Optimal Balance of Hydrophobic Content and Degree of Polymerization Results in a Potent Membrane-Targeting Antibacterial Polymer

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ABSTRACT: Globally, excessive use of antibiotics has drastically raised the resistance frequency of disease-causing microorganisms among humans, leading to a scarcity of efficient and biocompatible drugs. Antimicrobial polymers have emerged as a promising candidate to combat drug-resistance pathogens. Along with the amphiphilic balance, structural conformation and molecular weight ($M_n$) play an indispensable role in the antimicrobial potency and cytotoxic activity of polymers. Here, we synthesize cationic and amphiphilic methacrylamide random copolymers using free-radical copolymerization. The mole fraction of the hydrophobic groups is kept constant at approximately 20%, while the molecular weight (average number of linked polymeric units) is varied and the antibacterial and cytotoxic activities are studied. The chemical composition of the copolymers is characterized by $^1$H NMR spectroscopy. We observe that the average number of linked units in a polymer chain (i.e., molecular weight) significantly affects the polymer activity and selectivity. The antibacterial efficacy against both of the examined bacteria, *Escherichia coli* and *Staphylococcus aureus*, increases with increasing molecular weight. The bactericidal activity of polymers is confirmed by live/dead cell viability assay. Polymers with high molecular weight display high antibacterial activity, yet are highly cytotoxic even at $1 \times$ MIC. However, low-molecular-weight polymers are biocompatible while retaining antibacterial potency. Furthermore, no resistance acquisition is observed against the polymers in *E. coli* and *S. aureus*. A comprehensive analysis using confocal and scanning electron microscopy (SEM) techniques shows that the polymers target bacterial membranes, resulting in membrane permeabilization that leads to cell death.

1. INTRODUCTION

Multi-drug-resistant microorganisms threaten our ability for effective prevention and treatment of infectious diseases.1–3 They complicate medical procedures and surgical therapies, increasing the health-care costs.5–7 This situation has urged researchers to explore new materials that can efficiently and selectively kill pathogens as an alternative to conventional antibiotics by which pathogens develop resistance. Antimicrobial polymers represent a class of robust, functional, and biocompatible agents derived from the principles of host defense antimicrobial peptides (AMPs) coupled with inexpensive, facile polymer chemistry that have emerged as promising candidates to combat drug-resistant microbes.8–10 Though designing membrane-active polymer molecules that can specifically distinguish between bacterial cells and mammalian cell membranes is a challenging task due to the complex and dynamic nature of cell membranes, tailoring the polymer’s structural parameters would make it possible to reproduce the chemical functionality and physicochemical features of AMPs. The principal structural determinants regulating polymers’ antimicrobial potency and hemolytic behavior relevant to host defense peptides are the net cationic charge at physiological pH, the average number of linked units in a polymeric chain, and amphiphilic balance.11,12 Tuning of amphiphilic balance (the average number of hydrophobic and cationic residues present in a polymer chain) is crucial in designing highly selective antimicrobial polymers.13 Polymers with strong cationic groups, for example, bind to the anionic constituents of bacterial cell surfaces through electrostatic attraction, but are unlikely to be integrated into the membrane’s hydrophobic center, which restricts their action. Conversely, highly hydrophobic polymers make it possible to bind and penetrate the membranes of
human cells without the aid of electrostatic attraction, thus making them indiscriminately harmful to both human and microbial cells. When there is an appropriate balance of hydrophobic and cationic residues in antimicrobial polymers, they may specifically kill the bacteria without affecting human cells because of the selective attachment of polymers to bacterial cells.14–16

The mode of action and efficacy of the antimicrobial agents are regulated mainly by the physical and chemical properties of the active chemical molecule present in a macromolecular system. Thus, the structure, composition, and chemical moiety used to introduce hydrophobic and cationic units in the polymer chain dictate the biological properties of a polymer. Comprehensive optimization has been carried out in random polymers, dendrimers, and oligomer systems to achieve some selected compounds that demonstrate limited or no hemolytic activity with strong antimicrobial efficacy.17–21 Because of the diversity of these synthetic scaffolds in the context of conformation, sequence, and molecular size, they display a favorable activity profile. The benefits of synthetic polymers as antimicrobial agents include the incorporation of diverse functional groups, large-scale synthesis, low production cost, and reduced cytotoxicity.22,23 These antimicrobial polymers are usually short synthetic polymers with random monomer sequences that display the hallmarks of AMPs’ action, including fast bactericidal kinetics, a low chance of bacterial resistance, and a broad spectrum of activities.23

