Antimicrobial polymers as synthetic mimics of host-defense peptides

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Antibiotic-resistant bacteria 'superbugs' are an emerging threat to public health due to the decrease in effective antibiotics as well as the slowed pace of development of new antibiotics to replace those that become ineffective. The need for new antimicrobial agents is a well-documented issue relating to world health. Tremendous efforts have been given to developing compounds that not only show high efficacy, but also those that are less susceptible to resistance development in the bacteria. However, the development of newer, stronger antibiotics which can overcome these acquired resistances is still a scientific challenge because a new mode of antimicrobial action is likely required. To that end, amphiphilic, cationic polymers have emerged as a promising candidate for further development as an antimicrobial agent with decreased potential for resistance development. These polymers are designed to mimic naturally occurring host-defense antimicrobial peptides which act on bacterial cell walls or membranes. Antimicrobial-peptide mimetic polymers display antibacterial activity against a broad spectrum of bacteria including drug-resistant strains and are less susceptible to resistance development in bacteria. These polymers also showed selective activity to bacteria over mammalian cells. Antimicrobial polymers provide a new molecular framework for chemical modification and adaptation to tune their biological functions. The peptide-mimetic design of antimicrobial polymers will be versatile, generating a new generation of antibiotics toward implementation of polymers in biomedical applications. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

The need for new antimicrobial agents is a well-documented issue relating to world health.[1–6] The past 20 years have seen a dramatic increase in the development of antibiotic resistance harbored by relatively common pathogenic bacterial species. These resistances can arise from both natural evolution/selective pressure as well as from the improper use and administration of antibiotics at the clinical level. Regardless of the origin of the resistance, these new 'superbugs' require the development of newer, stronger antibiotics which can overcome these acquired resistances. Through the development of these new compounds, significant effort has been given to developing compounds that not only show high efficacy, but also those that are less susceptible to resistance development in the bacteria they target. Amphiphilic, cationic polymers have emerged as a promising candidate for further development as an antimicrobial agent with decreased potential for resistance development.[7–10] These polymers are designed using naturally occurring host-defense

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peptides (HDPs) or antimicrobial peptides (AMPs) as a 'molecular inspiration', building on the selectivity of these peptides imparted from their cationic, amphiphilic nature.[11] However, polymers exhibit significant advantages over the peptide antimicrobials in that polymers can be cost-effectively produced in much greater quantities, have greater compatibility with drug-delivery methodology, and provide a flexible framework for chemical modification and adaptation.

The chemical and structural diversity available to polymer chemists allow for great variability in the construction of cationic, amphiphilic molecules designed to function as antimicrobials. This diversity yields a significant amount of requisite investigation into mechanism of action, efficacy and selectivity profiles, resistance potential, as well as structure–activity relationships. In general, the cationic, amphiphilic polymers exhibit selectivity to bacterial targets through favorable electrostatic interactions between the polymer and the highly negatively charged bacterial cell surface.[11] This electrostatic interaction allows for broad-spectrum activity as most bacterial surfaces maintain anionic properties. Once bound, many polymers can significantly disrupt the bacterial membrane(s), presumably by inserting the hydrophobic moieties into the bilayer core.[12–14] While these are likely mechanisms, mounting evidence shows that simple membrane disruption may be only one component of a more complex series of effects bringing about bacterial cell death.[15,16]

BACKGROUND

Antimicrobial or antibiotic resistance is an emerging threat due to the inherent loss of efficacy of clinically prescribed antibiotics but also due to the slowed pace of development of new antibiotics to replace those that become ineffective.[1,2,5,6] The development of resistant organisms were first detected in hospital settings as nosocomial infections but have since spread and are now routinely agents of community acquired infections. Clinically isolated resistant organisms include bacteria, viruses, fungi, and parasites. While resistance in any pathogenic organism is problematic, the problem of bacterial resistance has taken a primary focus of interest due to widely publicized examples [methicillin-resistant *Staphylococcus aureus* (MRSA), for example] as well as the numerous different strains which have shown resistance development in a clinical setting including *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, MRSA, multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), Vancomycin-resistant

Enterococci (VRE), and Vancomycin-intermediate/ resistant *Staphylococcus aureus* (VISA/VRSA).[2] The rapid emergence of these strains coupled with the wide variety of antimicrobial agents that resistance has developed against dictates the need for new, highly effective antimicrobials that act on novel targets or critical bacterial components to which resistance cannot be evolved.

Host-Defense Antimicrobial Peptides

Host-defense AMPs have been extensively studied as new alternatives for antibiotics because of little or no susceptibility to the current resistance mechanisms of bacteria.[15–22] AMPs are a class of peptides in the innate immunity, which protect the body from invasion of microbes by quick killing action. The structural feature of AMPs includes a relatively small molecular size (10–50 amino acids) and cationic amphiphilicity exerted by cationic and hydrophobic side chains.[18] One of the classes is α-helical AMPs including magainin and LL-37, and these AMPs form helix upon binding to bacterial cell membranes, and the cationic and hydrophobic side chains are localized on different side of helix[15] (Figure 1(a)). AMPs attack bacterial cell membranes, which causes membrane permeabilization, leakage of cellular components, and breakdown of membrane potential, resulting in cell death. This contrasts the mode of action of conventional antibiotics, which are generally enzyme and DNA replication inhibitors. The activity of AMPs is selective to bacteria over mammalian cells. This is because of preferential binding of cationic AMPs to high net negative charge on bacterial surfaces over mammalian cells (Figure 1(b)). Several molecular mechanisms of membrane permeabilization by AMPs have been proposed: the peptides accumulate on the cell membranes and form discrete pores in the membrane (toroidal and barrel-stave models) or disrupt the membrane nonspecifically (carpet model)[15,19] (Figure 1(c)).

Although an in-depth discussion of antimicrobial mechanism of AMPs is beyond the scope of this review, recent studies on natural HDPs provided new insights into the mechanism of these membrane-active antimicrobials. Wong and coworkers investigated the role of lipids and topological changes in the membrane permeabilization by peptides and peptidomimetics using small-angle X-ray scattering (SAXS).[23] Natural peptides selectively permeabilized model bacterial membranes, while they do not destabilize model eukaryotic membranes. The HDP defensin generated a negative Gaussian (saddle-splay) curvature in model bacterial membranes composed of lipids that intrinsically induce negative curvature, which is necessary for

FIGURE 1 | α-Helical cationic antimicrobial peptide (AMP) and antimicrobial mechanism. (a) α-helical structure of magainin-2 (pdbID: 2MAG). Cationic residues are colored blue while hydrophobic residues are green. (b) Representation of the selectivity of AMPs to bacteria over mammalian cells based on coulombic attraction. Anioinc lipid head groups are shaded red, zwitterionic lipid head groups gray, and the peptide color scheme is the same as (a). (c) Proposed membrane-permeabilization models. (Reprinted with permission from Ref 15. Copyright 2005 Nature Publication Group)
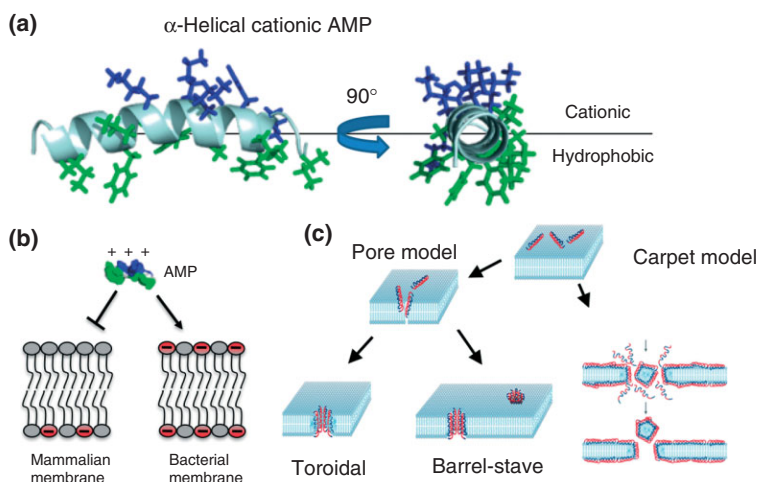
pore formation. The ability of peptides to selectively generate Gaussian curvature, or the Gaussian curvature selection rule is one of the key factors for rational design of AMPs. It would be of interest to investigate if this selection rule can be applied to polymers as a versatile mechanism. Weisshaar and coworkers studied the mode of antimicrobial action by human AMP LL-37 by real-time fluorescence spectroscopy.[24] The peptide binds to the *Escherichia coli* surfaces and translocates across the outer membrane (OM) after reaching a threshold concentration of peptide on the OM. Interestingly, the growth of *E. coli* was halted by the peptide translocation across the OM before the peptide was able to permeabilize the inner membrane (IM). The peptide preferentially attacked septating (dividing) cells and accumulated on the septum region, which seems to be the weakest link in membrane integrity. The peptide further diffused into the peptidoglycan layer and spread throughout the entire cell. The peptide also permeabilized the IM, which is likely to be the lethal step, although further investigation is necessary. The real-time observation of single live cells will be a strong tool to elucidate the mechanism of antimicrobial polymers. In addition, it has been postulated that the AMPs likely have multiple cellular targets simultaneously such as cell membranes, internal cellular components, and cell wall structures, which may combine to a synergistic effect on their activity.[15,16,25,26] The multiplicity of targets allow us classify AMPs as 'dirty drugs'[25,26] in contrast to conventional antibiotics which have a specific molecular target. Also, peptide binding generally exhibits lower affinity when compared to high affinity, binding of antibiotics to a specific target. The antibacterial mechanism of peptides utilizing these multiple low affinity targets may be responsible for the low susceptibility of peptides to the development of resistance mechanisms

