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Broad Spectrum Antibacterial and Antifungal Polymeric Paint Materials: Synthesis, Structure–Activity Relationship, and Membrane-Active Mode of Action

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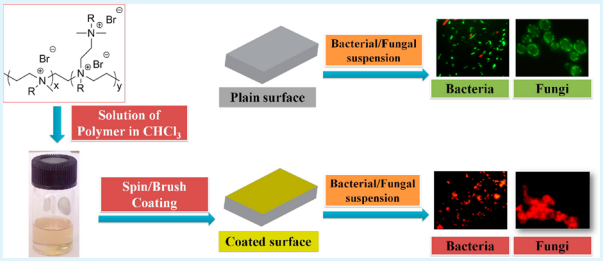
Supporting Information

ABSTRACT: Microbial attachment and subsequent colonization onto surfaces lead to the spread of deadly community-acquired and hospital-acquired (nosocomial) infections. Noncovalent immobilization of water insoluble and organo-soluble cationic polymers onto a surface is a facile approach to prevent microbial contamination. In the present study, we described the synthesis of water insoluble and organo-soluble polymeric materials and demonstrated their structure–activity relationship against various human pathogenic bacteria including drug-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and beta lactam-resistant *Klebsiella pneumoniae* as well as pathogenic fungi such as *Candida* spp. and *Cryptococcus* spp. The polymer coated surfaces completely inactivated both bacteria and fungi upon contact (5 log reduction with respect to control). Linear polymers were more active and found to have a higher killing rate than the branched polymers. The polymer coated surfaces also exhibited significant activity in various complex mammalian fluids such as serum, plasma, and blood and showed negligible hemolysis at an amount much higher than minimum inhibitory amounts (MIAs). These polymers were found to have excellent compatibility with other medically relevant polymers (polylactic acid, PLA) and commercial paint. The cationic hydrophobic polymer coatings disrupted the lipid membrane of both bacteria and fungi and thus showed a membrane-active mode of action. Further, bacteria did not develop resistance against these membrane-active polymers in sharp contrast to conventional antibiotics and lipopeptides, thus the polymers hold great promise to be used as coating materials for developing permanent antimicrobial paint.



KEYWORDS: *microbicidal paint, antibacterial activity, antifungal activity, contact-based noncovalent antimicrobial coating, bacterial resistance, water-insoluble and organo-soluble polymers, membrane-active mode of action*

1. INTRODUCTION

Infectious diseases are responsible for 10 million deaths representing almost 20% of all fatalities worldwide, and it is estimated that approximately 80% of the human infections occur from microbe-contaminated surfaces.[1] Attachment and subsequent colonization of bacteria and fungi onto surfaces of biomaterials/common objects lead to the spread of deadly community-acquired and hospital-acquired (nosocomial) infections.[2−4] In addition, growing microbial resistance with a diminishing antibiotic pipeline poses serious problems in many household and biomedical applications.[5] Thus, there is an ever-growing demand to prevent infections caused by the microorganisms.[6−8] The first step toward preventing these infections is inhibiting microbial colonization on surfaces. The use of water-soluble antimicrobial compounds to prevent infections leads to rapid emergence of resistant strains and causes environmental problems.[9−13] Hence, there is an urgent need for materials that can kill pathogenic microorganism, e.g., bacteria and fungi, but stay bound to surfaces and are thus less likely to lead to the development of microbe resistance.[14−16]

Cationic antimicrobials are well-known for the development of self-sterilizing surfaces and used for a number of applications such as at hospital surfaces, surgical equipment, protective clothes in hospitals, medical implants, wound dressings, food packaging materials, and everyday consumer products.[17−21] Among the most commonly used cationic antimicrobials, cationic polymers with quaternary ammonium groups show great promise in the field of antimicrobial coatings.[22−26] In order to achieve this, a number of polycations possessing antimicrobial properties, such as *N*-alkylated poly(4-vinyl-

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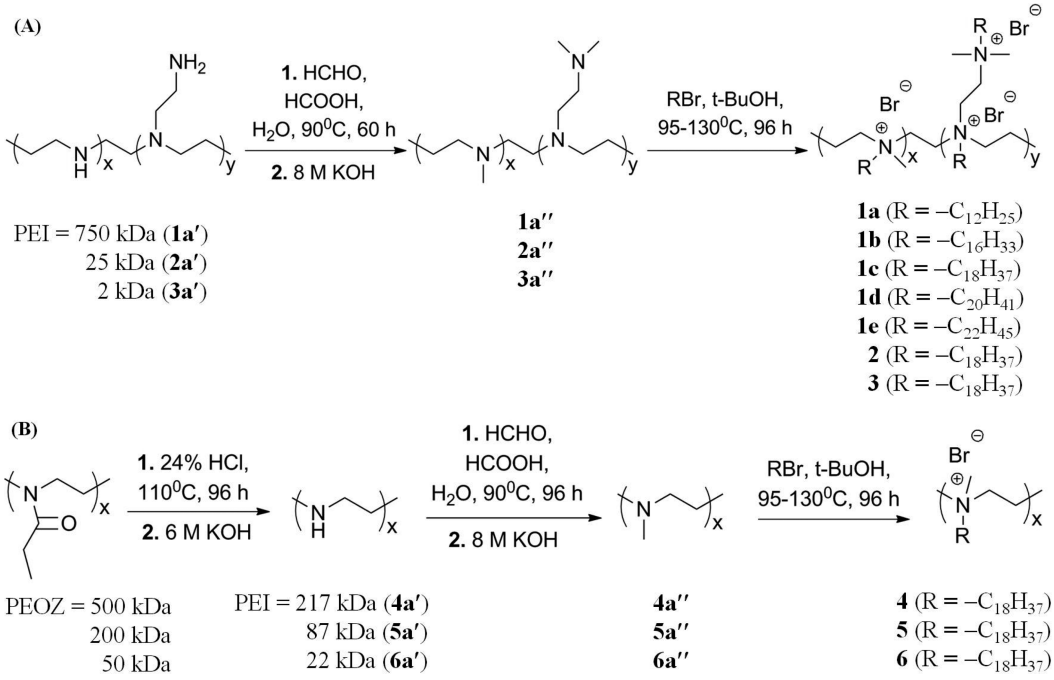
Scheme 1. Synthesis of Water Insoluble and Organo-Soluble PEI Derivatives[a]



[a](A) Synthesis of branched *N*-alkyl-*N*-methyl PEIs by *E. Clarke* methylation of branched PEIs and subsequent quaternization with alkyl bromide. (B) Synthesis of linear *N*-alkyl, *N*-methyl PEIs by deprotection of PEOZs, deprotonation of PEIs, *N*-methylation of deprotonated PEIs, and subsequent quaternization of *N*-methyl PEIs with alkyl bromide.

pyridine), quaternized polyethylenimine, quaternary derivatives of acrylic acid, poly(ester-carbonate), cellulose, etc., were applied covalently or noncovalently onto the surfaces.[27−31] However, covalent immobilization requires different chemical modification strategies for different surfaces. The harsh reaction conditions required for such modifications may alter the surface properties of the biomaterials/common objects such as mechanical properties, transparency, etc. Further, the requirement of multiple synthetic steps in this method limits its practical usage.[32,33] To overcome these difficulties, water insoluble and organo-soluble cationic antimicrobial polymers have been developed, and the solutions of these polymers in suitable organic solvents are coated onto surfaces in the form of paint.[34−36] The noncovalent strategy to create "microbicidal paint" is thus facile, straightforward, and easy to apply.

Despite evidence of antimicrobial activity of polymers coated noncovalently, there are no clear-cut reports on the structure−activity relationship of the quaternary polymers used for antimicrobial coatings. It is therefore necessary to understand the structure−activity relationship and mechanism of action of the polymers at the molecular level. Also, microbial resistance, one of the major threats at the present time, is yet to be understood against the water-insoluble and organo-soluble antimicrobial polymers. Further, the antimicrobial activity of cationic polymers has been mostly investigated against bacteria although the fungal infections remain as one of the key hazards to human health. Moreover, fungal biofilms are known to allow the formation of bacterial colonies on their surface and even lead to enhanced bacterial resistance to antibiotics.[37] When associated with the bacterial infections, fungal proliferation has been shown to induce an increased frequency or severity of diseases.[38,39] Hence, it is meaningful to develop polymers with both antibacterial and antifungal activity and study their structure−activity relationship for the future development of broad spectrum multifunctional coating materials.

