binary copolymers showed higher antimicrobial activity against *E. coli* (MIC = 7.8 – 10 μg/mL or 2.8 – 4.4 μM) than the parent ternary copolymer (MIC = 31 μg/mL or 10 μM), but these binary copolymers were very hemolytic (HC_{50} = 35 – 90 μg/mL or 13 – 47 μM) (Table 2). This result suggests that HEMA component reduced the antimicrobial activity of copolymers slightly, but it can mitigate their hemolytic activity significantly. The result also suggests that, because these binary and ternary copolymers have the similar numbers of cationic (AEMA) and

hydrophobic (EMA) groups, the cationic and hydrophobic monomer compositions are not the only factors to determine their antimicrobial and hemolytic activities. We speculate that the HEMA component (hydroxyl side chains) has active roles in the membrane-active mechanism of copolymers, which will be discussed later.

Antimicrobial activity against *Staphylococcus aureus* and *Streptococcus mutans*.

In the previous sections, we used *E. coli* as a model bacterium to examine the effects of monomer compositions on the antimicrobial activity of copolymers. Here we extended this study to exam the antimicrobial activity of copolymers against Gram-positive bacteria *S. aureus* and *S. mutans* since the broad spectrum of activity is one of the hallmarks of AMP's properties. *S. mutans* is an oral cariogenic bacterium which produces acid to cause tooth decay[29] as we are interested in the applications of antimicrobial polymers for dental products[30]. Interestingly, the series of copolymers with ~30 mole % of AEMA did not show any substantial activity against *S. aureus* (MIC ≥ 500 μg/mL), although the copolymers with more than 30 mole % of EMA were active against *E. coli* (MIC ≤ 125 μg/mL) (Table 1). This result suggest that the copolymers are selective to *E. coli* over *S. aureus*. In addition, *S. mutans* was susceptible to the copolymers, and the MIC values were even smaller than those for *E. coli* or comparable, although the copolymers were not effective against *S. aureus* (Table 1). The MIC values decreased as the mole % of EMA increased, indicating that the hydrophobicity of copolymers is responsible for the antimicrobial activity against *S. mutans*, in similar to *E. coli*.

DISCUSSION

In this study, we prepared ternary statistical copolymers to separately control the compositions of cationic and hydrophobic monomers and investigated the effect of each component on the antimicrobial and hemolytic activities of copolymers. Our data indicated increasing the number of cationic groups of copolymers to be more than the 30 mole % did not increase their antimicrobial activity against *E. coli*. All the series of copolymers with different EMA contents showed the same threshold values of AEMA in their MIC values. This suggests that the role of cationic groups in the antimicrobial mechanism is independent from that of hydrophobic EMA groups. On the other hand, the MIC values of copolymers with > 30 mole % of AEMA depend on only the mole % of EMA. These results suggest that the roles of cationic and hydrophobic groups could be controlled independently in the ternary copolymers studied here, which meets our challenge to decouple the roles of these groups by polymer design.

Based on the results presented in this study, we propose the antimicrobial mechanism of copolymers against *E. coli*, depicted in Figure 5. Because the MIC values were decided by the mole % of EMA, the electrostatic binding of copolymers to bacterial cell membranes is not the MIC-determining step once the number of cationic groups in the copolymers is sufficient. Therefore, the copolymers are likely to be attracted from solution to the surface of bacterial cell membranes by electrostatic interactions, but we speculate that the copolymer chains may not cause any substantial disruption in *E. coli* cell membranes for antimicrobial activity at this stage. The 30 mole % of cationic AEMA or 5.5–7.7 positive charges may be

sufficient for weak electrostatic association with the inherent surface density of negative charge of *E. coli* cell membranes. Further increase in the positive change of copolymers may not significantly increase the polymer concentration on the cell membranes anymore or the following hydrophobic insertion of copolymers into the membranes may be the determining step for membrane binding. As our previous study using molecular dynamic simulations demonstrated,[14] followed by the electrostatic binding, the hydrophobic ethyl groups would be inserted into the cell membranes, and the polymer chains possibly form membrane-active conformations in which the cationic and hydrophobic groups are segregated to the opposite side of polymer backbone. Because the MIC values decreased as the mole % of EMA increased, We speculate that this hydrophobic insertion and following membrane disruption are increased as the hydrophobicity of copolymers (the mole % of EMA) was increased, and thus we proposed that these actions involving the hydrophobic interactions would be MIC-determining.