in bacteria. These results indicate that the classical model of killing by membrane permeabilization may be incomplete. There appears to be a multifactorial mechanism of action utilized by AMPs which would also contribute to the lack of resistance seen against these molecules. Which combinations of these killing methods are utilized may also be dependent on the specific peptide, bacterial target, and organismal environment where the interaction occurs. Overall, this creates the need for much more in-depth study of the specific of mechanism of action of AMPs as well as how these mechanisms can be recapitulated in small molecules or in polymer frameworks.

Although the AMPs exhibit numerous beneficial antimicrobial characteristics, there are also significant hurdles to the large-scale implementation of AMPs in a clinical setting.[17,18,27,28] One of the primary sets of issues with implementation of AMPs as therapeutics relates to activity and availability once delivered.[18,29,30] The function and activity of AMPs are often determined in controlled laboratory environments in restricted, well-defined media sets. This often leads to a decrease in functional activity when the peptides are exposed to the complex serum milieu, including numerous proteins and extracellular structures that can nonspecifically sequester AMPs. The delivery of AMPs is also a challenge in that oral-availability is low due to the gastrointestinal tract (GI) tract efficiently digesting peptide/protein material. This fact necessitates additional carriers or adjuvants to increase half-life and availability of orally delivered peptides. Alternatively, intravenous delivery is possible but limits the broad applicability for antimicrobial applications, especially when compared to the currently available small molecule antibiotics. Another major issue is a high cost of manufacturing peptide therapeutics on a large scale.[17,18] Owing to

their nature as antibacterial, large scale fermentations and biologically based production are incompatible with these molecules. The length and complexity of most AMPs are also incompatible with solution-phase chemical methodology, requiring time- and reagent-intensive solid phase methods for production.

PEPTIDE-MIMETIC DESIGN

To address these obstacles of AMPs, synthetic polymers have been designed as mimics of AMPs. In one of the peptide studies, an all D-enantiomer magainin homologs showed the same level of activity compared to natural magainin.[31] This indicated that the antimicrobial action is not receptor-dependent, that is, based on a specific protein–protein interaction. In addition, many AMP structures share the same physicochemical properties of a net positive charge and contain several hydrophobic amino acid residues, but yet no common consensus sequences or motifs have been found.[18] While a significant number of known AMPs utilize the α-helical conformation to create a facially amphiphilic, active structure, recent evidence from the investigation of diastereomeric peptides indicate that the stable helix structure may not be necessary for activity.[19,32] In this study, several amino acids were substituted with D-enantiomers, which disfavors helix formation. However, the peptides were still found to adapt amphiphilic conformations with segregation of cationic and hydrophobic residues, even without stable helix formation, when bound to lipid membranes.[19,32] These results suggest that the cationic, amphiphilic properties, the three-dimensional conformation of AMPs, or some balance of these two factors are likely the key determinants for antimicrobial activity rather than the exact sequence, stereochemistry, and/or stable secondary structure. Accordingly, the new design of antimicrobial polymers has been focused to reflect the cationic amphiphilic structures of AMPs on the synthetic polymer platform rather than to mimic the secondary structural conformations such as the α-helix.

In general, AMPs are relatively small, and the cationic functionality is imparted by the primary ammonium groups of lysine. Accordingly, polymer mimics of AMPs have been generated to have low molecular weights (MWs) (a few thousands) and primary ammonium side chains.[11] Hydrophobic comonomers are also incorporated to mimic the amphiphilic property of AMPs, producing amphiphilic random copolymers. Several examples of antimicrobial polymers are depicted in Figure 2. Polymers with quaternary ammonium side chains (polycations) have been widely utilized as polymeric
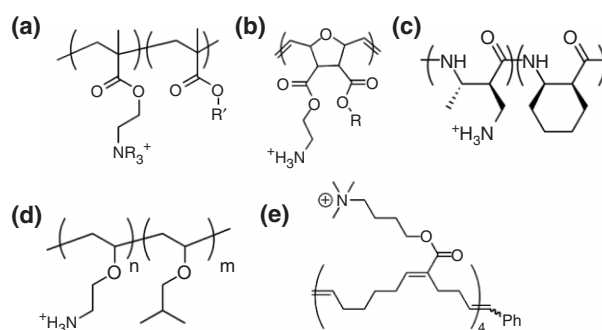

FIGURE 2 | Several examples of antimicrobial peptide (AMP)-mimicking polymers based on (a) methacrylate,[37] (b) norbornene,[38] (c) nylon,[39] (d) vinyl ether,[40] and (e) alternating ring-opening metathesis polymerization (ROMP) copolymers[41].

disinfectants and have high MWs in general.[8,33–36] Considering the net positive charge of the amphiphilic polymers, AMP-mimetic polymers can be also classified as polycations, but their low MW, primary ammonium side chains, and hydrophobic components provide new functionalities and characteristics including polymer–lipid interactions and altered biological activity. The roles of these factors are discussed below.

In the following sections, we discuss the antimicrobial activity and toxicity of polymers by focusing the recent results from our laboratories.[40,42–50] These polymers build on the fundamental amphiphilic, cationic properties found in most AMPs and extend them to platforms which are compatible for antimicrobial applications as well as being more scalable for production. Many research groups are using different classes of polymers, different assay conditions, and different strains of target bacteria, which result in different measures of activity and data analysis. In other words, antimicrobial effectiveness and toxicity of polymers depend on assay conditions. Therefore, direct comparisons of biological activities of polymers including MIC, IC_{50}, and cytotoxicity in literature may be ambiguous, and can be qualitative at best. This review discusses results from our work with the peptide-mimetic design of antibacterial polymers and their potential as new antimicrobials rather than focusing on comparisons of efficacy with other classes of polymers. The readers are advised to refer other excellent review articles for more detailed discussions on synthesis and mechanistic studies of other antimicrobial polymers.[7–10,33,51]

ANTIMICROBIAL ACTIVITY

Antimicrobial Assays

The activity of antimicrobial polymers against bacteria is evaluated as inhibition effect on bacterial growth

as well as bactericidal effect. The inhibition effect is commonly measured as a minimum inhibitory concentration (MIC) of polymers, which is the concentration that completely inhibits bacterial growth. A micro-dilution method using a microplate has been popular because the assay protocol is relatively easy and suitable for testing large number of compounds on a small scale. The standard and modified assay protocols are available in literature.[52,53] In general, known concentrations of bacteria are incubated with a range of concentrations of polymers overnight, and bacterial growth is determined by increase in the turbidity of solution or optical density. It should be noted that this assay determines no growth of bacteria, which indicate the inhibition of bacterial growth by polymers, but not necessarily due to killing of bacteria by polymers. When the polymers inhibit the bacterial growth without killing (bacteriostatic effect), the bacteria are able grow again once the polymers are removed by dilution or washing. The bacteriostatic and bactericidal effects of polymers should be distinguished for proper data and mechanism analysis. In addition, the micro-dilution assay is generally performed by using a plastic multi-well microplate. It has been reported that the activity of AMPs depends on the properties of microplate plastics.[54] AMPs showed low activity in polystyrene (PS) microplate compared to a polypropyrene (PP) microplate because amphiphilic peptides can bind nonspecifically to the walls of a PS plate, sequestering them from the bacterial solution.[53,54] Considering the amphiphilic property of antimicrobial polymers, it is reasonable to assume that the selection of microplates affects the results of antimicrobial assay. On the other hand, bactericidal effect is evaluated as the number of residual viable bacteria at given polymer concentrations or the polymer concentrations necessary to kill certain number of bacteria, typically >99.9% or 3-log reduction. The number of viable cells is usually reported by colony forming units (cfu) determined through plating assays.