Antimicrobial polymers interact with the bacteria and their environment in multiple ways, including interaction with hydrophilic/hydrophobic groups, suspension of the movement of substances across the membrane, or membrane permeabilization, disrupting the integrity of the plasma membrane and resulting in cell lysis.24–26 Studies on antibacterial polymers27–29 with cationic and hydrophobic groups show that the cationic group of the polymer interacts with the anionic bacterial surface via electrostatic interaction, and the hydrophobic group of the polymer is inserted inside the hydrophobic core of the membrane, resulting in permeabilization and, eventually, cell lysis. The mechanism by which these polymers perform their function reduces the potential for bacterial resistance development. The structural architecture and molecular weight (MW) of the polymers play a significant role in their antimicrobial efficacy, apart from the amphiphilic balance that enables the permeation of polymers into the bacterial lipid bilayers.13,15,16 Regulating the polymers’ average molecular weight (MW) allows adjustment of the total number of bioactive molecules working collectively in each polymer unit.22,30 Nylon-3 polymers of low MW (1–4 kDa) showed high antimicrobial activity with reduced hemolysis.31–34 A study on random methacrylate copolymers showed that increasing the molecular weight (MW) results in decreased cell-type selectivity.35,36 Various researchers have examined the role of polymer characteristics in their antimicrobial efficacy.14,15,37–44 However, it is quite arduous to develop a universal correlation because antimicrobial efficiency depends on many factors, including nature and the number of functional groups present in a polymer, polymer structure/architect, composition, and the type of bacterial strain studied. Therefore, a judicious design and synthesis of well-defined polymer molecules is essential to provide a comprehensive understanding of the polymer structure/activity relationship.

We previously explored the structural parameters that affect polymer activity and selectivity by systematically varying the mole fraction of hydrophilic/hydrophobic groups (amphiphilic balance) in methacrylamide-based polymers, allowing us to obtain an optimum polymer composition suitable for use as nontoxic antimicrobials.29 The innovative feature of this research was the use of an aromatic benzyl group to incorporate hydrophobicity in the methacrylamide-based scaffold. We observed that the aromatic benzyl units influenced polymer activity and selectivity. The polymer with approximately 20% hydrophobic benzylmethacrylamide units was nonhemolytic and displayed high antibacterial efficacy against both of the examined bacteria (Escherichia coli and Staphylococcus aureus). These outcomes motivated the current research of investigating the role of polymer molecular weight (an average number of linked polymeric units), while keeping the hydrophobic groups constant (~20%), in regulating antibacterial and cytotoxic activities. The minimum inhibitory concentration values are determined to find out the polymers’ antibacterial activity against S. aureus and E. coli using broth microdilution assay. Polymer toxicity is examined using human juvenile foreskin fibroblast cells via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results show that the average number of linked units in a polymer chain i.e., average molecular weight (Mn), significantly influences antibacterial and cytotoxic activities. The antibacterial efficacy increases as the average molecular weight of the polymers increases. However, polymers having Mn ≥ 5 kDa are highly cytotoxic. Polymers with a degree of polymerization (DP) of 17 and 27 display the best combination of high antibacterial efficiency with low cytotoxicity. Both these polymers display bactericidal activity and attack the bacterial membrane, as evident by confocal and scanning electron microscopy (SEM) experiments. In addition, a serial passage study shows no observable resistance in E. coli and S. aureus.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization. By way of free-radical copolymerization, the polymer molecular weight (i.e., the average number of linked polymeric units) was varied by keeping the hydrophobic groups constant and selecting suitable initial monomer concentrations as described in the

![Figure 1. Synthesis of amphiphilic, cationic methacrylamide random polymers with varying average molecular weight, keeping the hydrophobicity constant (~20%). AIBN: 2,2’-azobis(2-methylpropionitrile); MMP: methyl mercaptopropionate; MeOH: methanol; EtOH: ethanol.](image-url)
experimental section (Figure 1). Aminopropyl methacrylamide (APMA) induces hydrophilicity as it remains protonated at physiological pH, thereby supplying cationic charge, while benzylmethacrylamide (BMA) is chosen for providing hydrophobicity due to the presence of the aromatic hydrophobic benzyl group.

