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Highly potent antimicrobial polyionenes with rapid killing kinetics, skin biocompatibility and *in vivo* bactericidal activity

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ABSTRACT

Effective antimicrobial agents are important arsenals in our perennial fight against communicable diseases, hospital-acquired and surgical site multidrug-resistant infections. In this study, we devise a strategy for the development of highly efficacious and skin compatible yet inexpensive water-soluble macromolecular antimicrobial polyionenes by employing a catalyst-free, polyaddition polymerization using commercially available monomers. A series of antimicrobial polyionenes are prepared through a simple polyaddition reaction with both polymer-forming reaction and charge installation occurring simultaneously. The compositions and structures of polymers are modulated to study their effects on antimicrobial activity against a broad spectrum of pathogenic microbes. Polymers with optimized compositions have potent antimicrobial activity with low minimum inhibitory concentrations of 1.95 −7.8 μg/mL and high selectivity over mammalian cells. In particular, a killing efficiency of more than 99.9% within 2 min is obtained. Moreover, the polymers demonstrate high antimicrobial efficacy against various clinically-isolated multidrug-resistant microbes, yet exhibit vastly superior skin biocompatibility in mice as compared to other clinically used surgical scrubs (chlorhexidine and betadine). Microbicidal activity of the polymer is mediated *via* membrane lysis as demonstrated by confocal microscopy. Unlike small molecular antibiotics, repeated use of the polymer does not induce drug resistance. More importantly, the polymer shows excellent bactericidal activity in a *P. aeruginosa*-contaminated mouse skin model. Given their rapid and efficacious microbicidal activity and skin compatibility, these polymers have tremendous potential to be developed as surgical scrubs/hand sanitizers to prevent multidrug-resistant infections.

1. Introduction

Since penicillin was discovered, many antibiotics have been identified that exert antibacterial effects by inhibiting various targets within microbial biosynthetic pathways [1–3]. An unexpected drawback of the high therapeutic specificity of these antibiotics is the subsequent development of drug resistance mediated through sequential mutations [2,4,5]. Antimicrobial compounds are extensively used in personal care products to prevent infections and prolong product shelf-life [6–8]. Most antimicrobial agents found in personal care goods have molecular weights below 600 Da and include triclosan, chlorhexidine and benzalkonium chloride. Triclosan is amongst the most extensively used compounds and is present in many personal care products such as cosmetics,

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deodorant and soap [9,10]. Though efficacious against Gram-positive bacteria, its effectiveness against Gram-negative bacteria and mould has been found wanting [9,11,12]. For the antimicrobial agents as aforementioned, there has been documented resistance against various strains of bacteria. More importantly, there is a major concern with the development of cross- and co-resistance with clinically-used antibiotics, further complicating the resistance dilemma.

Therefore, there exists an urgent need to discover more effective antimicrobial agents against multidrug-resistant (MDR) pathogens, which would mitigate drug resistance development. In spite of the pervasive exploitation of cationic antimicrobial peptides (AMPs) in our innate immune system, these AMPs have still been largely protected from the development of bacterial resistance [13]. This is due in large part to the fact that these AMPS utilise less selective cationic amino acids which interact with bacterial membranes electrostatically. Secondary conformational changes, which occur after electrostatic membrane association, allow the hydrophobic regions within these AMPS to integrate with the bacterial cell membranes [14,15]. These cell membranes are destablised once these hydrophobic regions are inserted into the lipid bilayers, eventually resulting in cell lysis and death. As opposed to antibiotics that inhibit specific intracellular microbial processes, these AMPs exert their antimicrobial activity *via* non-specific electrostatic and hydrophobic interactions. This unique mode of action largely shields AMPs from the development of the antimicrobial resistance.