Broad Spectrum of Activity

Antimicrobial polymers generally show broad-spectrum activity against both Gram-positive and negative bacteria. In contrast, conventional antibiotics are usually more specific because their unique cellular targets such as enzymes or DNA/RNA replication inhibitors. The polymers show slightly different activity against bacteria, but there seems to be no general trend for preference in terms of Gram-positive and negative strains. Methacrylate copolymers displayed antimicrobial activity against

drug-resistant *S. aureus* (MRSA) with the same level of activity with the drug-susceptible laboratory strain[47] (Table 1). The polymer is effective against *A. baumannii*, which has developed significant resistance against conventional antibiotic drugs. Recently, *A. baumannii* infections are found among patients treated at overseas military medical facilities.[55] The broad-spectrum activity of antimicrobial polymers can be attributed to the membrane-disruption mechanism, which is likely to be less sensitive to the specific cell wall structures and lipid compositions in cellular membranes.

Amphiphilic Polymer Structures and Activity

The antimicrobial activity of polymers in general increases with increasing hydrophobic content in the side chains. This is likely because of the increased hydrophobicity of polymers enhancing insertion of polymers into the hydrophobic region of cell membranes. Our recent computational investigation on methacrylate polymers indicated that the hydrophobic side chains of methacrylate random copolymers are inserted into lipid bilayers (Figure 3(a) and (b)).[47] It could be speculated that the polymer insertion to cell membranes would be enhanced by the hydrophobicity of side chains. In the same study, methacrylate random copolymers are more deeply inserted into lipid bilayers when the spacer arms in the cationic side chain are elongated. This is because of the snorkeling effect, in which the cationic ammonium groups are bound to the anionic phosphate lipid head groups,

TABLE 1 | Activity Spectrum of Representative Methacrylate Copolymer (E4$_{29}$). (Reprinted with permission from Ref 47. Copyright 2012 American Chemical Society)

Bacteria or Human Cell	MIC or HC$_{50}$ (µg/mL)		
	E4$_{29}$	Melittin	Magainin-2
Gram-positive			
Staphylococcus aureus	63	6	>250
Staphylococcus aureus (CA-MRSA)[1]	31	n.d.	n.d.
Enterococcus faecalis	31	16	>500
Bacillus subtilis	8	6	63
Gram-negative			
Escherichia coli	21	12	125
Acinetobacter baumannii	10	n.d.	n.d.
Pseudomonas aeruginosa	16	280	500
Salmonella enterica	16	140	500
Human RBC (HC$_{50}$)	1300	2	>250

n.d., not determined.
[1]Community acquired methicillin-resistant *S. aureus* (MRSA) strain LAC 1236.

(a) Model polymers

(b)

E2　　　　　　　　　　E4　　　　　　　　　　E6
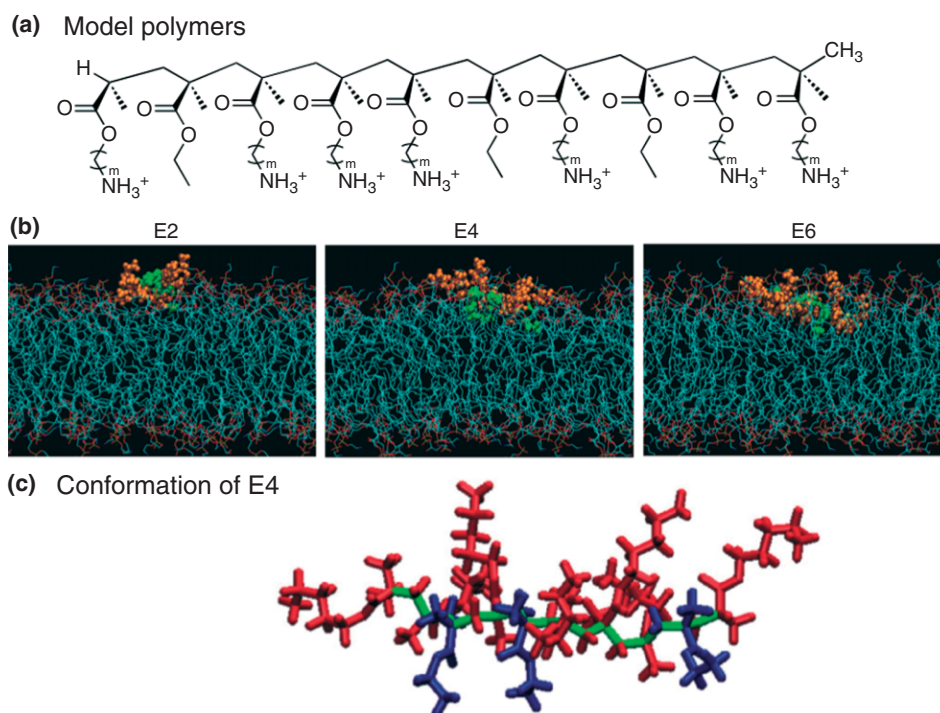
(c) Conformation of E4

FIGURE 3 | Simulated binding and insertion of methacrylate copolymers into lipid bilayers. (a) Model polymer structures with different cationic spacer arm lengths, $m = 2$ (E2), 4 (E4), and 6 (E6). (b) Snapshots of polymers inserted into phosphatidylethanolamine/ palmitoyloleoylphosphatidyl glycerol (POPE/POPG) lipid bilayers. (c) Conformation of E4 in the lipid bilayer. The polymer structures are presented by green (polymer backbone), red (cationic side chains), and blue (hydrophobic ethyl side chains) colors. (Reprinted with permission from Ref 47. Copyright 2012 American Chemical Society)

and the long arm or spacer enables the polymer chains to separate from the membrane surface and sink into the lipid bilayer (Figure 3(c)). The concept of snorkeling effect has been developed originally in the peptide science. Transmembrane peptides have been modified to have extended spacer arms in ammonium (lysine and guanidine) side chains. These long arms with cationic groups can reach to the water–lipid interface, which stabilizes the hydrophobic peptide helices in the hydrophobic domains of lipid bilayers. Therefore, the snorkeling effect can modulate the position and orientation of transmembrane peptides.[56–63] The similar effect was found in our methacrylate copolymers discussed above (Figure 3). Interestingly, the polymer chain is stretched when they are bound to the lipid bilayer, and the cationic groups and hydrophobic (ethyl) side chains of comonomers are segregated relative to the polymer backbone. This amphiphilic structure is similar to AMPs such as magainin, which have also cationic and hydrophobic side chains segregated into the different side of helix. Similarly, Yethiraj and coworkers demonstrated that random copolymer models of β-peptides bind to lipid bilayers and form the segregated amphiphilic structures.[64] The amphiphilic structure of polymers and peptides when

bound to cell membranes may be the key determinant rather than the identity and heterogeneity of chemical structure and conformation.