A series of random copolymers are synthesized by controlling the molecular weight ranging from 1.9 to 11.8 kDa to determine the structure–activity relationship, keeping the hydrophobic groups constant (~20%). The DP values are in the range of 10–66 repeat units. All of the polymers are characterized by their proton NMR spectra using a Bruker-500 MHz NMR spectrometer (Figure 2 shows the representative spectrum). Monomer purity is confirmed by taking 1H NMR spectra for aminopropyl methacrylamide in D$_2$O and benzylmethacrylamide in CDCl$_3$. Table 1 represents the molecular characteristics of the synthesized random copolymers. The polymers synthesized here have a well-defined end-group structure, and the resonance signals from different monomers can be differentiated clearly; therefore, the chemical composition, degree of polymerization (DP), and average molecular weight ($M_n$) were determined by integrating the proton NMR peaks using end-group analysis.

Table 1. Characterization of Cationic Amphiphilic Random Copolymers

| polymer    | N1 | N2 | DP | $M_n$ (kDa) |
|------------|----|----|----|-------------|
| (AB-20)$_{10}$ | 0.20 | 0.8 | 10  | 1.9         |
| (AB-20)$_{17}$ | 0.23 | 0.76 | 17  | 3.1         |
| (AB-20)$_{27}$ | 0.19 | 0.81 | 27  | 4.9         |
| (AB-20)$_{49}$ | 0.22 | 0.78 | 49  | 8.8         |
| (AB-20)$_{66}$ | 0.21 | 0.79 | 66  | 11.8        |

* N1 (mole ratio of BMA). * N2 (mole ratio of APMA). * DP (degree of polymerization). * $M_n$ (average molecular weight) values were determined by integrating the proton NMR peaks using end-group analysis.

All synthesized random copolymers were assigned AB nomenclature, which stands for poly[(APMA)-ran-(BMA)]: for example, (AB-20)$_{10}$, where the number within the brackets indicates the mole percent of the hydrophobic group and the subscript number indicates the average number of linked polymeric units, i.e., DP.

2.2. Antibacterial Activity. The antibacterial efficacy of polymers was investigated against E. coli and S. aureus, responsible for various nosocomial and community-acquired infections. The minimum inhibitory concentration of polymers (MIC) was determined spectrophotometrically and was identified as the lowest concentration of polymers that prevents bacteria from growing. All of the synthesized random copolymers show activity against both the tested bacteria. The results are outlined in Table 2.

| polymer    | MIC (µM) | E. coli | S. aureus |
|------------|----------|---------|-----------|
| (AB-20)$_{10}$ | 25 ± 0.002 | 28 ± 0.12 |
| (AB-20)$_{17}$ | 12 ± 0.001 | 14 ± 0.05 |
| (AB-20)$_{27}$ | 6.5 ± 0.004 | 6.5 ± 0.018 |
| (AB-20)$_{49}$ | 5.2 ± 0.008 | 5.9 ± 0.02 |
| (AB-20)$_{66}$ | 2.7 ± 0.005 | 3.8 ± 0.07 |

* MIC is the minimum inhibitory concentration of polymers that completely prevents bacterial growth. MIC values are reported in units of µM. All data are represented as the mean of three replicates ± standard deviation (SD).

The molecular weight of the polymers had a profound effect on the antibacterial efficacy of both the examined bacteria. The MIC values decrease with increasing molecular weight, thereby signifying a potent antibacterial efficacy with high-MW polymers, as shown in Figure 3. For example, (AB-20)$_{10}$ with 1.9 kDa has MIC values of 25 ± 0.002 µM for E. coli and 28 ± 0.12 µM for S. aureus. However, (AB-20)$_{66}$ having 11.8 kDa...
shows MIC values of 2.7 ± 0.005 μM for E. coli and 3.8 ± 0.07 μM for S. aureus. The relative antibacterial efficacy using the average number of linked units in a polymer chain varying from 10 to 66 was (AB-20)_{66} > (AB-20)_{44} > (AB-20)_{27} > (AB-20)_{17} > (AB-20)_{10}. The possible explanation for the observed trend in MIC values could be the presence of a large number of lysine-mimicking cationic hydrophilic groups in high-MW (≥5 kDa) polymers that interact strongly with the anionic bacterial membrane via electrostatic interaction compared to the low-MW (≤5) polymers.