We wondered why the cationic and hydrophobic groups of methacrylate copolymers can independently behave when they act against the *E. coli* cell membrane. If we do the same experiments using AMPs, would the roles of amino acid residues be independent? In general, it is difficult to separate the effects of functional groups in AMPs because one change may cause significant changes in many properties due to strong interconnection between amino acid residues and side chains. For example, one amino acid replacement in the sequence may alter the overall properties of AMPs, including, for example, the propensity of formation of membrane-active helix and amphiphilic patterns. On the other hand, the methacrylate polymer chains are flexible and not designed to form any defined helical structures. However our previous dynamic molecular simulations suggest that upon binding to bacterial cell membranes, the copolymers adopt global amphiphilic conformations, in which the cationic and hydrophobic side chains are segregated to the opposite side of polymer backbone,[14] which recapitulate the formation of a-helices of AMPs. Because the polymer chains are flexible and do not have strong interactions between the monomers or side chains as compared to AMPs, the cationic and hydrophobic groups of copolymers may be able to independently act to adopt such amphiphilic conformations, and thus a change in the composition of one monomer would not affect the other in contrast to AMPs. However, we cannot completely rule out the interactions between the monomers. For example, the polymer chains of copolymers with high mole % of AEMA might be relatively extended due to electrostatic repulsion between the neighboring cationic side chains. Such a rigid conformation may not favor the binding to cell membranes, and thus it may reduce the antimicrobial and hemolytic activities of copolymers. However, the extend conformation may in turn facilitate the formation of membrane-active amphiphilic conformation with extended polymer backbone in membranes as described above, which might enhance the membrane-disrupting activities of copolymers. The functional roles of cationic and hydrophobic groups in the antimicrobial and hemolytic activities of copolymers are likely to be interactive; their correlation remains unclear in the field.

Regarding the aforementioned question on the minimal numbers of cationic and hydrophobic groups for potent antimicrobial activity and desired selectivity of copolymers, 5.5 – 7.7 net cationic charges are sufficient to exert their antimicrobial effect against *E. coli*, which are comparable to the net positive charge of natural AMPs, as described above. The

antimicrobial activity of copolymers was increased as the hydrophobic EMA composition was increased, but the high mole % of EMA caused significant hemolysis. Comparing the MIC values and percent hemolysis, we identified the copolymer AE34HE29E37 as potent antimicrobial polymers with selectivity, which have 6.5 positive charges and 7.0 hydrophobic ethyl groups in a polymer chain. Interestingly, the series of binary copolymer derivatives with the same number of cationic and hydrophobic groups with AE34HE29E37, but without the hydroxyl groups, showed higher antimicrobial and hemolytic activities. We initially postulate that the hydroxyl group would be an inactive spacer monomer to control the monomer compositions. However, these results suggest that the hydroxyl groups of HEMA in the copolymers have important roles in their mechanism of antimicrobial activity and selectivity to bacterial cell membranes over human cell membranes.

Here we propose that the HEMA component (hydroxyl side chains) have an active role in the interactions of copolymers with cell membranes as a structural spacer to distribute the cationic and hydrophobic groups in their monomer sequences. and (2) a modulator of the energy states (stability) of copolymer chains inserted in bacterial and human cell membranes. Specifically, the HEMA units separate and distribute the AEMA and EMA monomer units in the statistical monomer sequences of ternary copolymers, which reduces the formation of sequential domains of hydrophobic EMA monomers. The binary copolymers are likely to have such strong hydrophobic domains, which would increase the hydrophobicity of copolymers and thus enhance their ability of copolymers to disrupt membranes, resulting in higher antimicrobial activity. However, the strong hydrophobicity also causes non-specific interactions with human cell membranes, and the copolymers would show high hemolytic activity. These expected activities are in good agreement with the observed higher antimicrobial and hemolytic activities of the binary copolymers relative to the parent ternary copolymer (Table 2). Gellman and coworkers previously demonstrated that such a hydrophobic monomer cluster in a polymer chain is a cause of significant hemolysis of binary copolymers,[31] supporting our explanation. In addition, the cationic AEMA are also distributed separately owing to the HEMA units, which would facilitate the global amphiphilic conformations capable of disrupting bacterial cell membranes.[14] Traditionally, the mole % of cationic and hydrophobic groups and/or the number of these groups have been considered as primary structural determinants because of their roles in the antimicrobial mechanism of copolymers. However, our results also suggest that the distribution or sequential arrangement of functional monomers is also an important factor to determine the membrane-active mechanism of copolymers.