To mimic the functionality of AMPs, antimicrobial polymers were prepared to have low MWs and primary ammonium side chains, although this design lacked any published experimental data to support the design. To that end, we investigated the structure–activity relationship of methacrylate random copolymers. To study the role of cationic side chains, we hypothesized that the antimicrobial activity of copolymers would not depend on the chemical structures of amine groups if the cationic net charge of polymers were only essential in their underlying antimicrobial mechanism. To test this hypothesis, we investigated the effect of chemical structure of amine groups (primary, tertiary and quaternary ammonium) on the antimicrobial and hemolytic activities of methacrylate copolymers (Figure 4(a)). The study indicated that the methacrylate copolymers with primary ammonium groups showed the highest activity while the copolymers with quaternary ammonium groups are not active. We speculate that the ammonium groups form complexes with phosphate lipid heads through a combination of electrostatic attraction
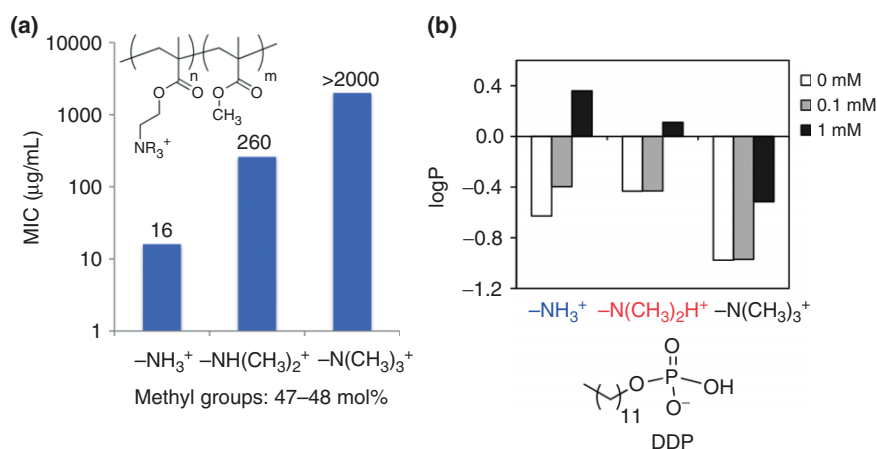
FIGURE 4 | Methacrylate copolymers with different chemical structures of ammonium groups. The mole percentage of methyl groups is 47–48%. (a) Antimicrobial activity and (b) effect of amine groups on polymer partition between octanol and aqueous buffer (pH 6) in the presence of phospholipid-mimic surfactant (Dodecylphosphate). Partition coefficient P is defined as $P = [Polymer]_{in\ octanol}/[Polymer]_{in\ buffer}$. (Reprinted with permission (a) from Ref 44. Copyright 2009 American Chemical Society; (b) from Ref 45. Copyright 2011 American Chemical Society)

and hydrogen bonding, enhancing the affinity of copolymers for the lipid membrane and facilitating membrane disruption.[45] In addition, the methacrylate copolymers are designed to have low MWs to mimic AMPs. The MIC values of copolymers did not depend on their MW significantly, but the copolymers showed adverse hemolytic activity against human RBCs as the MW was increased (Figure 5). The hemolytic activity can be described by the partitioning of hydrophobic side chains into lipid bilayers. Therefore, the high MW copolymers have large number of hydrophobic side chains, likely resulting in high hemolytic activity.
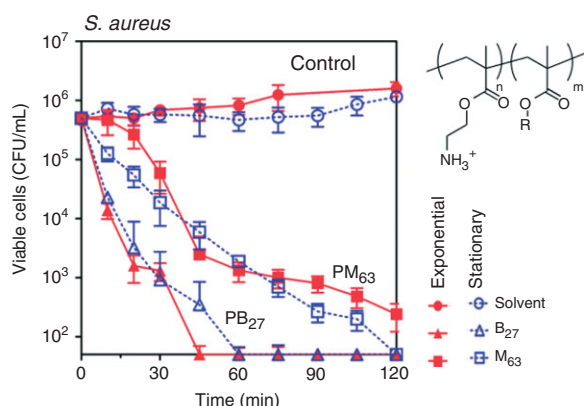

FIGURE 5 | Killing kinetics. The polymers are PB$_{27}$ (R = butyl, 27 mol. %) and PM$_{63}$ (R = methyl, 63 mol. %). The copolymers are tested at two times minimum inhibitory concentration (MIC) against *Staphylococcus aureus* sub-cultured from the exponential (*red closed markers and solid lines*) or stationary (*blue open markers and dashed lines*) phase of *S. aureus*. The MICs of PB$_{27}$ and PM$_{63}$ are 16 µg/mL and 250 µg/mL, respectively. (Reprinted with permission from Ref 49. Copyright 2011 MDPI)

These results indicate that tuning the balance of amphiphilicity and MW of polymers is a key strategy to generate nontoxic polymers with potent antimicrobial activity. The hemolytic activity and mechanism are discussed in detail below.

Cell Specificity

In general, antimicrobial polymers are good antimicrobial agents with a broad spectrum of activity because their mechanism targets bacterial lipid membranes, a cellular structure present in all bacterial species. However, this ubiquitous target in turn makes it difficult to target only specific strains by design. However, several antibacterial polymers are known to be selective against *S. aureus* over other strains, although there is no report on polymers with selective activity to bacteria other than *S. aureus*. Cationic polymers including polynorbornenes,[38] oligolysins,[65] and chitosan[66] all showed selective activity against *S. aureus* over *E. coli*. Although the chemical structures of these polymers are quite different from each other, the cationic functionality appears to be in common, which may be the key determinant in the activity against *S. aureus*. Recently, our laboratories also demonstrated that commercially available unmodified polyethyleneimines (PEIs) with branched structures (MW = 500–12,000) also are antimicrobial with selective activity against *S. aureus* over *E. coli*.[67] Interestingly, the natural AMP magainin did not show significant activity against *S. aureus* while the peptide is activity against *E. coli* under the same conditions. Branched PEIs induced no membrane depolarization of *S. aureus* even at high concentrations well above MIC. It is possible that the antimicrobial action of

PEIs is not to disturb the cell membranes, but act in the cell wall.[65,66] The cationic polymers may also be trapped by the peptidoglycan layer of *E. coli*, inhibiting polymer diffusion to the cytoplasmic membrane.[68] Therefore, the selective activity of cationic polymers may be linked to the structural difference in the bacterial cell walls as well as differences in the antimicrobial mechanism. Of note, since the tested strains are limited, it is not clear at this time that the activity of these polymers is specific to Gram-positive strains general or specifically for *S. aureus*. Although the mechanism of cationic polymers for anti-*S. aureus* activity and the selective activity is not clear yet, it would be of interest for further investigation, which will provide new insight into molecular design of next generation antimicrobials.

Biocidal Kinetics

In order to assess the timeframe required for antimicrobial polymers to kill bacteria, the number of viable bacterial cells is determined as a function of exposure time. In an assay, bacteria are incubated with polymers, and an aliquot of this treated culture is taken and diluted in buffer solution to remove the effect of polymers. Then the diluted solution is spread in an agar plate and incubated overnight to allow formation of bacterial colonies. The number of colonies on the agar plate is then counted. The assay solution needs to be appropriately diluted such that the final polymer concentration is much lower than effective concentrations of polymers, otherwise, carryover of polymers may inhibit colony formation. But, the bacteria concentration needs to be high enough to give colonies in countable numbers for accuracy. In principle, one bacterial cell forms one colony. Therefore, the number of viable cells after incubation with polymers can be calculated by taking the dilution factors into account and reported as a colony-forming unit (cfu). Typically these cfu values are converted into the units cfu per a given volume (typically cfu/mL) based on the dilution factors used and the volume of assay solution that was applied to the plate.

In general, antimicrobial polymers display quick bactericidal effect against bacteria: they kill more than 99.9% within an hour at the MIC, which is the same level of effect with antibacterial peptides. In our study on methacrylate copolymers, the effect of growth phases of bacteria was also investigated.[49] The copolymers displayed no significant difference in the killing rate against *E. coli* and *S. aureus* in the stationary and exponential growth phases (Figure 5). This indicates that the mechanism of antimicrobial action of the copolymers does not rely on the metabolic physiological activity associated with bacterial growth phases. Bacteria are less metabolically active when they are in biofilms or exposed to antibiotics, which is one of the bacterial defense mechanisms against antibiotics which can then lead to the development of antibiotic resistance. The copolymers we studied would likely be effective against bacteria in the dormant state or in biofilms as well as being less susceptible to resistance development through dormancy. It should be noted that activity against bacteria with decreased metabolic activity, as in biofilms, is not the only challenge in efficacy against biofilms. One of these challenges is that the cationic polymers may become 'trapped' in the biofilm matrix composed of anionic biopolymer exopolysaccharide (EPS), which would limit the polymer diffusion and reducing their efficacy. However, compared to conventional antibiotics, the potential activity of polymers against dormant bacteria is poised to be a major advantage for the development of these types of antimicrobial polymers effective against bacterial biofilms and biofilm-associated infections.