A number of synthetic cationic polymers that mimic the amphiphilic structure and antimicrobial functionalities of AMPs have been explored as highly tunable and broad spectrum antimicrobials [16–18]. For examples, polyacrylates [19–23], polynorbornene systems [23], polyethyleneimines [24,25], polyarylamides [18], metallopolymers [26], poly-β-lactams [27], poly-α-amino acids [28,29], and polycarbonates [30,31] have shown minimum inhibitory concentration (MIC) values of 4 to >1000 μg/mL. In general, the overall hydrophobic/hydrophilic balance of the polymers affects their antimicrobial activity and bacteria selectivity [32,33]. An alternative class of polymer electrolytes is the polyionenes, wherein the quaternized nitrogens are located in the polymer backbone in contrast to the above examples where the distal charges are pendent to the backbone [34,35]. Charge density and hydrophobicity of polyionenes can be readily tuned as compared to polycarbonates and polyacrylates by choosing appropriate commercially available monomers [36,37]. Polyionenes have been studied as antimicrobials. For example, Agarwal and coworkers investigated alkyloxyethylammonium ionenes with varying pendent groups and alky spacers in the main chain as biocides [38]. By employing polyaddition reaction of *N,N,N′,N′* tetramethyldiamines and α,ω-dibromoalkanes, Tiller's group also found that such polyionenes had excellent antimicrobial activity, and were not hemolytic towards red blood cells [39]. In general, the antimicrobial efficacy of polyionenes has been shown to be heavily dependent upon chain rigidity and alkyl groups of varying length, which were used to modulate hydrophobicity [38,39]. Our group has recently designed a class of antimicrobial polycarbonates and found that use of long alkyl chains led to high toxicity towards mammalian cells, while use of aryl group or cyclic group mitigated toxicity [33,40]. Moreover, there are limited studies reported on activity of polyioenes against clinically-isolated MDR microbes, skin biocompatibility and *in vivo* bactericidal activity. Therefore, in this manuscript, aryl groups were used in the synthesis of polyionenes to optimize hydrophobicity for high potency and *in vivo* skin biocompatibility.

Specifically, a new series of antimicrobial polyionenes has been synthesized through a simple polyaddition reaction, whereby both the polymer-forming reaction and the installation of the charge occur simultaneously (see Scheme 1, polymers **1**–**6**, polymers **1a**–**1c**, and polymers **4a**–**4c**). Moreover, the use of commercially available, low-cost starting materials, as well as simplified polymerization and isolation conditions are anticipated to lower the cost of final products. The objective of this study is to focus on efficacy and killing kinetics. The compositions and structures of the polymers were modulated to study their effects on antimicrobial activity against pathogenic microbes, including *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*, and their selectivity towards mammalian cells (e.g. rat red blood cells). The killing kinetics of the polymers were investigated, and the antimicrobial mechanisms were explored by visualizing the changes in the membrane integrity of microbes after polymer treatment. Possibility of drug resistance development in bacteria was evaluated by monitoring MIC changes after repeated use of polymers at sub-lethal doses. In addition, the antimicrobial activity of the polymer with the optimized composition was examined against clinically isolated multidrug-resistant bacterial strains including multidrug-resistant *S. aureus*, *MRSA*, *E. coli*, *A. baumannii* and *K. pneumonia,* as well as fungi *C. albicans* and *C. neoformans*. Finally, the *in vivo* bactericidal activity and dermal toxicity of the polymer were evaluated using a mouse model.

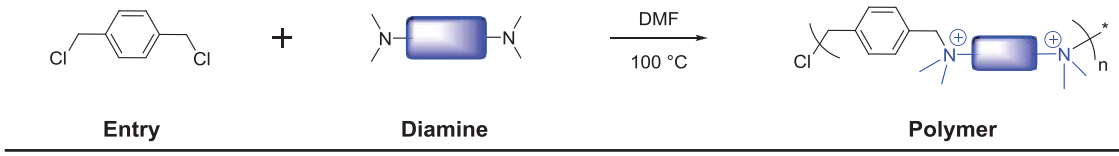
2. Materials and methods

2.1. Materials

All chemical reagents were provided by Sigma-Aldrich, U.S.A. and used as received unless specified otherwise. Micorbial broths were prepared using Muller Hinton Broth (MHB) powder (BD Diagnostics). *S. aureus* (ATCC No. 6538), *E. coli* (ATCC No. 25922), *P. aeruginosa* (ATCC No. 9027) and *C. albicans* (ATCC No 10231) were obtained from ATCC, U.S.A. Clinically isolated multidrug-resistant Gram-positive bacteria *S. aureus* and MRSA, Gram-negative bacteria *E. coli*, *A. bacumanii* and *K. pneumoniae*, and fungi *C. albicans* and *C. neoformans*, were extracted from patients' phlegm or cerebrospinal fluid (multidrug-resistant *C. neoformans*) and kindly provided by Dr. Jifang Sheng, Department of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, P. R. China. 3-[4,5-Dimenthylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was prepared in phosphate-buffered saline (PBS, pH 7.4) at 5 mg/mL. This solution was subsequently subjected to filtration using a filter (0.22 μm) so as to remove blue formazan crystals before usage. Fetal bovine serum was procured from Invitrogen Corporation. Rat red blood cells (RBCs) from Vistar rats (Invivos, Singapore) were employed for hemolysis study. Primary human dermal fibroblasts (HDFs) were obtained from ATCC (PCS-201-012).