In addition to the structural spacer role, we also propose that the hydroxyl groups of HEMA may also modulate the stability of copolymer conformations when they are inserted into cell membranes. We initially postulated that the hydroxyl group is polar, but not charged, so that it would not contribute to the electrostatic binding and hydrophobic insertion of polymers to membranes. However, the previous study on energy potentials for peptide insertion to membranes indicated that the hydroxyl groups of serine were accommodated in the apolar environments of lipid membranes rather than only the membrane surface in which polar/ionic amino acid residues such as lysine are located.[32] Therefore, the hydroxyl groups of copolymers may also interact with the hydrophobic domains of cell membranes. Such (mildly) polar hydroxyl side chains in cell membranes may not be so favorable for the

formation of amphiphilic conformation of copolymer chains in which the hydrophobic groups of copolymers tightly interact with the hydrophobic core of cell membranes. Alternatively, the insertion of the hydroxyl groups would cost energy, which disfavors the polymer insertion to the membranes. As a result, the antimicrobial and hemolytic activities of copolymers are reduced, which is in good agreement with the reduced antimicrobial and hemolytic activities of copolymers when EMA was reduced from 46 to 37 mole % by increasing HEMA (Table 1). However, we do not have any experimental data to directly indicate that the hydroxy groups are located in the hydrophobic domains of membranes nor the polymer insertion to bacterial membranes are altered due to the hydroxyl groups of polymers. It is not possible to determine what is the exact role of HEMA (hydroxyl groups) in the antimicrobial mechanism, however, these proposed roles of HEMA (spacer and modulator) are not mutually exclusive. We need further investigations using biophysical methods to elucidate the nature of hydroxy groups of polymers in their interactions with membranes.

It is interesting that the effect of hydroxyl groups seems to be more significant to the hemolytic activity of copolymers as compared to their antimicrobial activity; the HC_{50} value was increased by orders of magnitude from 42 to 1000 μg/mL, while the MIC value was increased from 13 to 31 μg/mL. Gellman and coworkers also reported the similar effect for their nylon-3 polymers.[19] This may in turn suggest that the *E. coli* cell membranes are more tolerant to the changes in the monomer compositions of hydrophobic and hydroxyl groups, but the human cell membranes are more sensitive. The difference in their response (MIC and HC_{50}) to the copolymers with different monomer compositions is likely to reflect their lipid compositions of *E. coli* and human cell membranes. More studies are needed to elucidate the role of lipids on the antimicrobial activity and selectivity of copolymers, which is a subject of our future research.

The copolymers showed selective activity against *E. coli* over *S. aureus* (Table 1). It has been previously reported that cationic amphiphilic polyurethanes showed selective activity against *E. coli* over *S. aureus*, although the reason is unknown.[33] On the other hand, cationic polynorbornens showed selective activity against *S. aureus* over *E. coli*.[34] Raaft et. al examined the antimicrobial activity of chitosan against *S. aureus* in detail and suggested a possibility that cationic chitosan polymers bind to anionic cell wall biopolymers and act in the cell wall, resulting in inhibitory effects and/or cell membrane destabilization as a secondary effect.[35] Our laboratory also demonstrated that cationic homopolymers of AEMA[36] and unmodified branched PEIs[37] were selective to *S. aureus* over *E. coli*. These results from our laboratory and the literatures appear to indicate that the cationic functionality of polymers is the key determinant for antimicrobial activity against *S. aureus*. Therefore, replacing cationic AEMA by HEMA might be responsible for the reduction of activity against *S. aureus*. However, the binary copolymers without HEMA showed potent activity against *S. aureus* (Table 2), which may suggest that only when the mole % of hydrophobic groups is high, the copolymers are active against *S. aureus*. This might be explained by the polymer hydrophobicity which caused disruption of *S. aureus* cell membranes. These results suggest that the copolymers may have multiple targets in *S. aureus* (cell wall, cell membranes, etc.), and the associated mode of action are driven by different structural factors of copolymers (cationic properties, amphiphilicity,

hydrophobicity, etc.). Therefore, the contribution of these different mechanisms to the overall antimicrobial activity of copolymers against *S. aureus* is likely dependent on their monomer compositions. More specifically, when the copolymers are highly cationic, the cationic functionality-driven mechanism targeting the cell wall might be dominant. For the copolymers have highly hydrophobic, membrane disruption is the primary mode of action. In other words, in order to kill S. aureus, polymers should be either very hydrophobic or very cationic. The copolymer AE34HE29E37, for example, was active against *E. coli* (MIC = 31 μg/mL), but not against *S. aureus* (MIC = 1000 μg/mL). This may suggest that this copolymer is neither hydrophobic or cationic enough to kill *S. aureus*. In addition to the cationic and hydrophobic finicalities of copolymers, the cell wall structure of *S. aureus* may be also responsible for lower activity of copolymers to *S. aureus* as compared to *E. coli*. It may be possible that the copolymer chains might be trapped by the thick peptidoglycan layer of *S. aureus* due to strong interactions such as possible hydrogen bonds between the hydroxyl groups of copolymers and cell wall biopolymers. This would reduce the effective polymer concentrations on the cell membranes, resulting in low activity against *S. aureus*. Although the details of antimicrobial mechanisms of copolymers against *S. aureus* is beyond the scope of this study, future study may reveal the polymer design to create antimicrobial polymers with specificity to *S. aureus*.