2.3. Cytotoxicity Study. The cytotoxicity of polymers to human juvenile fibroblast cells was studied using a colorimetric MTT assay. The principle is based on the action of the mitochondrial reductase enzyme that converts the soluble MTT tetrazolium dye into the insoluble purple formazan crystal. The formazan crystals thus formed were solubilized in dimethyl sulfoxide (DMSO). The absorbance value of the dissolved solution is measured spectrophotometrically.

Cytotoxicity was measured by the decrease in oxidoreductase enzyme activity of the metabolically active cells. The growth of cells did not alter the results; therefore, cells were grown in serum-free media when treated with a polymer, and thus, after 24 h of exposure, less than 75% of cell confluence was observed. The metabolic rate of the cells was greatly influenced by the molecular weight of the polymers. The high-MW polymers reduced the metabolic activity of the cells, as shown in Figure 4. (AB-20)_{10} (AB-20)_{17}, and (AB-20)_{27} polymers showed less than 5–10% of decline in metabolic activity at 1 × and 8 × MICs. However, with (AB-20)_{44} and (AB-20)_{66} polymers at 1 × MIC, approximately 70% reduction in metabolic activity was observed. These findings suggest that polymers with high molecular weight are extremely cytotoxic due to the presence of a large number of cationic units in (AB-20)_{44} and (AB-20)_{66} polymers, which corroborates with the literature that the cytotoxicity of polycationic compounds depends on the molecular weight. Furthermore, high-MW polymers contain more protonated units, which catalyze the acid hydrolysis of the ester linkage found in the phospholipid chains, forming single-chain lipids and fatty acids. Then, single-chain lipids quickly destabilize the membrane, leading to cytotoxicity.

These results show that the polymers (AB-20)_{17} and (AB-20)_{27} are most suitable, as they show high antimicrobial activity in combination with low cytotoxicity. Therefore, we choose these two polymers to carry out further studies.

2.4. Live/Dead Assay. Antimicrobial agents conduct their activities in two ways, either by inhibiting bacterial growth or by killing cells. Agents that prevent bacterial growth are bacteriostatic, whereas those that destroy bacteria are bactericidal. Polymer activity and membrane permeability assay were done by performing a viability assay using the commercially available LIVE/DEAD BacLight kit. The kit comprises two dyes: green-fluorescent SYTO-9, which enters the nucleic acid of every cell, and red-fluorescent propidium iodide (PI), which enters exclusively into those cells with an impaired membrane. E. coli and S. aureus cells exhibiting green fluorescence signals indicate live cells (Figure 5a-i, a-ii, a-iii, d-i, d-ii, and d-iii), while (AB-20)_{17} and (AB-20)_{27} polymer-treated cells generate red fluorescence signals, suggesting damaged and dead cells (Figure 5b-i, b-ii, b-iii, c-i, c-ii, c-iii, e-i, e-ii, e-iii, f-i, f-ii, and f-iii). These observations demonstrate that the polymer showed a bactericidal activity. The observed bactericidal activity is due to the structural disturbance in the bacterial morphology, which is compatible with the SEM results.

The commercially available Live/dead bacterial viability assay kit is used for membrane permeability assay. The procedure employs two intercalating dyes: propidium iodide (PI), a red fluorescing membrane-impermeable dye, and SYTO-9, a green fluorescing membrane-permeable dye. SYTO-9 can access the nucleic acids of all cells, which results in green fluorescence, while propidium iodide penetrates only compromised or damaged membranes and substitutes SYTO-9 from the nucleic acid of the compromised membrane, resulting in red fluorescence due to change in emission properties of the dye.