TOXICITY TO HUMAN CELLS

Hemolysis

Lytic activity to red blood cells or hemolytic activity has been used as a measure of first assessment of cytotoxicity of antimicrobial polymers. In a hemolysis assay, the polymers are incubated with a suspension of RBCs for a few hours. After centrifugation of assay solution, red blood cells sediment to the bottom of vessels or wells, and the amount of hemoglobin released in the supernatant is determined spectroscopically. The hemolytic activity of polymers is quantified by the percentage of released hemoglobin relative to 100% lysis of all RBCs by, typically, a surfactant. As the polymers are supposed to act on bacterial cell membranes, damages to human cells are the first assessment for cell selectivity. One of the reasons for popularity of hemolysis is likely due to the easy assay procedure compared to other cytotoxicity assays using *proliferable* cells. It should be noted that there is no standard protocol for hemolysis available which yields difficult comparisons between experimental systems and molecular species in question. Hemolytic activity is known to be very sensitive to the assay conditions as isolated RBCs so care should be taken to minimize experimental variability. One should employ extreme caution when attempting to directly compare specific values for hemolytic activity from the literature and instead should primarily focus on behavioral trends.

In general, when methacrylate copolymers contain a large amount of hydrophobic mass (either by number of groups, size of groups, or both), the

polymers behave as hydrophobic and hemolytic.[50] The polymers are expected to bind selectively to bacterial cell surfaces over human cells because of electrostatic interaction. However, when the hydrophobicity of polymers dominates the cellular binding of polymers, the polymers start to bind nonspecifically to both cell-types and cause hemolytic activity. As the hydrophobic interaction is also the driving force to enhance their antimicrobial activity, the balance between hydrophobicity and cationic functionalities is a key determinant for design of polymers with potent activity and selective activity against bacteria over human cells. Methacrylate copolymers with a range of lengths (C_1–C_6) and contents of alkyl side chains showed that the hemolytic activity can be described by portioning of alkyl groups into the hydrophobic domain of lipid bilayers (Figure 6).[50] In addition, for the copolymers with high contents of alkyl methacrylates, we observed polymer aggregates in water, which decreases the number of polymer chains active or accessible to cells, resulting in no enhancement of activity by further increases in hydrophobicity.

How can we remove the hemolytic activity from polymers without losing antimicrobial activity? This is a million dollar question in the field. We have several insights into the approach in polymer deign from our laboratory and the literature. From the same study on methacrylate copolymers,[50] short alkyl side chains seem to provide better selective activity compared to counterparts with longer alkyl side chains. One of the reasons may be because of small incremental changes in hydrophobicity allowing fine-tuning of polymer hydrophobicity for optimal balance between cationic and hydrophobic groups. In addition, the polymers

need more methyl groups to get the same level of activity as longer alkyl groups. This may indicate that the distribution of hydrophobicity along the polymer chain may be important. Indeed, cationic amphiphilic vinyl ether block copolymers showed little or no hemolytic activity compared to random copolymers.[40] Sen and coworkers demonstrated that spatial separation of cationic charges and hydrophobic groups in amphiphilic pyridium-methacrylate random copolymers results in increased antimicrobial and hemolytic activities, indicating a higher membrane-disrupting ability.[36] Although the chemical structures of ammonium groups (primary ammonium vs pyridium) of these examples are different, the distribution of hydrophobicity along the polymer chains and overall amphiphilic sequences are one of the key determinants to control the antimicrobial and hemolytic activities. The polymeric amphiphilic structure with tuned hydrophobic distribution will be an interesting subject for further research. In addition, the amphiphilicity of polymers, polymer MW and MW distribution also affect the hemolytic activity of polymers. In general, low MW polymers are less hemolytic than high MW counter parts.[11,50,37] In the study on nylon polymers, copolymers were purified by dialysis and the resultant polymers are much less hemolytic.[39] This indicates that the high MW components in polydisperse polymer samples are more responsible for hemolysis.

Although hemolysis has been widely used to evaluate polymer toxicity, the hemolysis mechanism has not been studied in detail. In this regard, methacrylate copolymers have been reported to cause all-or-none hemolysis, in which a fraction of RBCs were lysed and released all hemoglobin into solution,
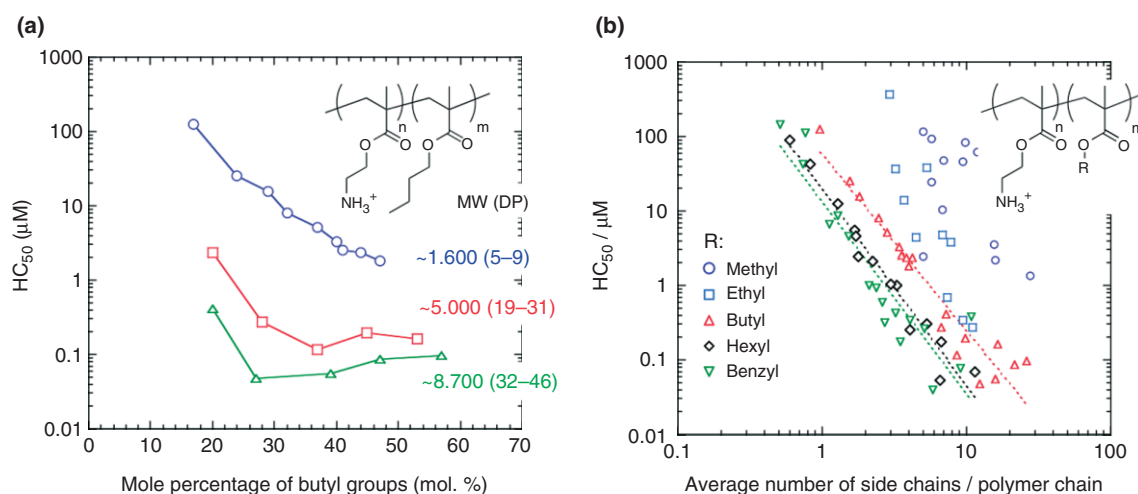

FIGURE 6 | Hemolytic activity of methacrylate copolymers. (a) Molecular weight (MW) dependence of hemolytic activity. High MW copolymers are more hemolytic. (b) Correlation between the number of hydrophobic groups in a polymer chain and HC_{50}. (Reprinted with permission from Ref 50. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA)
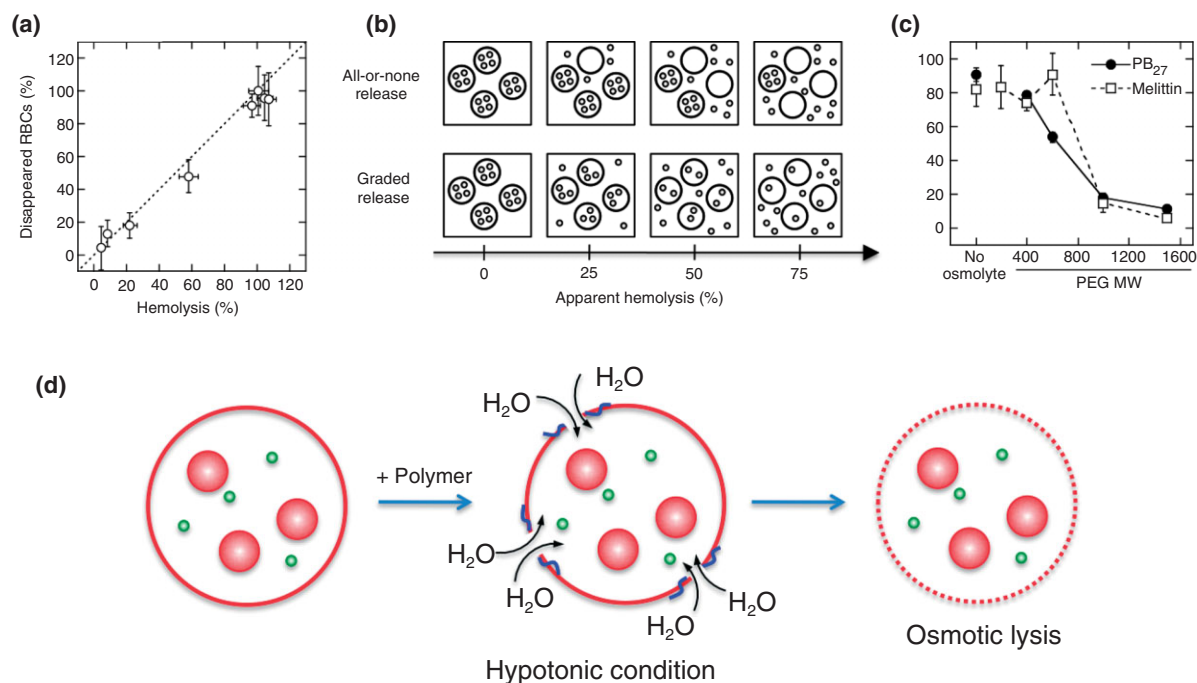
FIGURE 7 | All-or-none osmotic hemolysis induced by methacrylate polymer. The polymer PB$_{27}$ contains 27 mol. % of butyl groups. (a) The correlation between percentages of hemolysis (released hemoglobin) and disappeared cells. The good correlation suggests that the hemolysis caused by the polymer is an all-or-none type event. (b) Schematic presentation of all-or-none and graded release of hemoglobin from RBCs. (c) Osmotic protection by polyethylene glycols (PEGs). High molecular weight (MW) PEGs (> 1000) suppressed hemolysis by the polymer. The threshold MW of PEG for hemolysis inhibition is 500–1000, corresponding to 1.6–2 nm in diameter. (d) Schematic representation of the osmotic lysis mechanism of polymer action. (Reprinted with permission from Ref 48. Copyright 2011 American Chemical Society)