Herein, we describe the preparation of novel antimicrobial coating materials based on polyethylenimine (PEI) and study their structure−activity relationship and the mechanism of action at the molecular level. In the present work, for the first time, we report the synthesis of colorless water insoluble and organo-soluble PEI derivatives by *Eschweiler-Clarke methylation* of PEIs and subsequent quaternization of *N*-methyl PEIs with alkyl bromides. The antimicrobial activities of the polymer coated surfaces were evaluated against various human pathogenic bacteria and fungi, including drug resistant strains. The effectiveness of these polymers as antimicrobial coating was also evaluated along with the conventional polymers and commercial paint. Hemocompatibility of the polymeric coating was also evaluated with human erythrocytes. Bacterial propensity to develop resistance against these polymers was studied against both Gram-positive and Gram-negative bacteria.

2. EXPERIMENTAL PROCEDURES

2.1. Chemicals and Instrumentation. Branched PEIs (M_w values of 750, 25, and 2 kDa), poly(2-ethyl-2-oxazoline) (M_w values of 500, 200, and 50 kDa), 1-bromododecane, 1-bromohexadecane, 1-bromooctadecane, 1-bromoeicosane, and 1-bromodocosane were purchased from Sigma-Aldrich while formic acid (90% vol/vol), formaldehyde (37−40% vol/vol), potassium hydroxide (KOH), dichloromethane, chloroform, acetone, methanol, ethanol, *n*-butanol, *tert*-butanol, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF) (all ACS grade) were purchased from SD-FINE, India. Bacterial and fungal growth media and agar were supplied by HIMEDIA, India. Propidium iodide (PI) and SYTO 9 dyes were purchased from Sigma-Aldrich and Invitrogen, respectively. Bacterial strains, *S. aureus* (MTCC 737) and *E. coli* (MTCC 447), were obtained from MTCC (Chandigarh, India). *P. aeruginosa* (ATCC 424), vancomycin-resistant enterococci (VRE)

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(ATCC 51559), beta lactam-resistant *K. pneumoniae* (ATCC 700603), and methicillin-resistant *S. aureus* (MRSA) (ATCC 33591) were obtained from ATCC (Rockville, MD). The fungal strains used in this study are *C. albicans* (SC5314), *C. dubliniensis* (CD36), *C. tropicalis* (MYA3404), *C. neoformans var. grubii* (serotype A) (H99), *C. gattii* (serotype B) (WM276), and *C. neoformans var. neoformans* (serotype D) (JEC21). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker AMX-400 instrument. FT-IR spectra of the solid compounds were recorded on Bruker IFS66 V/s spectrometer using KBr pellets. IR spectra of all the liquid compounds were recorded on the same instrument using a NaCl crystal. Elemental analysis was performed in a Thermo Finnigan FLASH EA 1112 CHNS analyzer. A WS5000 spin coater, Techno India, India, was used to prepare polymer coatings. A Leica DM2500 fluorescent microscope was used for bacterial imaging whereas an Olympus microscope (Model BX51) and Olympus DP71 camera were used for the fungal imaging. Scanning electron microscope images were obtained using Quanta 3D FEG, FEI field emission scanning electron microscopy. Thermogravimetric analyses were performed on a TGA 850C Mettler, Toledo thermogravimetric analyzer.

2.2. Synthesis of Hydrophobic Cationic Polymers. *2.2.1. Branched N-Methyl-PEIs (**1a**″, **2a**″, and **3a**″).* A 50% aqueous solution of PEIs (PEIs of 750, 25, and 2 kDa; **1a**′, **2a**′, and **3a**′; 10 g, 0.12 mol/repeating unit) was transferred to a round-bottom flask to which 90% formic acid (24.5 mL, 0.48 mol) was added followed by 37% formaldehyde (29.3 mL, 0.36 mol) and 20 mL of water (Scheme 1A). The reaction mixture was stirred at 90 °C for 60 h. After cooling down to room temperature, the pH of the reaction mixture was adjusted to 11 using an 8 M KOH solution. The deprotonated *N*-methylated PEI was extracted several times by chloroform, and the entire organic solution was subjected to repeated water wash. Chloroform was then removed to yield a yellow viscous product with 100% degree of methylation.

*2.2.2. Branched N-Alkyl N-Methyl PEIs (**1a**−**1e**, **2**, **3**).* Branched *N*-methylated-PEIs (1 g, 17.5 mmol/repeating unit) were dissolved in 75 mL of *tert*-butanol in a screw-top pressure tube, and 1-bromoalkanes (70 mmol) were added to it (Scheme 1A). The reaction mixture was heated at 95 °C (for 1-bromododecane), 105 °C (for 1-bromohexadecane), 120 °C (for 1-bromooctadecane), 125 °C (for 1-bromoeicosane), and 130 °C (for 1-bromodocosane) for 96 h. After the reaction, the solvent was removed to one-tenth of it's initial volume. Then, an excess of acetone (200 mL) was added to the reaction mixture, and the precipitate was filtered off. The precipitate was dissolved in $CHCl_3$, and acetone was added to reprecipitate the product for further purification. Finally, excess solvent was decanted off, and the precipitate was dried using a high vacuum pump to yield colorless branched *N*-alkyl *N*-methyl PEI polymers (**1a**−**1e**, **2**, and **3**).

*2.2.3. Deacetylated Linear PEIs (**4a**′, **5a**′, and **6a**′).* Fully deacylated linear PEIs were prepared by the acid-catalyzed hydrolyses of the commercial PEOZs (500, 200, and 50 kDa).[40] Typically, 10.0 g of the PEOZs was added to 400 mL of 24% (wt/vol) HCl, followed by refluxing for 96 h. The POEZ crystals dissolved completely in 2 h, but 3 h later, a white precipitate appeared. The precipitate in each case was isolated by filtration and then air-dried. The resultant white powders were confirmed to be pure PEI hydrochlorides by NMR and elemental analysis. Protonated linear PEIs were then deprotonated using aqueous KOH solution. Briefly, 10 g of protonated linear PEIs was dissolved in distilled water (50 mL), and 6 M KOH was added until the pH of the solution became ∼11. Deprotonated PEIs as white precipitates were filtered and washed repeatedly with distilled water until it became neutral (pH ∼7). Separately, 217, 87, and 22 kDa linear PEIs without *N*-acyl groups were obtained from commercial PEOZs of 500, 200, and 50 kDa.

*2.2.4. Linear N-Methyl PEIs (**4a**″, **5a**″, and **6a**″).* Linear *N*-methyl PEIs were prepared from linear PEIs following a similar procedure for branched *N*-methyl PEIs (Scheme 1B).

*2.2.5. Linear N-Alkyl N-Methyl PEIs (**4**, **5**, and **6**).* Linear *N*-alkyl *N*-methyl PEIs were prepared from linear *N*-methyl PEIs following a similar procedure for branched *N*-alkyl *N*-methyl PEIs (**4**, **5**, and **6**; Scheme 1B).

2.3. Solubility of *N*-Alkyl *N*-Methyl PEIs. A small portion (10 mg) of all the PEI derivatives was added to 1 mL of various organic solvents (chloroform, dichloromethane, methanol, ethanol, *n*-butanol, *N*,*N*-dimthylformamide, dimethyl sulfoxide, tetrahydrofuran), vortexed for about 10−15 min, and observed visually to check the solubility. After vortexing, the solubility limit of the polymers was also determined visually for different amounts (10, 20, 50, and 100 mg) of the PEI derivatives in 1 mL of solvent. However, to test water solubility, 10 mg of the PEIs derivatives in 1 mL of water was vortexed for 10−15 min and kept for 12 h. The aqueous part was decanted and subjected to freeze-drying. ^{1}HNMR spectra were recorded with the freeze-dried sample in $CDCl_3$.

2.4. Preparation of the Film. PEI derivatives were first dissolved/suspended in *n*-butanol (50 mg/mL), and the solutions were serially diluted (2-fold serial dilution). Solutions of different concentrations (20 μL) were added into wells of a 96-well plate. The solution was first air-dried and finally dried in a vacuum oven at 50 °C to prepare film on the flat-bottom surface of the wells of a 96-well plate. Each concentration was applied in triplicate for all the polymers for antimicrobial activity studies. In order to make films, e.g., a microbicidal coating, on the surface of a microscopic glass slide, a solution of PEI derivatives (in $CHCl_3$, 350 μL) was coated using a spin coater. An antimicrobial coating of these derivatives was prepared along with the medically relevant polymers such polylactic acid (PLA) using a spin coater from the solutions (350 μL) of PEI derivatives (of different concentrations) and PLA (10 mg/mL) in chloroform. In order to prepare microbicidal paint, different amounts of PEI derivatives were brush coated along with the commercial paint solution (100 and 50 mg/mL PEI derivatives **1c** and **6**, respectively, in paint solution).