The LIVE/DEAD confocal microscopic assessment is simplified to differentiate between normal healthy cells and cells with impaired membrane.
Figure 5. Confocal laser scanning microscopy (CLSM) images of *E. coli* (first, second, and third panels from a-i to c-iii) and *S. aureus* (fourth, fifth, and sixth panels from d-i to f-iii) stained with SYTO-9/propidium iodide provided with a Live/Dead cell viability kit. Green dots indicate live cells with an intact membrane, while red dots indicate dead cells with a compromised membrane. The first and fourth panels show control cells without polymer treatment: *E. coli* in a-i, a-ii, and a-iii, and *S. aureus* in shown in d-i, d-ii, and d-iii. The second, third, fifth, and sixth panels show (AB-20)$_{17}$ and (AB-20)$_{27}$ polymer-treated cells: *E. coli* in b-i, b-ii, b-iii, c-i, c-ii, and c-iii, and *S. aureus* in e-i, e-ii, e-iii, f-i, f-ii, and f-iii. Fiji ImageJ software is used to analyze all CLSM images. Scale bar = 5 μm.
2.5. Scanning Electron Microscopy (SEM) Study. The traditional scanning electron microscopy (SEM) is used to analyze the characteristic morphological modifications induced by the antimicrobial action of the synthesized polymer on the bacterial membrane’s surface. SEM micrographs provide direct visualization of the cells before and after AB polymer treatment. *E. coli* and *S. aureus* cells were treated with *(AB-20)_{17}* and *(AB-20)_{27}* polymers, and samples were taken and
prepared at various time intervals of polymer incubation, i.e., 1, 2, and then 4 h. Control cells of \textit{E. coli} and \textit{S. aureus} without polymer treatment present a bright, smooth, and undamaged membrane surface, as depicted by the SEM micrographs (Figure 6a-i, b-i, c-i, a-ii, b-ii, and c-ii). \((\text{AB-20})_{17}\) and \((\text{AB-20})_{27}\) polymer-treated \textit{E. coli} cells show a roughed wrinkled membrane, with some blebs at the surface after 1 h of incubation. However, after 2 h of incubation, the intensity of the blebs increases, and multiple blebs of various shapes and sizes are observed. A completely collapsed membrane was seen in the SEM micrographs after increasing the incubation time for 4 h, as evident in Figure 6f-i and i-i. SEM micrographs of \textit{S. aureus} cells displayed numerous protrusions of discrete blebs at the membrane surface. However, aggregation of \textit{S. aureus} cells takes place when the incubation time of the polymer treatment is increased to 4 h. Aggregated cells showed damaged cell walls leading to cellular collapse, as depicted in Figure 6f-ii and i-ii.

Figure 7A shows control and \((\text{AB-20})_{17}\) polymer-treated \textit{E. coli} and \textit{S. aureus} cells in higher magnification to visualize the effect of a polymer at the surface of the bacterial membrane after 2 h of polymer incubation. These defects caused by polymers at the surface of the bacteria demonstrate a similar mechanism as displayed by membrane-active antimicrobial agents, including antibiotics and antimicrobial peptides.

Furthermore, a control experiment was carried out, where negatively charged silica particles and AB polymer were incubated for 2 h and then viewed using scanning electron microscopy. SEM images of silica treated with polymer indicate that there is no coating of polymer on the silica particles (Figure 7B-ii). This experiment suggests that the blebs formed at the surface of the bacterial membrane are due to the polymer interacting with the membrane rather than it coating the cell surface.

In summary, we conclude that AB polymers are capable of causing significant characteristic modifications to the bacterial membrane surface that influence the overall morphology of both the examined bacteria, as shown by SEM micrographs leading to membrane disruption accompanied by leakage of intracellular contents such as RNA, DNA, or proteins, resulting in cell death.

2.6. Resistance Study. \((\text{AB-20})_{17}\) and \((\text{AB-20})_{27}\) polymers are chosen for resistance study. Susceptible strains of \textit{E. coli}
and *S. aureus* were serially passaged at subinhibitory polymer concentration and determined any change in MIC values. Ciprofloxacin, belonging to the fluoroquinolone class of antibiotics, was used as a control. No incremental change in MIC values was observed with (AB-20)17 and (AB-20)27 polymers in 25 and 30 days of serial passage (Figure 8). However, with ciprofloxacin, a 256-fold change in the MIC values was observed with *E. coli* and 64-fold with *S. aureus* (Table 3). This reduction in susceptibility correlates to a high level of resistance development with ciprofloxacin. In contrast, no resistant mutants were observed with (AB-20)17 and (AB-20)27 polymers against both the examined bacteria. These findings are of particular importance due to the emergence of resistance to conventional antibiotics in these bacteria.