CONCLUSION

In this study, we investigated the antimicrobial and hemolytic activities of ternary copolymers consisting of cationic primary ammonium, neutral hydroxyl, and hydrophobic ethyl groups in their side chains. Our data indicated that the ternary copolymer system can decouple the cationic and hydrophobic effects, and therefore, we are able to independently control the functional roles of these groups in the antimicrobial activity and selectivity by design. We also propose that the HEMA component (hydroxyl side chains) may have two active roles: (1) as a structural spacer to distribute the cationic and hydrophobic groups in monomer sequences and (2) as a modulator of copolymer chain insertion in bacterial and human cell membranes. The natural AMP sequences present more functional amino acids than cationic, hydrophobic, and neutral groups, which provide specific molecular interactions with cell membranes.[38–41] These AMP functions are likely stemmed from a result of molecular evolution through the peptide-cell membrane interactions. The copolymers with ternary or more monomer components will provide a molecular platform to explicit such evolutionally optimized AMP functionaries for the development of new antimicrobial agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors acknowledge the National Institute of Health (U01DE023771), Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, and MCube (Cost-effective novel antibiotics to combat multidrug-resistant bacteria), University of Michigan. L.F. was supported by National Science Graduate Research Fellowship (No. DGE-1315231). We thank Dr. Christopher Fenno at University of Michigan School of Dentistry for providing *S. mutans*, and Dr. Robertson Davenport at University of Michigan Hospital for providing RBCs.

REFERENCES:

1. Kenawy ER; Worley SD; Broughton R, Biomacromolecules 2007, 8 (5), 1359–1384. [PubMed: 17425365]
2. Timofeeva L; Kleshcheva N, Appl. Microbiol. Biotechnol 2011, 89 (3), 475–492. [PubMed: 20953604]
3. Engler AC; Wiradharma N; Ong ZY; Coady DJ; Hedrick JL; Yang YY, Nano Today 2012, 7 (3), 201–222.
4. Ganewatta MS; Tang CB, Polymer 2015, 63, A1–A29.
5. Krumm C; Tiller JC, Chapter 15 Antimicrobial Polymers and Surfaces - Natural Mimics or Surpassing Nature? In Bio-inspired Polymers, The Royal Society of Chemistry: 2017; pp 490–522.
6. Zubris DL; Minbiole KPC; Wuest WM, Curr. Top. Med. Chem 2017, 17 (3), 305–318. [PubMed: 27572084]
7. Ergene C; Yasuhara K; Palermo EF, Polym. Chem 2018, 9 (18), 2407–2427.
8. Uppu DSSM; Samaddar S; Hoque J; Konai MM; Krishnamoorthy P; Shome BR; Haldar J, Biomacromolecules 2016, 17 (9), 3094–3102. [PubMed: 27442617]
9. Coady DJ; Ong ZY; Lee PS; Venkataraman S; Chin W; Engler AC; Yang YY; Hedrick JL, Adv. Healthcare Mater. 2014, 3 (6), 882–889.
10. Locock KES; Michl TD; Stevens N; Hayball JD; Vasilev K; Postma A; Griesser HJ; Meagher L; Haeussler M, ACS Macro Letters 2014, 3 (4), 319–323.
11. Tew GN; Scott RW; Klein ML; Degrado WF, Acc. Chem. Res 2009, 43 (1), 30–39.
12. Takahashi H; Caputo GA; Vemparala S; Kuroda K, Bioconjugate Chem. 2017, 28 (5), 1340–1350.
13. Palermo EF; Vemparala S; Kuroda K, Antimicrobial Polymers: Molecular Design as Synthetic Mimics of Host-Defense Peptides. In Tailored Polymer Architectures for Pharmaceutical and Biomedical Applications, Scholz C; Kressler J, Eds. 2013; Vol. 1135, pp 319–330.
14. Palermo EF; Vemparala S; Kuroda K, Biomacromolecules 2012, 13 (5), 1632–1641. [PubMed: 22475325]
15. Porter EA; Weisblum B; Gellman SH, J. Am. Chem. Soc 2002, 124 (25), 7324–7330. [PubMed: 12071741]
16. Hu K; Schmidt NW; Zhu R; Jiang YJ; Lai GH; Wei G; Palermo EF; Kuroda K; Wong GCL; Yang LH, Macromolecules 2013, 46 (5), 1908–1915. [PubMed: 23750051]
17. Zasloff M, Nature 2002, 415 (6870), 389–395. [PubMed: 11807545]
18. Yang X; Hu K; Hu GT; Shi DY; Jiang YJ; Hui LW; Zhu R; Xie YT; Yang LH, Biomacromolecules 2014, 15 (9), 3267–3277. [PubMed: 25068991]
19. Chakraborty S; Liu RH; Hayouka Z; Chen XY; Ehrhardt J; Lu Q; Burke E; Yan YQ; Weisblum B; Wong GCL; Masters KS; Gellman SH, J. Am. Chem. Soc 2014, 136 (41), 14530–14535. [PubMed: 25269798]
20. Hancock RE W. Hancock Laboratory Methods. http://cmdr.ubc.ca/bobh/methods.htm.
21. Giacometti A; Cirioni O; Barchiesi F; Del Prete MS; Fortuna M; Caselli F; Scalise G, Antimicrob. Agents Chemother. 2000, 44 (6), 1694–1696. [PubMed: 10817731]
22. Baul U; Kuroda K; Vemparala S, J. Chem. Phys 2014, 141 (8), 84902.
23. Kuroda K; Caputo GA; DeGrado WF, Chem.-Eur. J 2009, 15 (5), 1123–1133. [PubMed: 19072946]
24. Palermo EF; Kuroda K, Biomacromolecules 2009, 10 (6), 1416–1428. [PubMed: 19354291]
25. Michl TD; Locock KES; Stevens NE; Hayball JD; Vasilev K; Postma A; Qu Y; Traven A; Haeussler M; Meagher L; Griesser HJ, Polym. Chem 2014, 5 (19), 5813–5822.
26. Wei G; Liu X; Yuan L; Ju XJ; Chu LY; Yang LH, J. Biomater. Sci., Polym. Ed 2011, 22 (15), 2041–2061. [PubMed: 21029518]
27. Fernandes HP; Cesar CL; Barjas-Castro Mde L, Rev. Bras. Hematol. Hemoter 2011, 33 (4), 297–301. [PubMed: 23049321]
28. Palermo EF; Lee DK; Ramamoorthy A; Kuroda K, J. Phys. Chem. B 2011, 115 (2), 366–375. [PubMed: 21171655]