2.5. Antibacterial Activity. *2.5.1. Determination of Antibacterial Activity by the Spray Method (Airborne Bacteria).* Bacteria were grown for 6 h in suitable nutrient media at 37 °C under constant shaking. The 6 h grown bacteria (1 mL) was centrifuged down at a speed of 12 000 rpm for 1 min. The bacterial pellet was then washed twice with 1× PBS (pH 7.4). The final concentration of the bacterial solution was then adjusted to 10^7 CFU/mL for *S. aureus* and 10^6 CFU/mL for *E. coli*, and the volume was adjusted to 10 mL. The bacterial solution was then sprayed onto the polymer coated glass slides (2.5 cm × 5.5 cm) at a spray rate of approximately 10 mL/min. The sprayed slides were then carefully transferred into petri dishes and dried in air for about 2 min. A slab of nutrient agar was then placed onto the glass slide, and the Petri dish was sealed and kept at 37 °C until visible colonies developed.[41] The coated and noncoated slides were imaged using a Cell Biosciences Gel Documentation instrument. Images were captured under white light and processed using Alpha-imager software. A similar experiment was performed with (PLA/Paint + **1c**) and (PLA/Paint + **6**) coated glass slides, i.e., the slides coated with the polymers **1c** and **6** along with PLA and paint individually, and antibacterial efficacy of the coated slides was evaluated similarly.

2.5.2. Determination of Antibacterial Activity (Waterborne Bacteria). To determine the antibacterial activity of the polymers, 200 μL of 10^5 CFU/mL bacteria was added to the polymer coated wells of a 96-well plate following the coating procedure as described above. Two controls were made: in one control, no solvent was added to the wells (blank wells) containing an equal volume of bacteria (10^5 CFU/mL), and the other was solvent-dried wells with 10^5 CFU/mL bacteria. The plates were then were incubated at 37 °C for 24 h. The visual turbidity was recorded before and after incubation to determine the minimum inhibitory amount (MIA; μg/dm^2 present in the well). In order to differentiate between bacteriostatic and bactericidal effects, aliquots (20 μL) from wells that appeared to have little or no bacterial growth were plated on suitable nutrient agar plates. The plates were then incubated at 37 °C for 24 h, and then, colonies were observed.

2.5.3. Antibacterial Kinetics. A 96-well plate was coated with the polymers **1c** and **6** at two different concentrations: MIA and 6× MIA for both *S. aureus* and *E. coli*, respectively, following the coating procedure as described above. A bacterial suspension (200 μL) containing approximately 4.9 × 10^5 CFU/mL *S. aureus* in nutrient broth and 5.1 × 10^5 CFU/mL *E. coli* in Luria−Bertani (LB) broth

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were added, and the plates were incubated at 37 °C under constant shaking. The initial time of addition of the bacteria to the wells was taken as zero, and 10 μL aliquots were withdrawn from each of the wells at definite time intervals. These aliquots were then added to 90 μL of 0.9% saline. These solutions were further diluted by a factor of 10, and 20 μL of the final solutions was plated on nutrient or LB agar plates immediately. The plates were incubated at 37 °C for 24 h, and bacterial colonies were counted. A plot of colony forming unit (CFU) in logarithmic scale versus time ($\log_{10}$ CFU/mL vs min) was then plotted to determine the bactericidal kinetics.

2.5.4. Antibacterial Activity in the Mammalian System. Blood (sodium heparin as anticoagulant) was donated by a healthy human donor. Plasma was isolated by centrifugation of the blood at 3500 rpm for 5 min. Serum was obtained by using an SST II *Advance* plus blood collection tube (BD vacutainer; ref 367956) containing human blood and then centrifuging at 3500 rpm for 5 min. Methicillin-resistant *S. aureus* (MRSA) was grown at nutrient media for 6 h (~10^9 CFU/mL). Finally, MRSA was diluted in minimum essential medium (MEM) and mixed with all three mammalian systems individually to obtain 10^5 CFU/mL in 50% serum, 50% plasma, and 10% blood. 200 μL of 50% serum, 50% plasma, and 10% blood containing 10^5 CFU/mL MRSA was added to the wells of a 96-well plate coated with the polymers (**1c** and **6**). Likewise, for the the MIA experiment, two controls were made: in one control, no solvent was added to the wells (blank wells), and the other one was a solvent-dried well. The plates were then placed in an incubator at 37 °C for 24 h. After incubation, aliquots from each well (20 μL) were plated on nutrient agar plates. The plates were then incubated at 37 °C for 24 h, and colonies were observed.

2.6. Mechanism of Antibacterial Action. *2.6.1. Cytoplasmic Membrane Depolarization Assay.* The 96-well plates were coated with the PEI polymers following the similar coating procedure as mentioned previously to give an amount of 1.56 μg/dm^2 for *S. aureus* and 15.6 μg/dm^2 for *E. coli*. Mid log phase bacterial cells (*S. aureus* and *E. coli*) were harvested, washed with 5 mM HEPES and 5 mM glucose, and resuspended in 5 mM glucose, 5 mM HEPES buffer, and 100 mM KCl solution in a 1:1:1 ratio (10^8 CFU/mL).[42] Measurements were made in black 96-well plates containing 200 μL of a bacterial suspension and 2 μM $diSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide). The fluorescence of the dye was allowed to quench for 20 min for *S. aureus* and 40 min for *E. coli*, and 0.2 mM EDTA was used in the case of *E. coli* to allow dye uptake through the outer membrane. The bacterial suspension was then placed in a polymer coated 96-well plate (black plate, clear bottom with lid), and fluorescence intensity was measured at every 2 min interval for 25 min at excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 20 nm).

2.6.2. Fluorescence Microscopy. Bacterial suspensions (200 μL, 10^9 CFU/mL and 10^8 CFU/mL for *S. aureus* and *E. coli*, respectively) were added to the wells of a 96-well plate coated with polymer **6** (at 6× MIA). A control was made similarly in noncoated wells. The 96-well plate was placed in an incubator at 37 °C under constant shaking for 4 h. After the incubation, the bacterial suspension was harvested and washed with PBS twice and finally resuspended in 100 μL of PBS. Then, 10 μL of the bacterial suspension was combined with 20 μL of a fluorescent probe mixture containing 3.0 μM green fluorescent nucleic acid stain SYTO 9 (Invitrogen, USA) and 15.0 μM red fluorescent nucleic acid stain propidium iodide (PI) (Sigma-Aldrich, USA) (1:1 v/v). The mixture was incubated in the dark for 15 min, and a 5 μL aliquot was placed on a glass slide, which was then covered by a coverslip, sealed, and examined under a fluorescence microscope. Excitation was done for SYTO 9 at 488 nm and at 543 nm for PI, respectively. Emission was collected using a band-pass filter for SYTO 9 at 500−550 nm and a long pass filter for PI at 590−800 nm. In all cases, a 100× objective was used with immersion oil, giving a total magnification of 1000×. Images were captured with a Leica DM 2500 fluorescence microscope.

2.6.3. Scanning Electron Microscopy. Bacterial suspensions (200 μL, 10^8 CFU/mL for both *S. aureus* and *E. coli*, respectively) in suitable nutrient media were added to the wells of a 96-well plate coated with polymer **6** (6× MIA). Bacteria were incubated at 37 °C for 2 h. After incubation, the suspension from the wells was transferred to a 1 mL eppendrof tube and centrifuged. The bacterial pellet was resuspended in 30% ethanol and subsequently dehydrated with 50%, 70%, 90%, and 100% ethanol. Finally, the bacteria were resuspended in 70% ethanol, and 5 μL of the bacterial suspension in ethanol was drop casted onto a silicon wafer and dried. The samples were sputter coated with gold prior to imaging using Quanta 3D FEG, FEI field emission scanning electron microscopy.