rather than all RBCs released a part of hemoglobin (graded leakage) (Figure 7(a) and (b)).[48] A hemolysis assay using osmoprotectants such as polyethylene glycols (PEGs) suggested that the copolymers form nano-sized pores (1–2 nm in diameter) in RBC cell membranes, causing osmotic lysis of RBCs due to osmotic imbalance between the outer buffer solution and the cytoplasm of RBCs (Figure 7(c) and (d)). The bee venom toxin melittin also formed nano-pores (∼2 nm), followed by osmolysis of RBCs. Although the peptide and polymers have distinctive difference in their chemical structures, they seem to share the same underlying mechanism.

Cytotoxicity Assays

In addition to hemolysis, other standard cytotoxicity assays have been used for assessment of toxicity of antimicrobial polymers to mammalian cells, although the number of reports is limited. One assay method is to monitor a lease of lactate dehydrogenase (LDH) from the cytoplasm, which reports damage to the cell membrane.[67] The released LDH is quantified by spectroscopically monitoring product formation catalyzed by the enzymatic reaction rather than the

direct detection of LDH, which contrasts to the hemolysis assay. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been also used for cell viability.[69] This method detects the metabolic activity of cells using a substrate which

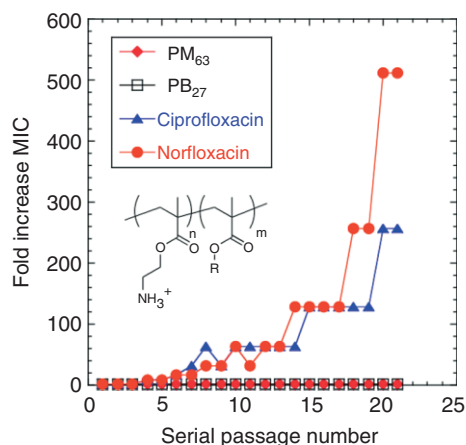**FIGURE 8** | Susceptibility of methacrylate copolymers and antibiotics to the development of resistance in *Escherichia coli*. The polymers are PB$_{27}$ (R = butyl, 27 mol. %) and PM$_{63}$ (R = methyl, 63 mol. %). (Reprinted with permission from Ref 49. Copyright 2011 MDPI)

produces distinctive color change when reduced by active components of normal cellular metabolism. Recently, a water-soluble version of substrate, XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium), has been used for the same assay to facilitate the procedure.[67] Combining these assay methods, we are able to characterize the cytotoxicity of polymers as well as giving insights into the mechanism of cytotoxicity. These assays have been utilized for cationic polymers.[69] In our recent report, antimicrobial branched PEIs with low MWs (MW = 500 and 1100) showed no release of LDH from human epithelial HEp-2 cells, but the cell viability was reduced significantly after 24-h incubation.[67] This indicates that PEIs do not damage the cell membranes, but may cause potential long-term toxicity to host cells. In the literature, the cytotoxicity of antimicrobial polymers has not been fully evaluated. It would be important to investigate the interaction between antimicrobial polymers and host cells toward clinical applications.

ANTIMICROBIAL RESISTANCE IN BACTERIA

Low susceptibility to the existing or emerging resistance mechanisms is one of the hallmarks of AMPs. Although the number of reports is limited, some antimicrobial peptidomimetics and polymers appear to be effective against strains already resistant to conventional antibiotics and do not contribute to the development of new antimicrobial resistance.[49,70] We have investigated the occurrence of resistance in *E. coli* using methacrylate copolymers (Figure 8).[49] Methacrylate copolymers at half the MIC were incubated with *E. coli*, and the solution was cultured overnight. Then the copolymers were tested against the bacterial cells, and MIC was determined. The bacteria were cultured again at half of the new MIC, and MIC was determined after overnight incubation. In this assay, if the bacteria develop resistance, the MIC increases and thus the amount of antibiotics increase at the subsequent culture. Therefore, this condition is likely to stimulate and accelerate the resistance development in bacteria. After 21 passages of this process, the MIC values of conventional antibiotics Ciprofloxacin and Norfloxacin increased 200- and 500-fold higher, indicating significant resistance development. On the other hand, the MIC values of methacrylate copolymers did not increase, indicating no resistance development under this condition. The bacteria incubated with the copolymers were susceptible to the antibiotics Norfloxacin and Ciprofloxacin, and the copolymers were active against bacteria

resistant to these antibiotics. This demonstrates that the copolymers do not contribute to cross-resistance development in bacteria.

FACTORS TO INFLUENCE ACTIVITY

It has been reported that environmental factors inhibit the activity of AMPs and polymers.[17] For the implementation of antimicrobial polymers in biomedical applications, it is important to evaluate the polymer activity in the physiological conditions and to understand the factors that affect the activity. These factors that affect efficacy can be widely variable, but a good starting point are the variables that relate to the mechanism of action of the polymers in question. In the case of the antimicrobial polymers described here, the polymer selectivity relies on electrostatic interactions while the membrane disruptive activity relies primarily on hydrophobic interactions. As such, the role of pH, ionic strength, and serum components that have some hydrophobic character are likely modulators of *in vivo* efficacy.

Solution pH

The polymers and peptides described have primary amine groups, which exhibits a pH dependence on ionization behavior. It has been demonstrated that solution pH changes polymer's antimicrobial activity (Figure 9). In the study on methacrylate copolymers, copolymers were tested for their activity against *E. coli* between pH 6 and 8.[44] The copolymers showed higher activity at higher pH. These data suggest that the copolymers are likely more hydrophobic due to the increased population of nonprotonated amine groups at higher pH, which are less hydrophilic (and hence more hydrophobic) than the charged versions of these moieties. It should be noted that uncharged primary
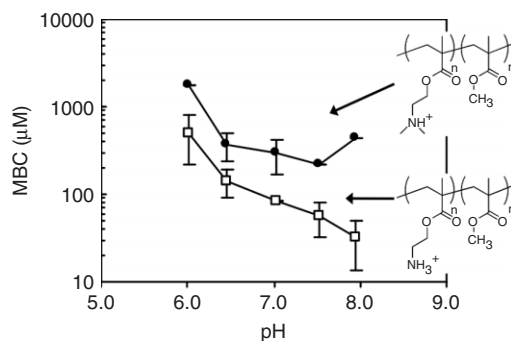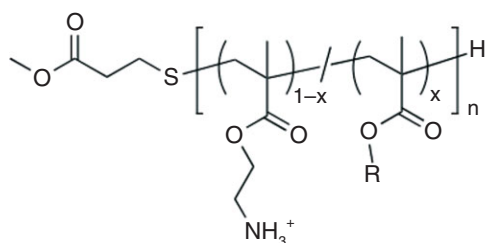
FIGURE 9 | pH effect on antimicrobial activity (MIC) of methacrylate copolymers with primary or tertiary ammonium side chains. (Reprinted with permission from Ref 44. Copyright 2009 American Chemical Society)
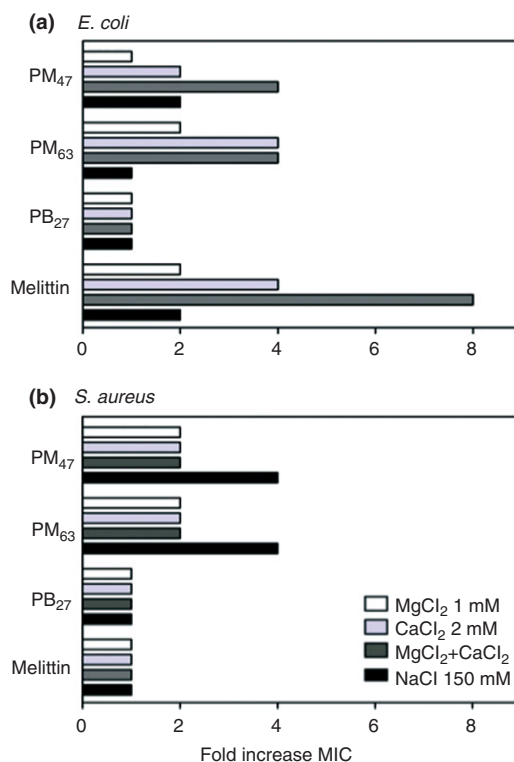
FIGURE 10 | Effect of physiological salts on the antibacterial activity of methacrylate copolymers (PB$_{27}$: 27% of butyl side chains, PM$_{63}$: 63% of methyl side chains) and melittin against *Escherichia coli* (a) and *Staphylococcus aureus* (b). (Reprinted with permission from Ref 49. Copyright 2011 MDPI)