2.2. Synthesis of polymers

The synthesis of polymer **4** is given as an example. To a round-bottom flask equipped with a nitrogen inlet was added 1,4-bis(chloromethyl)benzene (1.65 g, 9.43 mmol) and DMF (20 mL). A solution of bis[2-(*N*,*N*-dimethylamino)ethyl] ether (1.51 g, 9.43 mmol) in DMF (20 mL) was then added dropwise over 30 min at room temperature. The reaction is exothermic. After the addition was complete, the reaction mixture was heated to 85 °C and stirred for 18 h, at which point a precipitate was observed. The polymer was isolated by filtration and washed with ethyl ether (3 × 50 mL) to afford an off-white powder in near quantitative yield. ^{1}H NMR (400 MHz, D_2O): δ 7.58 (s, 4 nH, Ph*H*), 4.52 (s, 4 nH, PhCH_2), 3.98 (s, 4 nH, $CH_2CH_2N^{\oplus}(CH_3)_2$), 3.59 (s, 12 nH, $-N^{\oplus}(CH_3)_2$), 3.01 (s, 12 nH, $-N^{\oplus}(CH_3)_2$). $M_{n,\,GPC} = 7930$ Da, $Đ = 2.6$.



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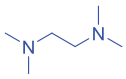
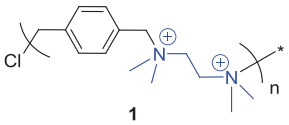
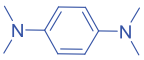
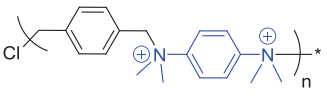
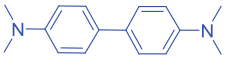
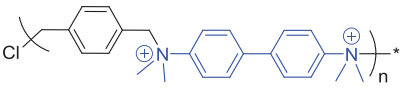
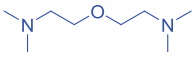
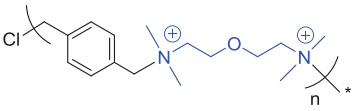
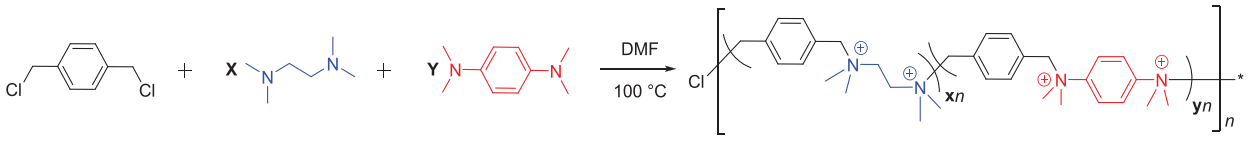
1		 1
2		 2
3		 3
4		 4



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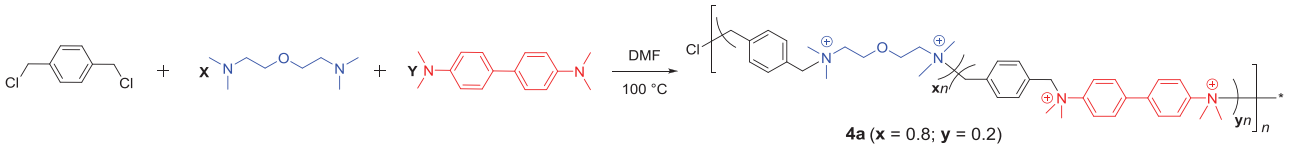
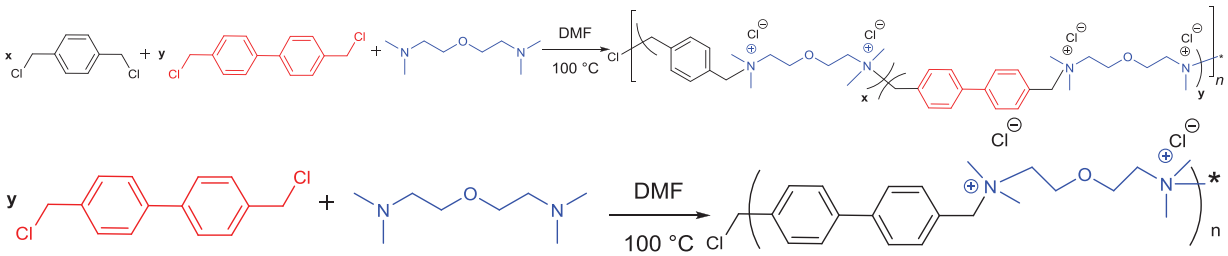
1b (x=0.6; y=0.4)
1c (x=0.4; y=0.6)