2.7. Antifungal Activity. *2.7.1. Minimum Inhibitory Amount (MIA).* The yeast cultures were grown overnight in 5 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose). The growth was measured by optical density (OD_{600}), and cells were diluted in fresh media to get a concentration of 10^5 cells/mL. After the dilution, 200 μL of the fungal cell suspension (equal to 2×10^4 cells) was added into the polymer coated wells of a 96-well plate. The plates were incubated at 30 °C for 20 h with shaking (180 rpm) to allow sufficient growth of the fungal cells. The growth of the fungi was measured at OD_{600} using YPD wells as a blank. The OD_{600} values were analyzed by calculating mean and standard deviation and further plotting the graph. The amount present in the well (μg/dm^2) where the OD of the test well matched with that of the blank well was taken as the MIA. To account for the optical density contributed by the polymer coating on the wells, only media (200 μL) was added in the coated wells and used as a blank for the respective concentrations.

2.7.2. Minimum Fungicidal Amount (MFA). To discriminate between the fungistatic and fungicidal effect of the polymers, aliquots from the wells that appeared to have less or no turbidity were plated on agar plates. For this, 3 μL of the culture from each well was taken after the MIA experiment and spotted onto the YPD agar plate. The YPD plates were then incubated for 24 h at 30 °C to allow the growth of the live cells. The amount of the polymers present in the well (μg/dm^2) for which no colony was observed on the agar plate was taken as the minimum fungicidal amount (MFA) for each fungus.

2.7.3. Kinetics of Antifungal Activity. To determine the rate of fungal killing by the polymer coated surfaces, a time course experiment was performed and the antifungal activity of the most active polymers from branched and linear groups (**1c** and **6**) was determined. For this, the cells were grown and added (10^5 cells/mL) into the polymer coated wells of a 96-well plate coated at two different concentrations (MIA and 8× MIA) as described above. At every 2 h interval, 3 μL of cells was spotted on a YPD plate starting at 0 h until 12 h. The plates were then incubated at 30 °C for the next 24 h, and growth was assayed to determine the time required to kill the fungus.

2.8. Mechanism of Antifungal Action. In order to have insights into the mechanism of antifungal activity by the polymer coatings, a fluorescence-based LIVE/DEAD assay was performed. *C. dubliniensis* cells were grown as described previously in polymer coated 96-well plates for 12 h with different amounts of a branched polymer **1c** and a linear polymer **6** (MIA or 8× MIA). The cells were then collected from all wells and pelleted. Following, the cells were washed with 1 mL of autoclaved water and finally resuspended in 100 μL of Milli-Q water. The live and dead cells were stained by adding 1 μL of propidium iodide (PI) and 1 μL of SYTO 9 (3.0 μM SYTO 9 and 15.0 μM PI). The mixture was then kept for incubation at room temperature for 30 min in the dark. The cells were then observed under a microscope for the fluorescence signal. Bright field imaging was done for DIC images; SYTO 9 fluorescence was monitored using the green emission filter, and PI fluorescence was determined in the red emission filter. The images were captured on an Olympus microscope (Model BX51) using an Olympus DP71 camera. The images were further processed using Image Pro-Plus software, ImageJ, and Adobe Photoshop.

2.9. Resistance Study. MIA values of polymers (**1c** and **6**) were determined against both *S. aureus* and *E. coli* following the procedure as described previously. After the initial MIA experiment, serial passaging was initiated by harvesting bacterial cells grown at the sub-MIA of the polymers (at [MIA]/2) and was subjected to another MIA assay. After a 24 h incubation period, cells grown in the sub-MIA of the polymers (at [MIA]/2) from the previous passage were once again harvested and assayed for the MIA.[43] The process was repeated for 20

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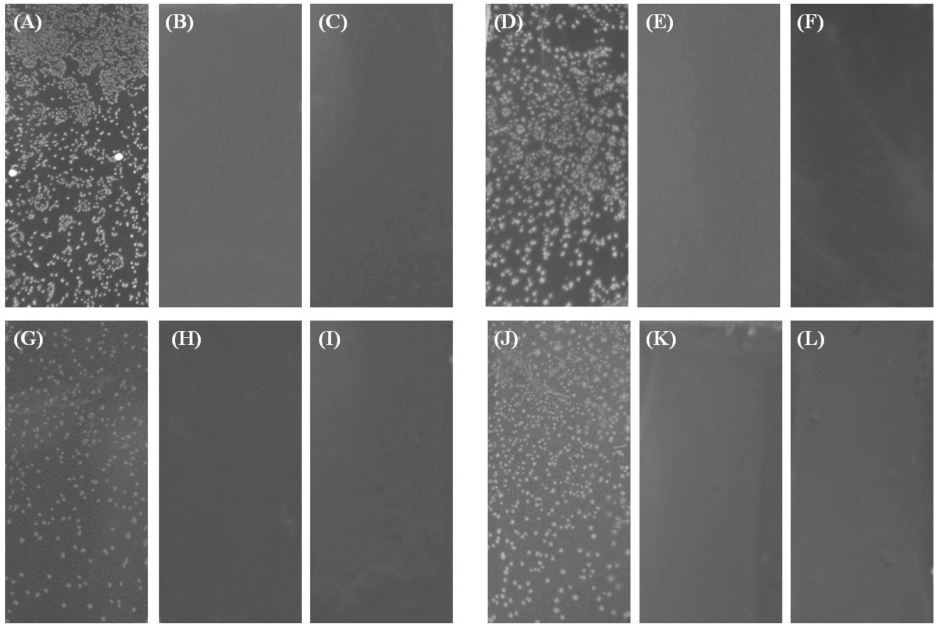

Figure 1. Antibacterial activity of polymer coated glass surface. Photographs of microscopic glass slides: (A) and (D) noncoated glass slides (controls); (B) and (E) slides coated with **1c** (0.4 and 12.5 μg/dm^2); (C) and (F) slides coated with **6** (0.2 μg/cm^2 and 6.25 μg/dm^2); (G) and (J) slides coated with PLA; (H) and (K) slides coated with **1c** along with PLA (0.4 μg/dm^2 + 2.5 μg/dm^2 PLA and 12.5 μg/dm^2 + 2.5 μg/dm^2 PLA); (I) and (L) slides coated with **6** along with PLA (0.2 μg/dm^2 + 2.5 μg/dm^2 PLA and 6.25 μg/dm^2 + 2.5 μg/dm^2 PLA). Slides A–C and G–I for *S. aureus* and slides D–F and J–L for *E. coli*, respectively. Each white dot corresponds to a bacterial colony grown from a single surviving bacterial cell.

passages. Norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively, as the controls. In case of norfloxacin and colistin, the initial MIC values were also determined against respective bacteria and the process was repeated for 20 passages following a similar procedure as discussed above. The fold increase in MIA/MIC values of the polymers and the controls was plotted against the number of passages.

2.10. Hemolytic Assay. Human red blood cells (hRBC) were isolated from freshly drawn, heparinized human blood and resuspended to 5 vol % in phosphate buffered saline (PBS; pH 7.4). Then, 200 μL of hRBC suspension (5 vol % in PBS) was added to the wells of a 96-well plate coated with polymers. Two controls were made, one noncoated well dried after using only solvent and another noncoated well with the solution of Triton X-100 (1 vol %). The plates were then incubated for 1 h at 37 °C and were centrifuged at 3500 rpm for 5 min. Following centrifugation, 100 μL of the supernatant from each well was transferred to a fresh 96-well plate, and absorbance at 540 nm was measured. Percentage of hemolysis was determined as $(A - A_0)/(A_{total} - A_0) \times 100$, where A is the absorbance of the test well (wells coated with polymers), A_0 is the absorbance of the wells with negative controls (wells without any polymer coating), and A_{total} is the absorbance of the well with 100% hemolysis (wells with Triton X-100, 1 vol %), all at 540 nm.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Cationic Hydrophobic Polymers. A new synthetic methodology has been introduced to prepare water insoluble and organo-soluble polymers from commercially available polyethylenimine (PEI) using the *Eschweiler-Clarke* reaction and subsequent quaternization (Scheme 1A,B). Branched PEIs (M_w = 750, 25, and 2 kDa) were first converted into *N*-methyl PEIs (**1a**″, **2a**″, and **3a**″) where all the primary and secondary amino groups were fully converted to the tertiary amines using excess formic acid and formaldehyde. The structures of the resultant polymers were confirmed by a combination of ^{1}H NMR and FT-IR spectroscopy. ^{1}H NMR spectra showed that the polymers had a peak at 2.2 ppm for the $-N(CH_3)-$ group in addition to the peaks from 2.3 to 2.6 ppm for the $-N(CH_2CH_2)-$ groups. However, complete methylation was confirmed from FT-IR spectra. IR spectra of all the branched *N*-methylated PEIs showed complete absence of the peaks at 3280 and 1600 cm^{-1} corresponding to the N–H bond stretching and bending, respectively, which were observed in the parent PEIs (Figures S1–S3, Supporting Information). To prepare various linear PEI derivatives, first commercially available poly(2-ethyl-2-oxazoline) (PEOZs; 500, 200, and 50 kDa) were deprotected by acid and then deprotonated with aqueous KOH. Finally, all the linear *N*-methylated PEIs (**4a**″, **5a**″, and **6a**″) were prepared from the linear PEIs (M_w = 217, 87, and 22 kDa) and characterized following a similar procedure as described for the branched *N*-methyl PEIs (Table S1 and Figures S4–S6, Supporting Information).