29. Metwalli KH; Khan SA; Krom BP; Jabra-Rizk MA, PLoS Path. 2013, 9 (10).
30. Takahashi H; Nadres ET; Kuroda K, Biomacromolecules 2017, 18 (1), 257–265. [PubMed: 27992189]
31. Zhang JH; Markiewicz MJ; Mowery BP; Weisblurn B; Stahl SS; Gellman SH, Biomacromolecules 2012, 13 (2), 323–331. [PubMed: 22168316]
32. Senes A; Chadi DC; Law PB; Walters RFS; Nanda V; DeGrado WF, J. Mol. Biol 2007, 366 (2), 436–448. [PubMed: 17174324]
33. Mankoci S; Kaiser RL; Sahai N; Barton HA; Joy A, ACS Biomater. Sci. Eng 2017, 3 (10), 2588–2597.
34. Lienkamp K; Madkour AE; Musante A; Nelson CF; Nusslein K; Tew GN, J. Am. Chem. Soc 2008, 130 (30), 9836–9843. [PubMed: 18593128]
35. Raafat D; von Bargen K; Haas A; Sahl H-G, Appl. Environ. Microbiol 2008, 74 (12), 3764–3773. [PubMed: 18456858]
36. Thoma LM; Boles BR; Kuroda K, Biomacromolecules 2014, 15 (8), 2933–2943. [PubMed: 25010735]
37. Gibney KA; Sovadinova I; Lopez AI; Urban M; Ridgway Z; Caputo GA; Kuroda K, Macromol. Biosci 2012, 12 (9), 1279–1289. [PubMed: 22865776]
38. Fan LL; Sun J; Zhou MF; Zhou J; Lao XZ; Zheng H; Xu HM, Sci. Rep 2016, 6. [PubMed: 28442741]
39. Pistolesi S; Pogni R; Feix JB, Biophys. J 2007, 93 (5), 1651–1660. [PubMed: 17496013]
40. Lee DK; Brender JR; Sciacca MFM; Krishnamoorthy J; Yu CS; Ramamoorthy A, Biochemistry 2013, 52 (19), 3254–3263. [PubMed: 23590672]
41. Sato H; Feix JB, Biochim. Biophys. Acta 2006, 1758 (9), 1245–1256. [PubMed: 16697975]