amines are not truly hydrophobic unlike hydrophobic side chain groups (methyl) in comonomers, which are expected to actively interact with the hydrophobic domains of lipid membranes. The deprotonation of ammonium groups reduces the solvation (hydration) of polymers, resulting in a shift of amphiphilic balance of polymers to a more hydrophobic character and thus increased antimicrobial and hemolytic activities as the solution pH increases. This supports the notion that the hydrophobicity of copolymers increased, which dominate the electrostatic attraction between the copolymers and bacterial cell surfaces, causing nonselective binding to both cells. The number of cationic protonated ammonium groups decreases as pH increases, and the affinity of copolymers to bacterial cell surfaces could be reduced. However, it is evident that the overall hydrophobicity of copolymers is more effective to increase the antimicrobial activity.

It has been known that the ammonium groups are cationic sources for selective binding to anionic bacterial cell wall, resulting in selective toxicity to bacteria over human cells. What if the anime groups are always charged? To this end, methacrylate copolymers with primary, tertiary, and quaternary ammonium groups in the side chains were prepared

and their activity was measured.[44] The copolymers with primary ammonium groups showed highest activity against *E. coli*. The activity was further tested in different pH conditions. According to separated potentiometirc titrations, most of amine groups are protonated at pH 6, suggesting all polymers have the same number of cationic groups in a polymer chain. It is interesting that the copolymers with primary ammonium groups showed the highest activity against *E. coli* again. These results suggest that the amine groups are not only cationic sources, but they are acting in the lipid–polymer interactions. The further investigation by examining partition of copolymers between water and octanol suggested that the primary ammonium groups are likely to form complex with phosphate lipid heads through hydrogen bonding and electrostatic interaction (Figure 4).[45] This complex formation might increase the affinity of copolymers with primary ammonium groups for bacterial cells as well as possibly enhances the antimicrobial mechanism of membrane disruption or pore formation.

Ionic Strength and Divalent Ions
As polymer binding to bacteria relies on electrostatic interaction, high salt concentration (ionic strength)
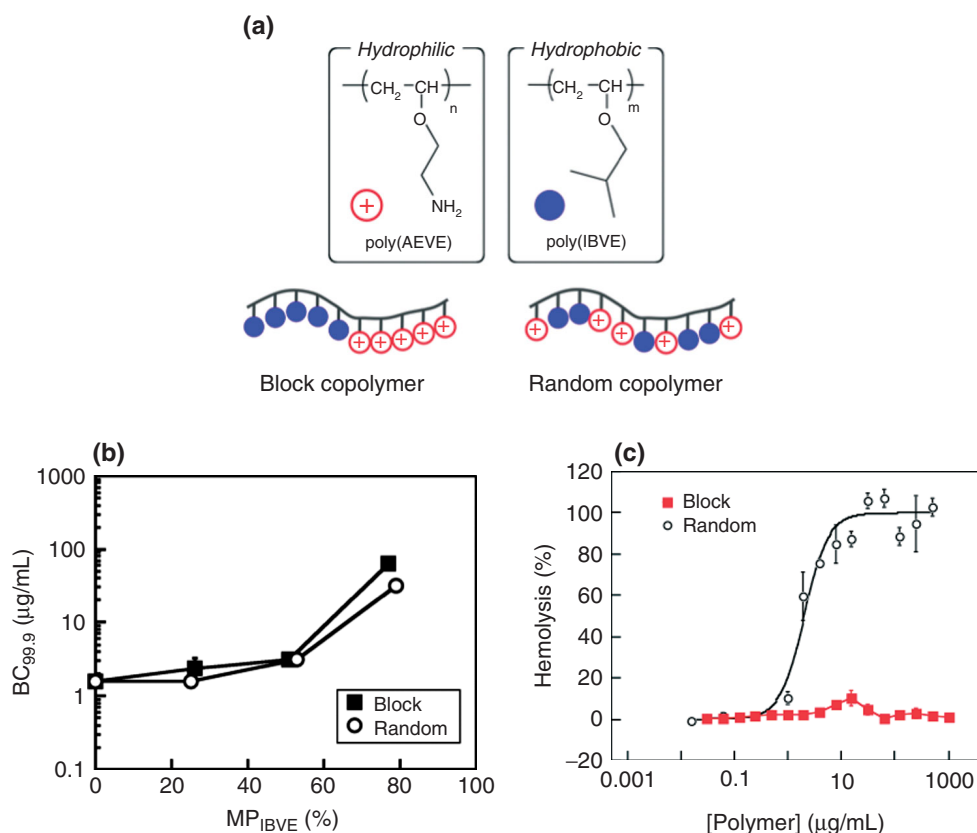
FIGURE 11 | Antimicrobial random and block copolymers. (a) Cationic amphiphilic block and random vinyl ether copolymers. (b) Biocidal activity of copolymers. BC$_{99.9}$ (biocidal concentration of polymers for 99.9% killing) is plotted as a function of MP$_{IBVE}$ (mol. % of IBVE units). (c) Hemolytic activity of block and random copolymers with ~25 mol. % of hydrophobic isobutyl vinyl ether (IBVE) units. (Reprinted with permission from Ref 40. Copyright 2012 American Chemical Society)

curtains the attractive interactions between polymers and bacteria, resulting in low affinity and low activity. The methacrylate copolymers showed twofold to fourfold increase in MIC against *E. coli* in Mueller Hinton (MH) broth with additional 150 mM NaCl (Figure 10).[49] Divalent cations Mg and Ca also reduce the activity of polymers.[49] The activity of methacrylate copolymers was twofold reduced in the presence of these cations (1 mM). These cations are structural ions, which stabilize the lipopolysaccharide (LPS) structures on the cell surfaces, preventing membrane disruption by the polymers. The high concentration of these cations may also reduce the replacement of cations in the LPS by the cationic groups of polymers, which prevent disintegration of membrane structure.