As the high microbicidal activity arises from not only high cationic charge but also substantial hydrophobicity (i.e., alkyl chain length), the modified polycations must possess certain hydrophilic/hydrophobic balance to ensure high activity and water insolubility. Further, in order to assess the structure–activity relationships of the antimicrobial polymers at the molecular level, a library of polymers with different molecular weights, different molecular architecture, and varying hydrophilic/hydrophobic balance was developed. All the tertiary amino groups of both the branched and linear *N*-methyl PEIs were quaternized by reacting with various alkyl bromides (Scheme 1). The degree of quaternization (DQ) was calculated from ^{1}H NMR spectra. Complete quaternization of the PEI derivatives was confirmed from NMR where the spectra showed no peaks at 2.2–2.6 ppm which correspond to the peaks for the $-N(CH_3)-$ and $-N(CH_2CH_2)-$ groups in *N*-methyl PEIs. The structure of the polymers was also confirmed by means of elemental analysis (Table S1, Supporting Information). By this way, for the first time, it was possible to determine structures of all the cationic PEI derivatives at the

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Table 1. Antibacterial (MIA) and Hemolytic (HA_{50}) Activities of Polymer Coated Surfaces

	MIA ($\mu g/dm^2$)						
	drug sensitive bacteria			drug resistant bacteria			
polymers	*S. aureus*	*E. coli*	*P. aeruginosa*	MRSA[a]	VRE[b]	*K. pneumoniae*[c]	HA_{50}[d] ($\mu g/dm^2$)
1c	0.98	15.6	3.9	0.98	0.98	15.6	>31
2	0.98	31	7.8	0.98	0.98	31	>31
3	0.98	31	7.8	0.98	0.98	>31	>31
4	0.24	7.8	1.95	0.24	0.24	7.8	>31
5	0.24	3.9	1.95	0.24	0.24	7.8	>31
6	0.24	3.9	1.95	0.24	0.24	7.8	>23

[a]MRSA = methicillin-resistant *S. aureus* (ATCC 33591). [b]VRE = vancomycin-resistant enterococci (ATCC 51559). [c]*K. pneumoniae* = beta lactam-resistant *K. pneumoniae* (ATCC 700603). [d]HA_{50} = amount of the polymer ($\mu g/dm^2$) coated in the well at which 50% hemolysis occurs.

molecular level. All the final cationic hydrophobic polymers were found to be almost colorless and thermally stable. Thermal stability of all the derivatives was measured by thermogravimetric analysis and was found to be stable even above 200 °C (Table S1 and Figure S15, Supporting Information).

As our goal was towards the development of water insoluble and organo-soluble noncovalent antimicrobial coating materials, the solubility of all the polymers was checked in water as well as in various organic solvents. The hydrophobic polycations **1a** (bearing $-C_{12}H_{25}$ alkyl chain and branched PEI of M_w = 750 kDa) and **1b** (bearing $-C_{16}H_{33}$ alkyl chain and branched PEI of M_w = 750 kDa) were found to be partially soluble in water whereas all other derivatives **1c** (bearing $-C_{18}H_{37}$ alkyl chain and branched PEI of M_w = 750 kDa), **1d** (bearing $-C_{20}H_{41}$ alkyl chain and branched PEI of M_w = 750 kDa), **1e** (bearing $-C_{22}H_{45}$ alkyl chain and branched PEI of M_w = 750 kDa), **2** (bearing $-C_{18}H_{37}$ alkyl chain and branched PEI of M_w = 25 kDa), **3** (bearing $-C_{18}H_{37}$ alkyl chain and branched PEI of M_w = 2 kDa), **4** (bearing $-C_{18}H_{37}$ alkyl chain and linear PEI of M_w = 217 kDa), **5** (bearing $-C_{18}H_{37}$ alkyl chain and linear PEI of M_w = 87 kDa), and **6** (bearing $-C_{18}H_{37}$ alkyl chain and linear PEI of M_w = 22 kDa) were found to be completely insoluble in water. However, all the quaternary hydrophobic polymers were soluble in chloroform, dichloromethane, *n*-butanol, *N,N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO; Table S2, Supporting Information). The polycations were soluble in chloroform up to the highest tested concentration of 200 mg/mL. Since the polymers **1a** and **1b** were partially soluble in water, no further experiments were performed with these polymers.

3.2. Antibacterial Activity of the Polymers. In order to evaluate the ability of these polymers to serve as antibacterial paint, glass slides were coated with solutions of the polymers in chloroform by brush coating/drop-casting/spin coating to establish the feasibility of the coating procedure. Further, to mimic the scenario of natural deposition of bacteria onto surfaces, bacteria were sprayed onto the polymer coated glass surfaces along with the noncoated glass surface (control). Bacterial growth was seen on noncoated glass surface as indicated by the presence of colonies, whereas no colony (at least 5 log reductions with respect to control) was observed on polymer coated surfaces (Figure 1). In order to evaluate the role of hydrophobicity and hence the hydrophobic/hydrophilic balance, surfaces coated with polycations (100% DQ with PEI of M_w = 750 kDa) of varying chain length (**1c** bearing $-C_{18}H_{37}$, **1d** bearing $-C_{20}H_{41}$, and **1e** bearing $-C_{22}H_{45}$) were assayed for antibacterial activity. These polymers with different hydrophobic/hydrophilic balance showed different activities (Figure S7, Supporting Information). Polymer **1c** coated surface showed 100% activity at 0.4 $\mu g/dm^2$ against *S. aureus* and at 12.5 $\mu g/dm^2$ against *E. coli*, respectively whereas polymers **1d** and **1e** showed 100% activity at 0.6 $\mu g/dm^2$ and 1.2 $\mu g/dm^2$ against *S. aureus* and did not show any activity until 12.5 $\mu g/dm^2$ against *E. coli*. Thus, polymer **1c** with $-C_{18}H_{37}$ chain length was found to be the most effective.

In order to evaluate the effect of molecular architecture and molecular weight of the polymers on antibacterial activity, bacteria were sprayed onto the glass surfaces coated with branched and linear polymers of different molecular weights having the same $-C_{18}H_{37}$ alkyl chain (**1c**, **2**, **3**, **4**, **5**, and **6**). It was observed that linear polymers were more active than the branched ones (e.g., surfaces coated with branched polymer **1c** exhibited 100% activity (5 log reductions) against *S. aureus* at 0.4 $\mu g/dm^2$ and against *E. coli* at 12.5 $\mu g/dm^2$ whereas surfaces coated with linear polymer **6** exhibited 100% activity against *S. aureus* at 0.2 $\mu g/dm^2$ and against *E. coli* at 6.25 $\mu g/dm^2$, respectively (Figures 1A–F and S8, Supporting Information). It was also observed that, among the branched polymers, **1c** with the highest molecular weight (PEI of 750 kDa) and, among the linear polymers, **6** with the lowest molecular weight (PEI of 22 kDa) were the most active (Figure 1A–F). These results clearly showed that different molecular architecture and molecular weight have a different impact on the antibacterial activity of the polymers even when coated onto the surface.