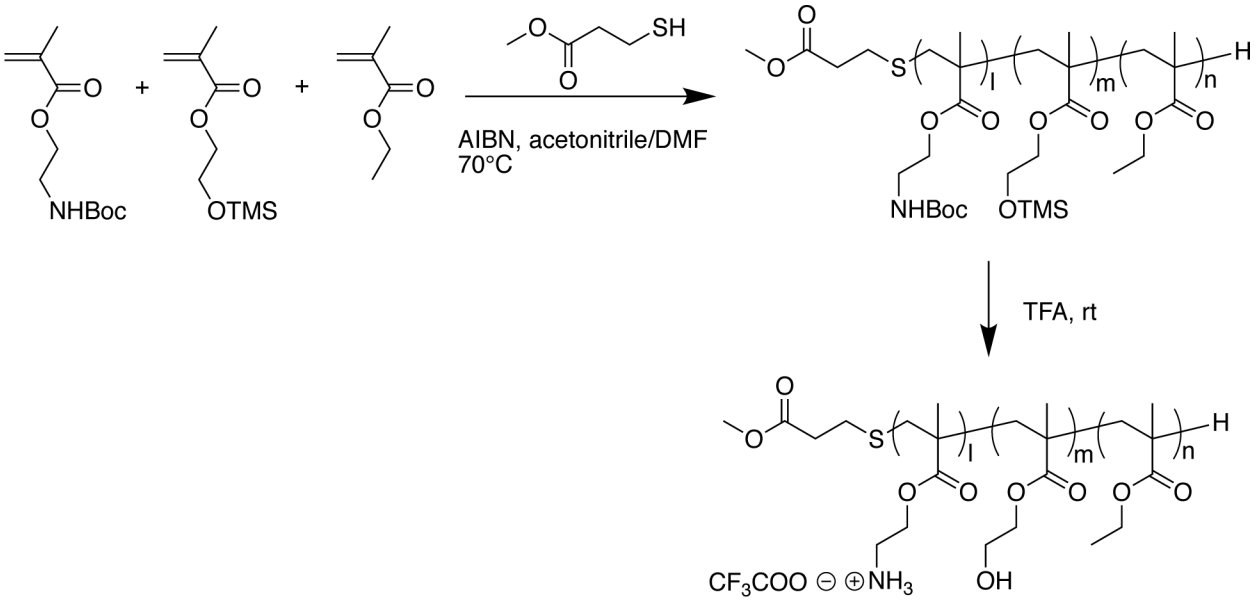


Figure 1.

Synthesis of ternary statistical copolymers. The statistical copolymers were prepared by free-radical polymerization in the presence of thiol chain transfer agent methyl 3-mercaptopropionate. The boc and TMS groups were removed using trifluoroacetic acid at room temperature.

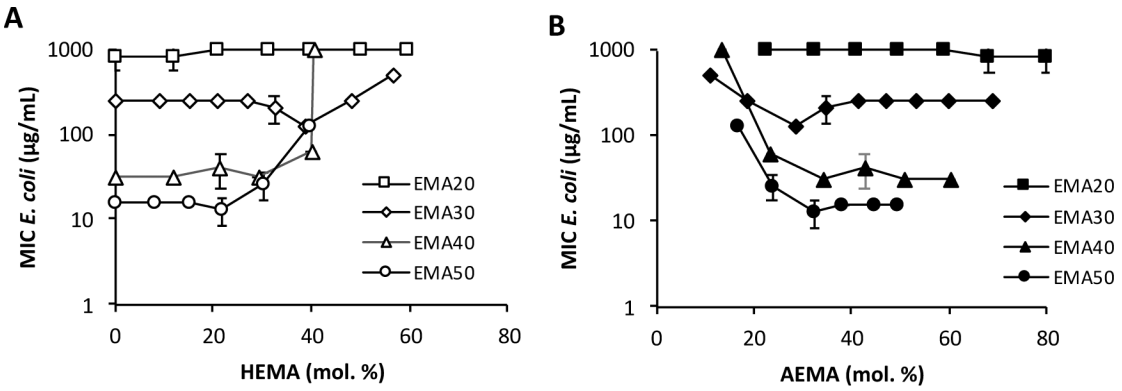


Figure 2.

Antimicrobial activity of copolymers against E. coli. The MIC values were plotted as a function of the mole percentage of (A) HEMA or (B) AEMA for each series of copolymers with different EMA compositions (20–50 mole %).

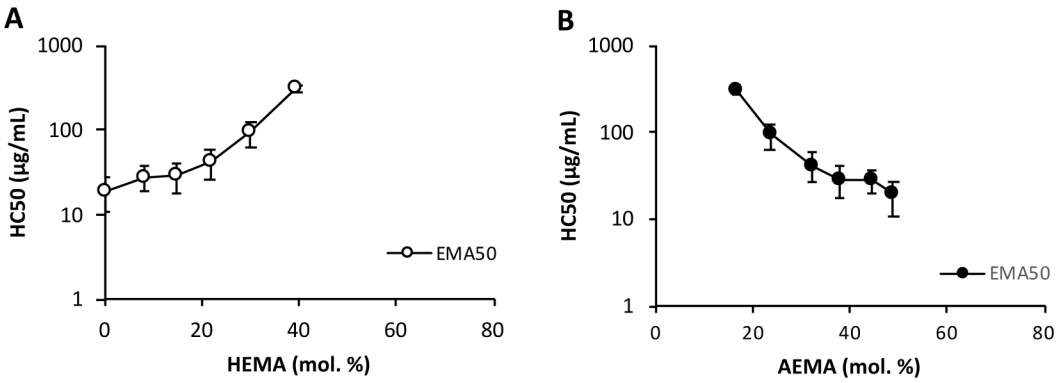


Figure 3.