3. CONCLUSIONS

The role of the average molecular weight of cationic random methacrylamide polymers in regulating the antibacterial efficacy and cytotoxic activity was investigated. Antibacterial susceptibility and cell viability assays explicitly demonstrated that MIC values and cytotoxicity are directly dependent on the average number of linked units present in a polymer chain, i.e., the average molecular weight. Higher-MW polymers having more cationic groups are highly toxic. The integrity of the cell membrane is compromised by these polymers, resulting in cell death (bactericidal activity), as exhibited by the confocal study. Our observations support the membrane-targeting mechanism as the first antibacterial effect on the cells by these polymers, predicted by protrusion and blebs formation on the surface of *S. aureus* and *E. coli* as viewed by SEM micrographs. (AB-20)17

| bacteria strain | antibacterial agent | number of passages | MIC (μM) | fold change in MIC |
|----------------|---------------------|--------------------|----------|--------------------|
| *E. coli*      | (AB-20)17           | 25                 | 0.188    | 48.28              | 256 |
|                | ciprofloxacin       | 25                 | 12       | 12                 | no resistance |
|                | (AB-20)27           | 30                 | 6.5      | 6.5                | no resistance |
|                | ciprofloxacin       | 30                 | 0.09     | 24.1               | 256 |
| *S. aureus*    | (AB-20)17           | 25                 | 0.75     | 48.28              | 64  |
|                | ciprofloxacin       | 25                 | 14       | 14                 | no resistance |
|                | (AB-20)27           | 30                 | 6.5      | 6.5                | no resistance |
|                | ciprofloxacin       | 30                 | 0.75     | 24.1               | 32  |

A 4-fold increase in MIC from the original MIC value is considered resistance development.
and (AB-20)$_{17}$ polymers can be developed as bio compatible antibacterial agents with MIC values ranging from 6.5 to 14 μM.

4. MATERIALS AND METHODS

4.1. Materials. N-Benzylmethacrylamide (Polysciences, Inc. Warrington), N-(3-aminopropyl) methacrylamide hydrochloride >98% (Polysciences, Inc. Warrington), methyl 3-mercaptopropionate (MMP, Sigma-Aldrich), 2,2′-azobisobutyronitrile (AIBN, Rankem), ethanol (Advent Chembio Pvt. Ltd., India), methanol (Sigma-Aldrich), benzoylated dialysis tubing (Sigma-Aldrich), and deuterium oxide (D$_2$O, Sigma-Aldrich). All chemical reagents were analytical grade and were used as purchased.

S. aureus (ATCC 25923; Gram-positive bacteria) and E. coli (ATCC 25922; Gram-negative bacteria) were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, (ATCC 25922; Gram-negative bacteria) were used as purchased.

Aldrich), Corning 96-well plates (polypropylene, non-treated, fl)

butyronitrile (AIBN, Rankem), ethanol (Advent Chembio Pvt.

6. Hydrophobic benzyl units is kept constant (0.2) to determine the impact of molecular weight on the biological activities.

4.3. Antimicrobial Susceptibility Assay. The antibacterial activity of polymers was determined using a standard microdilution assay by finding the minimum inhibitory concentration (MIC) following CLSI guidelines with some modifications proposed by Hancock, particularly for cationic antimicrobial agents. Bacterial glycerol stock was plated on sterile agar plates and incubated for 24 h at 37 °C.

The next day, an individual colony was picked from the agar plate and transferred into 10 mL of Muller–Hinton (MH) broth followed by overnight incubation at 37 °C under shaking conditions. The overnight bacterial inoculum was 100-fold diluted in MH broth and incubated for 2–3 h until the mid-logarithmic stage was reached. Mid-logarithmic bacterial cells were appropriately diluted to 10$^6$ CFU/mL in MH broth and mixed with polymer stock solution (prepared in aqueous 0.001% acetic acid) to a final volume of 100 μL in an individual well of a 96-well polystyrene microtiter plate (Corning no. 3379). MIC plates were incubated overnight at 37 °C, followed by optical density (OD) reading at 630 nm. A Biotek microplate reader was used to record the optical density (OD). Negative control comprises the Muller–Hinton broth, whereas positive control comprises bacterial cells without polymer treatment. For each experiment, biological replicates are used, and each experiment runs in triplicates, which correspond to the polymer solubility limits in the MHB. The rate of bacterial survival was calculated using the formula

\[
\text{bacteria survival rate (\%) } = \left( \frac{\text{OD}_{\text{test sample}} - \text{OD}_{\text{negative control}}}{\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}} \right) \times 100
\]

4.4. Cell Culture. Human fibroblast cells were grown in DMEM media supplied with 1% antibiotic solution consisting of penicillin and streptomycin and 10% fetal bovine serum (FBS) in a humidifying environment at 37 °C with 5 percent of carbon dioxide. Trypsinization of cells was done with 0.05% trypsin-EDTA, after obtaining ~80% confluence, and counted using a hemocytometer.