Further, in order to find the utility of these polycations to be used as antimicrobial coating materials for household and biomedical applications, the polymers were coated along with commercial paint and medically relevant polymer PLA, and the coated surfaces were similarly assayed for antibacterial activity. When polymers **1c** and **6** were brush coated onto the glass surface along with the commercial paint solution (10 and 5 wt %, wt/vol, for **1c** and **6**, respectively), both the branched and linear polymers showed 100% antibacterial activity even after loading with the paint against both *S. aureus* and *E. coli* (Figure S9, Supporting Information). Further, **1c** and **6**, when coated onto the glass surface along with PLA, retained antibacterial activity against both *S. aureus* and *E. coli*. Surfaces coated with branched polymer **1c** along with PLA (2.55 $\mu g/dm^2$) exhibited 100% activity against *S. aureus* at 0.4 $\mu g/dm^2$ and against *E. coli* at 12.5 $\mu g/dm^2$ whereas surfaces coated with linear polymer **6** along with PLA (2.55 $\mu g/dm^2$) exhibited 100% activity against *S. aureus* at 0.2 $\mu g/dm^2$ and against *E. coli* at 6.25 $\mu g/dm^2$, respectively (Figure 1G–L). These results thus indicated that these polymers could potentially be used to develop self-defensive paint and biomaterials.

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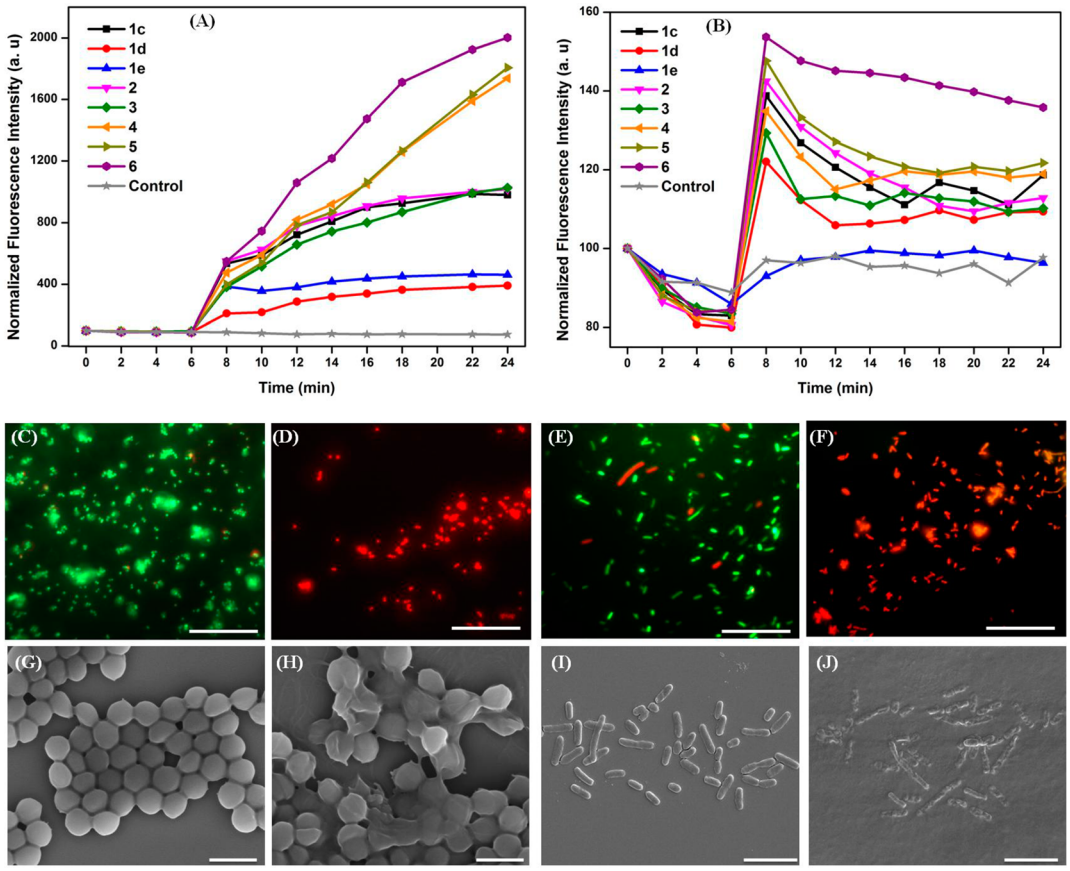

Figure 2. Mechanistic investigation of antibacterial activity of polymer coatings. Membrane depolarization for *S. aureus* (A) and *E. coli* (B), respectively. Fluorescence microscopy images of bacteria untreated (C: *S. aureus*; E: *E. coli*) and treated with the surface coated with **6** (D: *S. aureus*; F: *E. coli*) after staining with SYTO 9 and PI. Each image is a result of superposition of an image taken for the green fluorescent dye (SYTO 9) and red fluorescent dye (PI) using the appropriate filters. Scale bar is 20 μm. Scanning electron microscopy images of *S. aureus* (G and H) and *E. coli* (I and J) cells after a 2 h exposure to the uncoated surfaces (G and I) and surfaces coated with the polymer **6** (H and J). Scale bar is 1 μM for G and H and 5 μM for I and J.

To simulate the natural conditions of waterborne bacteria and to establish the efficacy of polymer coatings against these bacteria, antibacterial efficacy of the polymers was also evaluated with bacterial suspensions. The polymer coated surfaces were found to inhibit both *S. aureus* and *E. coli* (Table 1), and the minimum inhibitory amounts (MIAs) per square decimeter area of polymer coated surfaces were determined by measuring the optical density (OD) at 600 nm and also by inspecting visual turbidity (the minimum amount of polymer coated onto surface at which no visible bacterial growth observed was taken as minimum inhibitory amount). Likewise, the spray method polymer with $-C_{18}H_{37}$ alkyl chain (**1c**) was found to be most active among the polymers obtained from PEI of 750 kDa with varying chain length (**1c**, **1d**, and **1e**; Figure S10A, Supporting Information). When polymers with different molecular architecture and weights having the same $-C_{18}H_{37}$ alkyl chain were evaluated against both Gram-positive and Gram-negative bacteria, linear polymers were found to be more active than the branched ones. The MIAs for the branched polymers **1c**, **2**, and **3** were 0.98, 0.98, and 1.95 μg/dm^2, respectively, whereas MIAs for the linear polymers **4**, **5**, and **6** were 0.24 μg/dm^2 each against *S. aureus*. The MIAs for **1c**, **2**, and **3** were 15.6, 31, and 31 μg/dm^2 whereas those for **4**, **5**, and **6** were 7.8, 3.9, and 3.9 μg/dm^2 against *E. coli*, respectively (Table 1). However, all the polymers were more active toward Gram-positive *S. aureus* than the Gram-negative *E. coli*. This might be due to the high hydrophobic character of these polymers conferred by higher alkyl chain length which causes polymer aggregation, which in turn causes poor interactions with the cell surface of the Gram-negative bacteria composed of an additional outer lipid membrane made up of lipopolysaccharides.[22,44] Further, these polymers were also found to be active against *P. aeruginosa*, a bacterium which forms biofilm onto the surface most frequently and is difficult to treat (Table 1). When tested against drug-resistant bacteria, these polymers were found to inhibit MRSA, VRE, and beta lactam-resistant *K. pneumoniae* (Table 1), thus showing their broad spectrum antibacterial activity. The MIA values of the most potent polymer **6** were found to be 0.24 μg/dm^2 against MRSA and VRE and 7.8 μg/dm^2 against *K. pneumoniae*. Next, to find out whether these polymers are bacteriostatic or bactericidal, aliquots from the wells that appeared to have less or no turbidity were plated on an agar plate and minimum bactericidal amounts (MBA, μg/dm^2) were determined where no colony was observed. These polymers showed bactericidal effect against both Gram-positive and Gram-negative bacteria including drug-resistant ones (Table S3, Supporting Information). The MBAs of the most potent polymer **6** were found to be 0.24 μg/dm^2 against *S. aureus* and 7.8 μg/dm^2 against *E. coli*, respectively.

To establish how fast these polymers kill bacteria upon contact and also to understand the effect of molecular

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Table 2. Antifungal Activity (MIA) of Polymer Coated Surfaces

	MIA ($\mu g/dm^2$)					
	Candida spp.			*Cryptococcus spp.*		
polymers	*C. albicans*	*C. dubliniensis*	*C. tropicalis*	*C. neoformans Ser A*	*C. neoformans Ser B*	*C. neoformans Ser D*
1c	10	3.8	3.8	0.98	0.98	0.49
2	>31	>31	5.0	0.98	0.98	0.98
3	>31	>31	10	0.98	0.98	1.95
4	10	1.25	0.63	0.49	0.48	0.49
5	10	0.63	0.63	0.25	0.25	0.49
6	2.5	0.63	0.63	0.13	0.25	0.13

architecture on killing rate, the rates of action were investigated toward both *S. aureus* and *E. coli* using branched polymer **1c** and linear polymer **6** at the respective MIAs and 6× MIAs. Linear polymer **6** killed *S. aureus* (~5 log reduction) at 30 min at 6× MIA whereas branched polymer **1c** killed *S. aureus* (~5 log reduction) at 90 min at 6× MIA. Polymer **6**, on the other hand, killed *E. coli* (~5 log reduction) at 60 min at 6× MIA whereas **1c** killed *E. coli* (~5 log reduction) at 240 min at 6× MIA (Figure S11, Supporting Information). These results thus indicated that the cationic hydrophobic polymer coatings have remarkably high killing rates against both the bacteria making them suitable for efficient antimicrobial coating. Further, linear polymer has a higher killing rate than branched polymer against both Gram-positive and Gram-negative bacteria.