Hemolytic activity of copolymers against human red blood cells. The HC50 values were plotted as a function of (A) HEMA or (B) AEMA for the copolymers with 50 mole % of EMA.

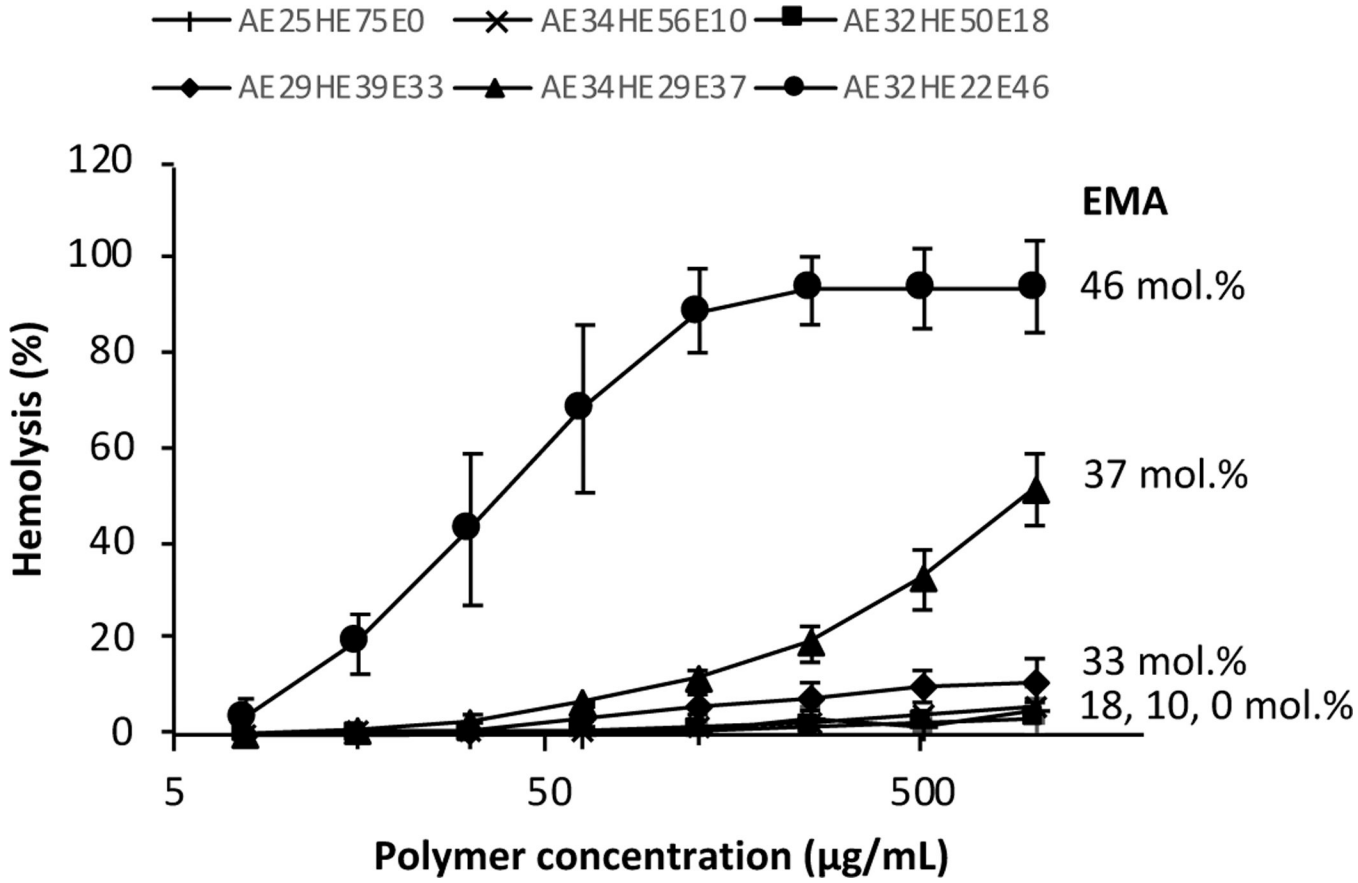


Figure 4.
Hemolytic activities of ternary copolymers with ~30 mole % AEMA.