4.5. Cytotoxicity Assay. The MTT assay is used to assess in vitro cytototoxicity using human juvenile fibroblast cells. A 96-well cell culture plate is used to seed the cells (10$^5$ cells/well). Growth media were replaced after obtaining 60–70% cell confluence with serum and antibiotic-free DMEM media consisting of varying polymer concentrations. Positive control consists of Triton-X-100, while negative control consists of PBS buffer. The plate was incubated at 37 °C for 24 h in a CO$_2$ incubator. After 24 h of polymer exposure, the old media was replaced with fresh media from the culture plate. Sterile filtered MTT stock solution (5 mg per mL in PBS buffer; 10 μL/well) was added to each well, achieving a final 0.45 mg/mL MTT concentration, followed by incubating the plate at 37 °C for 4 h in an incubator (supplied with 5 percent CO$_2$). Hundred microliters of the solubilizing agent, dimethyl sulfoxide, was added to each well after removing old media to dissolve the formazan crystals. All experiments were carried out in triplicate. A Biotek cytation 5 plate reader was used to record
the absorbance reading at 570 nm. The following formula is used to calculate the percentage of cell viability:

\[
\text{cell viability (\%)} = \left( \frac{\text{OD}_{570} \text{(test sample)} - \text{OD}_{570} \text{(negative control)}}{\text{OD}_{570} \text{(positive control)} - \text{OD}_{570} \text{(negative control)}} \right) \times 100
\]

(2)

where \( \text{OD}_{570} \) = absorbance at 570 nm, (test sample) is the average of the observed results of 3 wells, (negative control) is the average of 8 control wells with PBS buffer, and (positive control) is the average of 8 control wells lysed with 3% Triton-X 100.

4.6. Scanning Electron Microscopy (SEM). For microscopic evaluation, a high cell density is required (10^9–10^10 CFU/mL). The MIC values for high cell density are therefore determined together with the usual cell density of 10^6 CFU/mL. Mid-logarithmic-phase bacteria were appropriately diluted to the desired cell density, followed by centrifugation and PBS washing. The cell suspension was treated with (AB-20)_{17} and (AB-20)_{27} polymers and incubated at 37 °C. Control cells consist of bacteria without the treatment of polymer. After 1 h, 2 h, and 4 h, the cells were taken out from the control and polymer-treated samples, centrifuged, and washed with PBS buffer, followed by fixation with 2.5% glutaraldehyde overnight. The next day, cells were resuspended in water after washing with water. Graded ethanol series, i.e., 25, 45, 65, 85, 90, and 100%, were used to dehydrate the cells. Samples were coated with a small amount of platinum to prevent charging in a scanning microscope (CLSM), TCS SP8, Leica, using FS), and IIT Gandhinagar, India.

4.7. Bacterial Viability Assay Using a Live/Dead Kit. The effect of the polymer on bacterial cells is studied using a live/dead viability kit. The kit consists of two vials, A and B, with different concentrations of propidium iodide (PI) and SYTO-9 stains. S. aureus and E. coli cells in the mid-exponential phase were cultured and diluted with appropriate cell density in the Muller–Hinton broth pelleted by centrifugation and suspended in 0.9% salt solution. (AB-20)_{17} and (AB-20)_{27} polymers were incubated with bacteria for 4 h at 37 °C. Bacteria without polymer treatment serve as the control. Control and polymer-treated cells were centrifuged and resuspended in 0.9% salt solution. Then, 1 μL of stain from each vial (A and vial B) was added to the cell suspension (1 mL), followed by 15 min incubation at room temperature. Dyes are sensitive to light; therefore, an aluminum foil is used to protect the samples from light. At the center of the microscope slide, 5 μL of cell suspension was mounted between the coverslips and examined by a confocal laser scanning microscope (CLSM), TCS SP8, Leica, using appropriate filters as specified by the manufacturer.

4.8. Resistance Study. E. coli and S. aureus overnight cultures were appropriately diluted and grown with (AB-20)_{17} and (AB-20)_{27} polymers' subinhibitory MIC concentrations for developing resistant mutants by sequential passaging. The clinically used antibiotic Ciprofloxacin is used as a control. After 24 h of incubation, the bacterial inoculum with the highest polymer concentration (below MIC value) was 100-fold diluted with sterile media and added to tubes containing sub and supra 2-fold MIC concentrations of polymer. This procedure was performed every day for 25 and 30 days. Agar plates were used to passage cultures that displayed high MIC values, followed by their MIC determination.

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Notes

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