A serious concern of the health industry is the infections caused due to contamination of surfaces of medical implants and devices, which upon implantation come in direct contact with body fluids.[45] Hence, the antibacterial activity of the polymers in various mammalian fluids (50% serum, 50% plasma, and 10% blood) was investigated against MRSA, a Gram-positive drug resistant bacterium that causes many infections in humans undergoing implant surgery.[45] Both **1c** and **6** were found to be active in 50% serum, 50% plasma, and 10% blood. The MBA values of **1c** and **6** were 0.98 and 0.49 $\mu g/dm^2$ in 50% serum; 1.95 and 0.98 $\mu g/dm^2$ in 50% plasma, and 7.8 and 1.95 $\mu g/dm^2$ in 10% blood, respectively (Figure S12, Supporting Information). The above results thus suggest that these polymers could possibly be used *in vivo* as antimicrobial coatings in various medical implants and devices.

3.3. Modes of Antibacterial Action. To establish the mode of antibacterial action, disruption of cytoplasmic membrane potential was performed with the polymer coated surfaces against both *S. aureus* and *E. coli* using potential sensitive dye $diSC_3(5)$ by fluorescence spectroscopy. The dye was first added to bacterial cells, and as the dye accumulated in the membranes, the fluorescence intensity decreased because of self-quenching.[46] Upon disruption of the membrane potential by the polymer coated surfaces, an increase in fluorescence intensity was observed due to diSC3(5) being displaced into the solution. All the polymer coated surfaces except **1d** and **1e** were found to depolarize the membrane potential of both bacteria (Figure 2A,B). Interestingly, surfaces coated with linear polymers (**4**–**6**) were found to be more active in depolarizing the membrane potential compared to the surfaces coated with branched polymers (**1c**, **2**–**3**). Further, polymer **6**, which is most active among all the polymers, showed maximum membrane dissipation effect. Also, surfaces coated with polymers **1d** and **1e** having $-C_{20}H_{41}$ and $-C_{22}H_{45}$ alkyl chain, respectively, were unable to dissipate the membrane potential thus proving to be ineffective against bacteria as also reflected from their high MIA values.

The mechanism of antibacterial activity of the polycation coated surfaces against *S. aureus* and *E.coli* was further established by fluorescence microscopy.[47] Fluorescence microscopy images showed the cell viability in the case of the control samples as clearly seen by green fluorescence (Figure 2C,E for *S. aureus* and *E. coli*, respectively), and the cells treated with the polymer coated surface showed complete membrane permeabilization as seen by red fluorescence (Figure 2D,F for *S. aureus* and *E. coli*, respectively). Since PI is membrane impermeable and penetrates only when the bacterial membrane is compromised, the microscopy technique thus indicated that the cationic polymer interacted and subsequently disrupted the membrane integrity of the bacteria.

In order to have better insight into the mechanism of action of the polymer coated surfaces, scanning electron microscopy (SEM) was also used to visually characterize the morphology of bacteria upon contact with polymer coated surfaces. The micrographs presented for noncoated wells (control) showed that bacteria retained well-defined morphology and surface smoothness characteristic of unperturbed cells (Figure 2G,I for *S. aureus* and *E. coli*, respectively). In contrast, once treated with polymer (**6**) coated surface, bacteria exhibited morphological deformations (Figure 2H,J for *S. aureus* and *E. coli*, respectively). The appearance of irregularly shaped bacteria indicated a loss of structural integrity, thereby suggesting a specific and undermining interaction of hydrophobic polycations with the negatively charged bacterial cell membrane.[48] Since the polymers are water insoluble and coated onto surfaces, it is best expected that, when painted onto surfaces, the polymers killed bacteria on contact due to rupturing of bacterial cell membranes by erect fragments of the polymer chains.

3.4. Antifungal Activity. In order to find the utility of the cationic polymers developed herein to also be used as antifungal coating materials, the antifungal efficacy of all the branched and linear polymers were evaluated against different pathogenic *Candida* spp. (*C. albicans* SC5314, *C. dubliniensis* CD36, *C. tropicalis* MYA3404) and *Cryptococcus* spp. (*C. neoformans var. grubii* (serotype A) H99, *C. gattii* (serotype B) WM276, and *C. neoformans var. neoformans* (serotype D) JEC21). The cationic polymer coated surfaces were found to inhibit completely all the human pathogenic fungi species tested (Table 2). Linear polymers were found to be more active compared to branched polymers (e.g., MIA values of linear polymers **4**, **5**, and **6** are 0.63 $\mu g/dm^2$ for each polymer whereas the values for **1c**, **2**, and **3** are 3.75, 5, and 10 $\mu g/dm^2$ against *C. tropicalis*). Among the branched polymers, the polymer **1c**, with the highest molecular weight, and among all the linear polymers, polymer **6** with the lowest molecular weight showed maximum activity (Table 2). However, among all the polymers, polymer **6** was found to be most active against all the fungi

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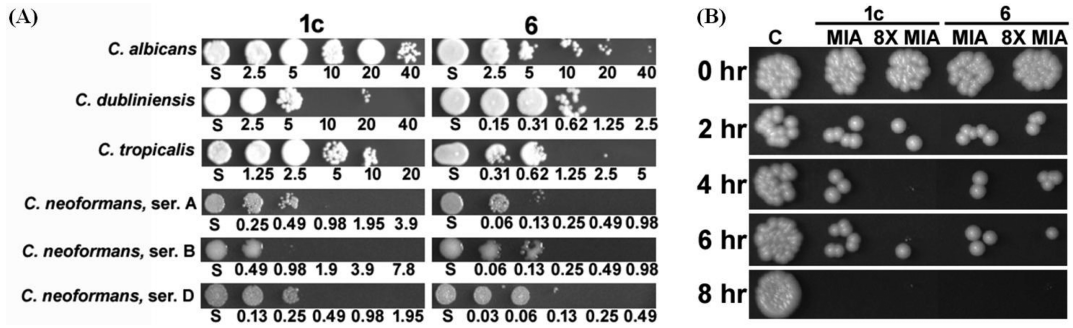

Figure 3. (A) Fungicidal activity (minimum fungicidal amounts): representative cross section of the YPD agar plate showing the growth of fungal colonies after treating with the polymers **1c** and **6** at different amounts for 20 h against various fungi (S stands for the well where only solvent was added and then dried as control, and numerical values are in $\mu g/dm^2$ of the well). (B) Antifungal kinetics of polymers **1c** and **6** coated surfaces at different concentrations (MIA and 8× MIA) against *C. dubliniensis*: representative cross section of the YPD agar plate showing the growth of fungal colonies at different times.

tested similar to the results obtained against bacteria. The MIA values for polymer **6** were 0.63−2.5 $\mu g/dm^2$ against *Candida* spp. and 0.13−0.25 $\mu g/dm^2$ against *Cryptococcus* spp.

The cationic hydrophobic polymers were not only fungistatic but also fungicidal (Table S4, Supporting Information). The minimum fungicidal amount (MFA) of the most potent polymer **6** was found to be 0.49 and 0.25 $\mu g/dm^2$ against *C. tropicalis* and *C. neoformans* Ser D, respectively. However, it is noteworthy that both branched and linear polymers were found to be less active against *C. albicans* as evident from their high MIA or MFA values (MIA value of polymer **6** was 1.56 $\mu g/dm^2$ against *Candida albicans*; Figure 3A).

To understand the effect of molecular architecture on fungal killing rate and to establish how fast these polymers kill fungi upon contact, the rate of action was investigated against *C. dubliniensis* using surfaces coated with both branched polymer **1c** and linear polymer **6**. Surface coated with polymer **1c** killed *C. dubliniensis* within 4−6 h at 8× MIA whereas surfaces coated with polymer **6** killed *C. dubliniensis* in 6−8 h at 8× MIA (Figure 3B). Even at their respective MIA values, polymers **1c** and **6** coated surfaces killed the fungal cells at 8 h. Therefore, it was observed that both branched and linear polymers have similar antifungal kinetics.