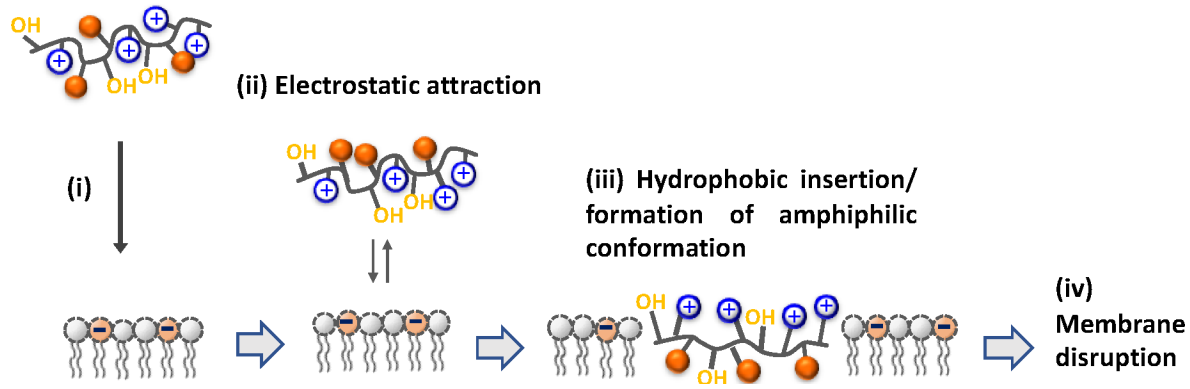


Figure 5.

Proposed antimicrobial mechanism of cationic amphiphilic copolymers, (i) Cationic polymer chains are attracted to the anionic *E. coli* cell membrane surface by electrostatic interactions, and (ii) associated with the membrane surface, (iii) The hydrophobic groups were inserted into the bacterial cell membranes, followed by the formation of membrane-active conformation and (iv) membrane disruption (MIC-determining step).

Table 1.

Characterization of ternary copolymers with ~30 mole % AEMA

Polymer[a]	The number of monomers/polymer chain			DP	M_n[b] (NMR)	M_n[c] (GPC)	M_w[c] (GPC)	$Đ$[c]	MIC (μg-mL^{-1})			HC_{50}[d] (μg-mL^{-1})
	AEMA	HEMA	EMA						*E.c.*	*S.a.*	*S.m.*	
AE25HE75E0	+2.5	7.5	0	10	1400	1210	1660	1.37	>1000	>1000	670 ± 290	(5.5 ± 4.9%)
AE34HE56E10	+7.1	11.8	2.1	21	2800	1680	2590	1.53	>1000	>1000	500	(6.1 ± 1.6%)
AE32HE50E18	+5.8	9.0	3.2	18	2400	1470	2670	1.81	1000	>1000	125	(3.3 ± 0.1%)
AE29HE39E33	+5.5	7.4	6.3	19	2500	n.d.	n.d.	n.d.	125	>1000	125	(11 ± 4%)
AE34HE29E37	+6.5	5.5	7.0	19	2500	1460	2720	1.86	31	1000	31	1000 (51 ± 8%)
AE32HE22E46	+7.7	5.3	11.0	24	3100	1800	2740	1.52	13 ± 5	500	n.d.	42 ± 16

a) The polymers are denoted as AExHEyEz, where the numbers x, y, z present the mole percentage of AEMA, HEMA, and EMA monomer unit in a polymer chain, respectively. The mole percentages of monomers were determined by 1H NMR analysis.

b) The molecular weight is based on the chemical formula without TFA salt.

c) The *Mn*, *Mw*, and *D* (PDI) were measured for the protected copolymers.

d) The hemolysis % at the highest polymer concentration (1000 μg/mL) is given in the parenthesis

Table 2.

Ternary vs. binary copolymers.

Polymer[a]	The number of monomers/polymer chain			DP	M_n[b] (NMR)	M_n[c] (GPC)	M_w[c] (GPC)	$Đ$[c]	MIC (μg-mL^{-1}/μM)			HC_{50}[d] (μg-mL^{-1}/μM)
	AEMA	HEMA	EMA						*E.c.*	*S. a.*	*S. m.*	
AE34HE29E3 (Parent)	+6.5	5.5	7.0	19	2500 (3200)	1460	2720	1.86	31/10	1000/310	31/10	1000/310
AE45HE0E55	+6.7	0	8.3	15	2000 (2700)	1460	2740	1.81	7.8/2.8	31/11	7.8/2.8	35 ± 8/13 ± 3
AE46HE0E56	+6.0	0	7.3	13	1600 (2300)	1260	2200	1.74	10 ± 5/4.4 ± 2.2	31/13	7.8/3	50 ± 10/22 ± 4
AE47HE0E53	+4.7	0	5.3	10	1400 (1900)	1340	1940	1.44	7.8/4.1	42 ± 18/22 ± 10	13 ± 4.5/6 ± 2	90 ± 11/47 ± 6

a) The polymers are denoted as AExHEyEz, where the numbers x, y, z present the mole % of AEMA, HEMA, and EMA monomers in a polymer chain, respectively.

b) The molecular weight is based on the chemical formula without TFA salt. The molecular weight including TFA is given in the parenthesis.

c) The M_n, M_w, and D (PDI) were measured for the protected copolymers.