Serum Proteins

When AMPs are tested in the presence of serum, the peptides' activity is significantly reduced.[17,71,72] This has been attributed to enzymatic degradation of peptide chains (proteolysis), high ionic strength, and nonspecific binding to serum proteins such as bovine serum albumin (BSA). It seems that activity of polymers in serum or physiological fluids have not been evaluated to the same degree, and of those studies available, many focus on the interaction of serum components with surface-attached polymers and *not* soluble structures as described herein. Similar to the case of AMPs, the increased ionic strength of the serum environment may screen the electrostatic attractive interactions between the cationic groups on the polymers and the intended bacterial surface targets. Alternatively, the nonpolar moieties present in antimicrobial polymers may drive nonspecific hydrophobic clustering with larger serum proteins. In the case of antimicrobial polymers, a majority of compatibility testing has relied on simple protein adsorption assays in which a standard serum protein, such as BSA, is used to test for nonspecific interactions with the polymers.[73,74] The complex serum environment engenders the need for future investigation of these complexities that may modify the efficacy and selectivity of antimicrobial polymers.
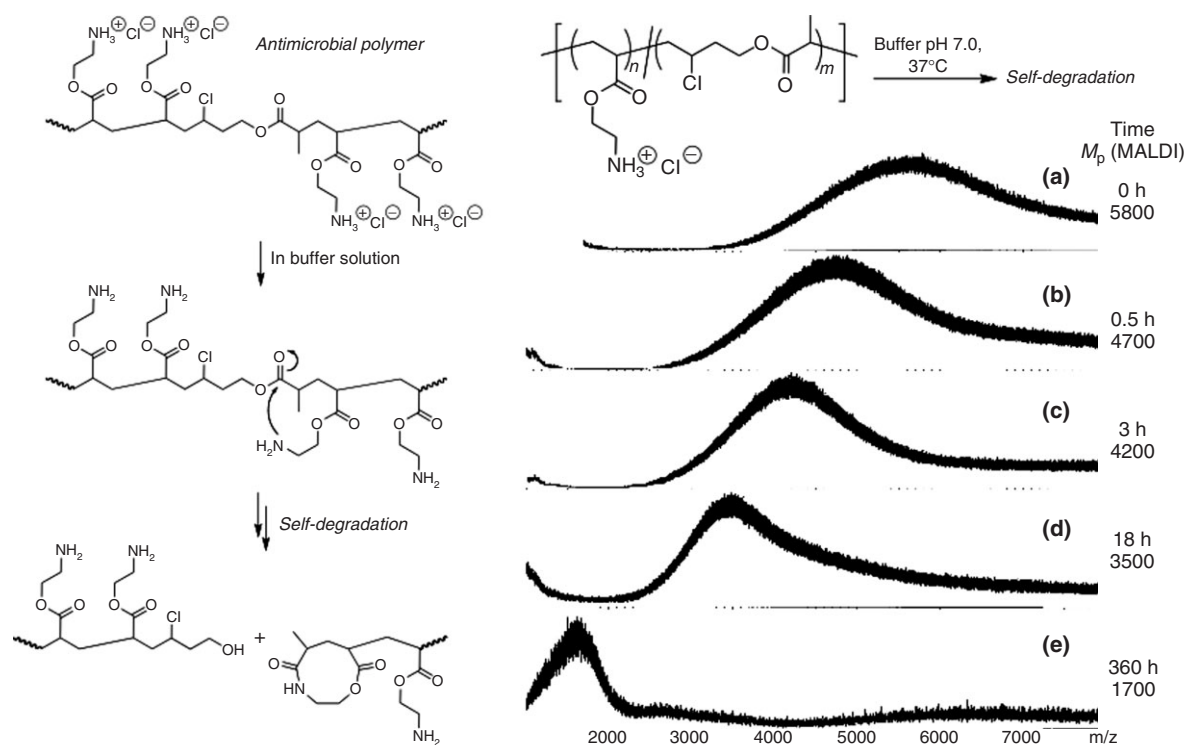
FIGURE 12 | Self-degrading copolymers. A polyester-based antimicrobial copolymer degraded into oligomers by self-degradation mechanism associated with amidation of ester linkages by primary ammonium side chains. (Reprinted with permission from Ref 43. Copyright 2012 American Chemical Society)

NEW DESIGN STRATEGIES FOR ANTIMICROBIAL POLYMERS

Amphiphilic Polymer Structures

Random copolymers have been traditionally used as a platform to generate antimicrobial polymers. The random copolymers may form conformations which display segregated or facially amphiphilic structures by separating cationic side chains and hydrophobic moieties in the membrane interface.[47,39] This mimics the amphiphilic structures of α-helical AMPs such as magainin and LL-37. On the other hand, antimicrobial and hemolytic activities of amphiphilic block copolymers have been investigated recently.[40,75] We have recently demonstrated that amphiphilic block copolymers are also good antimicrobial candidates with selective activity against bacteria over human cells (Figure 11).[40] Vinyl ether di-block copolymers with cationic and hydrophobic segments showed biocidal effect against *E. coli*, but cause little or no hemolysis. However, the random copolymers with the same ratio of cationic and hydrophobic moieties are highly hemolytic, although the polymers showed the same level of biocidal activity against *E. coli* as compared to the block copolymer counterparts. The block copolymers appear to form vesicles at

high concentrations. However, the block copolymers showed significant antimicrobial activity even below the critical micelle concentration of polymers, indicating the vesicle formation is not necessarily required for selective antimicrobial activity against *E. coli* over human RBCs. This suggests that the conformation of single polymer chains is responsible for the activity. In general, hydrophobic segments of amphiphilic copolymers aggregate in water and form hydrophobic domains which can be shielded from the aqueous milieu by the solvated hydrophilic segments, resulting in intramolecular or intermolecular micelle formation.[76] We speculated that the cationic segment of block copolymers wrapped around the hydrophobic segment, creating a low-energy structure in the aqueous environment, curtaining the hydrophobic domains and preventing nonspecific binding to human cells (see Ref 40 for the schematic presentation). This is a prevailing theory in the field regarding the structural conformations of hydrophobic/hydrophilic block copolymers.[76] On the other hand, the random copolymers would likely adopt a random-coil conformation because the hydrophobic groups are distributed along the polymer chains.[77] In these cases, many hydrophobic groups are likely located outside of any hydrophobic core and relatively exposed

to the solution, which can easily facilitate interaction with RBC cell membranes and cause hemolysis. This study provided a new insight into the important role which amphiphilic polymer structures play in their antimicrobial mechanism. In addition, it has been demonstrated that nanoparticles formed by amphiphilic block copolymers are effective against a broad spectrum of fungi and bacteria including a drug-resistant strain.[78,79] The molecular design for the next generation of antimicrobial polymers will be extended to include macromolecular structures and architectures that build on the increasing mechanistic understanding of both polymeric and peptide antimicrobials. These should take advantage of the known molecular components that contribute to specificity and efficacy, while additionally incorporating beneficial properties to aid in bioavailability and delivery.

Biodegradable Antimicrobial Copolymers

Antimicrobial polymers with synthetic biodegradable backbones including, for example, esters and carbonate groups have been reported.[78,80–82] Our research group has prepared polyester-based amphiphilic copolymers with cationic side chains by simultaneous chain- and step-growth radical polymerization (Figure 12).[43] The random copolymers contain primary ammonium groups in the side chains and ester linkages in the polymer backbone. The copolymers displayed antibacterial activity against *E. coli*, but degraded into oligomers at neutral pH, and the activity of copolymer decreased. The detailed investigation using control copolymers with acidic side chains instead of amine groups, we speculate that the primary amine groups in the side chains react with the ester groups of the polymer backbone, and the following amidation cleaves the polymer chains. This self-degradation mechanism will be useful for quick disintegration of polymer chains into inactive oligomers, avoiding potential toxicity after long-term exposure.

CONCLUSION

Antimicrobial polymers are potential candidates for antibiotics, which would be effective against drug-resistant bacteria and would not contribute to resistance development. This is likely because the polymers act in the cell membranes or cell walls as mimics of AMPs, although the molecular mechanism is not fully understood yet at this point. There are distinctive differences in their structures and conformations between AMPs and antimicrobial polymers. AMPs have homogeneous structures (defined sequence and molecular length) and are programmed to form a helix with regulated amphiphilicity (Figure 1). On the other hand, synthetic polymers are heterogeneous in terms of chemical structures (random sequences, MW distribution, and tacticity) and structural conformations (random coil). Despite these differences, antimicrobial polymers are effective against bacteria and selective to bacteria over human cells to the same degree as AMPs. This may indicate that the physicochemical properties of polymers and peptides are important for antimicrobial action rather than the structures while it remains still a question whether synthetic copolymers have the same molecular mechanism(s) of AMPs.

We envision that these polymers are most immediately useful for potential applications as topical antimicrobial agents including targets such as periodontal disease and dermatological applications. However, before this point, there remain many limitations and hurdles to overcome before moving to clinical applications. For example, there has been little investigation into potential side effects caused by the polymeric antimicrobials. While the selectivity for bacterial cells over mammalian cells is a positive indicator of compatibility, the long-term application of these molecules and their possible interactions with natural flora and/or interaction with complex physiological systems must be investigated. This is likely to be more involved than the peptidic counterparts as the potential for wildly different polymer backbones, compositions, and sizes will increase the need for individualized studies.

At this moment, there are only a few polymers identified as effective agents, and the translational studies of these polymers are still limited. However, the scientific reports on antimicrobial polymers have increased significantly over the last two decades, and new advanced chemical methodologies have been utilized to improve the polymer performance. Many different kinds of polymer structures have already been developed much like AMPs which also have large diversity in their chemical structures. This suggests that the AMP-mimetic design of polymers will continue to be versatile and applicable to numerous polymer types, which will in turn facilitate the implementation of antimicrobial polymers as a new generation of antibiotics.

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