3.5. Modes of Antifungal Action. It was observed that the cationic polymers interact with the cellular membrane of bacteria and thereby disrupt the membrane integrity causing cell death. To know whether these hydrophobic polycations interact with and damage the cell membrane of fungi, LIVE/DEAD assay was performed with the fungal cell using SYTO 9 and PI dyes by fluorescence microscopy.[49] *C. dubliniensis* was used as a model fungus and treated with both branched and linear polymer **1c** and **6** coated surfaces. The microscopy images showed the presence of viable cells in the case of control samples (plain surface) as observed by green fluorescence (Figure 4). The cells treated with the polymer coated surfaces on the other hand showed red color fluorescence of PI, thus indicating the compromised cell membrane (Figure 4). These results indicate that the cationic polymers interact with the fungal cell membrane and disrupt the integrity of the membrane presumably leading to cell death.

3.6. Hemocompatibility. Hemolysis is one of the major concerns for the application of cationic polymer-modified biomaterial surfaces and other surfaces for various biomedical apllications.[50] The hemocompatibility of the polymer coatings were thus evaluated with human red blood cells (hRBCs), and

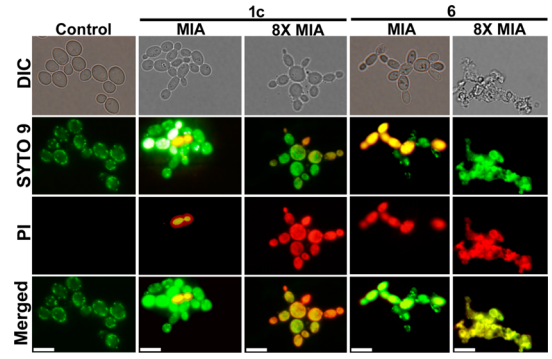

Figure 4. Mechanistic investigation of antifungal activity of polymer coatings. Fluorescence microscopy images of *C. dubliniensis* after staining with SYTO 9 and PI exposed to the uncoated surfaces (control), surfaces coated with the polymers **1c** and **6** at MIA and 8× MIA, respectively. Scale bar, 5 μm.

it was found that these polymers are nonhemolytic even up to 6.25 $\mu g/dm^2$. Only 25−35% hemolysis was observed at 31 $\mu g/dm^2$. Interestingly, the two most active polymers, **1c** and **6**, showed negligible hemolysis up to 6.25 $\mu g/dm^2$ which is 10−200 times more than their MIA values (Table 1). These results portrayed the nonhemolytic nature of these materials and enhanced their claim to be used as antimicrobial coatings in various biomedical and self-defensive biomaterial applications.

3.7. Propensity to Induce Bacterial Resistance. Due to the increasing growth of drug resistance in microorganisms, the potential emergence of bacterial resistance against these polymers was assessed by serial exposure of bacteria to the polymer coated surfaces. Both Gram-positive and Gram-negative bacteria were exposed to serial passages of branched polymer **1c** and linear polymer **6**, and the changes in MIA values were monitored over a period of 20 passages. After 20 serial passages, the MIAs of polymers **1c** and **6** remained the same whereas nofloxacin, a nucleic acid targeting antibiotic, showed an 850-fold increase in minimum inhibitory concentration (MIC) against sensitive *S. aureus* and colistin, a lipid II targeting lipopeptide, showed a 250-fold increase in MIC against sensitive *E. coli*, respectively (Figure 5). These membrane-active polycations were thus able to stall the development of bacterial resistance, and these studies demonstrated that these polymers could be used to create permanent microbicidal paint.

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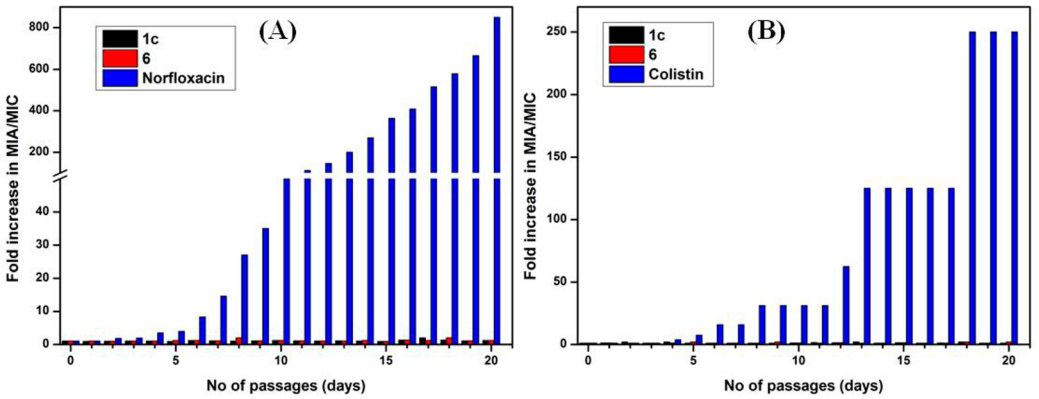

Figure 5. Development of bacterial resistance against: (A) *S. aureus* with polymers (**1c** and **6**) and ciprofloxacin; (B) *E. coli* with polymers (**1c** and **6**) and colistin.

3.8. Leaching Test. With a ^{1}H NMR study, it was found that the polycations **1a** and **1b** were partially soluble in water whereas all other polymers (**1c**−**1e**, **2**−**6**) were insoluble. However, to confirm further that these polymers kill bacteria on contact and not by leaching, a zone of inhibition test was performed with polymer coated filter papers. Filter papers (0.5 cm diameter) coated with polymers were placed onto an agar plate where ∼200 μL of 10^9 CFU/mL bacteria (*S. aureus* and *E. coli*) was spread by the spread-plate method. Polymers **1a** and **1b** showed a zone of inhibition against both *S. aureus* and *E. coli* thus confirming that these polymers are partially soluble in water and therefore leach from the coated surface. On the other hand, no zone of inhibition was found for **1c**−**1e** and **2**−**6**, thus suggesting that these polymers remained on the coated surface and kill microorganisms on contact (Figure S13, Supporting Information). In order to prove further, another experiment was performed by taking the two most active polymers (**1c** and **6**). If **1c** and **6** are soluble/partially soluble in water, enough of the polymer would be leached into the overlaid agar gels upon incubation. Further, the polymers would then leach out of gel slabs into the covered bacterial cells and kill bacteria upon placing these preincubated agar slabs onto noncoated glass surfaces sprayed with bacteria. However, the leaching test controls showed essentially as many colonies as the control slide (sprayed with bacterial suspension and covered with fresh agar gel; Figure S14, Supporting Information). Thus, it can be concluded that the leaching of the polymers from the coated surface is negligible in terms of their bactericidal activity.

4. CONCLUSIONS

In conclusion, we have demonstrated a simple method of developing water-insoluble and organo-soluble polymeric materials which, when painted onto surfaces, inactivate both airborne and waterborne bacteria completely, including drug-resistant ones and various pathogenic fungi. The polymer coated surfaces were found to be active in many complex mammalian fluids making them suitable for *in vivo* applications. The polymeric materials showed excellent compatibility with other polymers and commercial paint, thus could be useful for the development of self-defensive biomaterial and "microbicidal paint". Structure−activity relationship studies indicated that the linear polymers are more active and have a faster killing rate than the branched ones and an optimum alkyl chain length; hence, an optimum hydrophobic/hydrophilic balance is required for maximum activity of the water insoluble polymers. The cationic polymers were shown to inactivate bacteria and fungi by disrupting the membrane integrity, which stalled the development of microbial resistance. Further, the polymers showed negligible hemolytic activity at concentrations much higher than the MIAs and were therefore hemocompatible. Thus, the cationic polymeric materials developed herein could potentially be used as "microbicidal paint" for a wide variety of biomedical and household applications.

■ ASSOCIATED CONTENT

Supporting Information

Characterization of PEI derivatives; figures showing FT-IR spectra of PEI derivatives; figures showing the results of the spray methods; physical characterization, solubility, minimum bactericidal amount and minimum fungicidal amount tables of PEI derivatives; figures of antibacterial kinetics; figures showing the results of antibacterial activity in mammalian systems, zone of inhibition, and thermogravimetric analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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