4a (**x** = 0.8; **y** = 0.2)
4b (**x** = 0.6; **y** = 0.4)
4c (**x** = 0.4; **y** = 0.6)



5 (**x** = 0.8; **y**=0.2)
6 (**y** = 1.0)

Scheme 1. Direct polyaddition and concurrent introduction of charge in a single step. Counteranions are omitted for clarity.

2.3. Molecular weight determination

A Waters 2695 gel permeation chromatography (GPC) equipped with two ultrahydrogel columns: 300 mm × 7.8 mm in series and a Waters 2414 differential refractometer detector (MA, U.S.A.) were used to determine the molecular weights of the polymers synthesized. Details of the mobile phase used are as follows: 54/23/23 (v/v/v%) water/methanol/acetic acid, 0.5 M sodium acetate at a flow rate of 1.0 mL/min. Calibration was performed using a series of poly(ethylene glycol) standards of varying molecular weights (633–20,600) (Polymer Standard Service Inc., RI, U.S.A.). M_w and $Đ$ was subsequently calculated from the calibration curve.

2.4. Antimicrobial activity

2.4.1. MIC measurement

Bacterial and fungal samples were grown in MHB at 37 °C and room temperature, respectively, under shaking (100 rpm). They were subsequently incubated overnight so as to enter the log growth phase. The respective MIC of each polymer was determined using a broth microdilution method. Each microbial suspension (100 μL) was seeded into each well of a 96-well plate (3×10^5 colony forming units (CFU) ml^{-1}), to which 100 μL of broth containing a polymer at different concentrations was added. The plate was then subjected to incubation under shaking (100 rpm) for 18 h at 37 °C. MIC was taken to be the lowest polymer concentration at which microbial growth was completely inhibited by observation with a microplate reader (TECAN, Switzerland). Negative controls (broth containing only microbes without polymer treatment) were used. Six replicates were repeated for each experiment. The experiment was independently repeated at least three times.

2.4.2. Drug resistance study

E. coli were used as a model microbe for the drug resistance study. Drug resistance was induced by repeatedly treating *E. coli* (ATCC No 25922) with antimicrobial agents at sub-lethal doses [41]. The MIC of polymer **1** against *E. coli* was determined through 10 passages of growth. MIC was determined using the broth microdilution method described above. *E. coli* exposed to sub-MIC concentrations (1/8 of MIC at that particular passage) were allowed to re-grow and reach a logarithmic growth phase before being used for MIC measurement of the subsequent passage. Development of drug resistance in *E. coli* was evaluated by recording changes in the MIC normalized to that of the first cell passage.

2.5. Killing kinetics test

The microbes were treated with polymer **1** at various concentrations. At various time periods of treatment (0 min, 2 min, 10 min, 20 min, 1 h, 2 h, 10 h and 20 h), the microbial samples were diluted for plating (LB Agar, 1st Base). Incubation conditions: 18 h, 37 °C for bacteria; 42 h, room temperature for fungi. The colony-forming units were counted after the incubation period. The experiment was repeated in independent settings three times.

2.6. Confocal laser scanning microscopic (CLSM) analysis

Bacterial membrane permeability after polymer treatment was studied by CLSM analysis. Briefly, *S. aureus* and *P. aeruginosa* (10^8 CFU/mL) were incubated at 37 °C together with polymer **1** at various concentrations. Bacteria that were not treated with polymers were used as controls. For ease of visualization, a higher concentration of bacteria (10^8 CFU/mL) was used, instead of the usual 10^5 CFU/mL for MIC measurement. The bacteria cells were subjected to centrifugation at 3000 rpm for 5 min after 2 h of treatment. This was followed by re-suspension of the pellet in PBS and staining with PI (30 μM) or FITC-dextran (250 μg/mL, 100 kDa) at room temperature for 15 min. After being washed twice with PBS, the cells were observed under CLSM (Carl Zeiss LSM 510 META inverted confocal microscope, Germany). Similar conditions were maintained for all experiments.

2.7. Hemolysis assay

PBS was used to dilute the rRBCs suspension to 4% v/v blood content (2.64×10^8 RBCs/mL). Polymer solutions were prepared using PBS to obtain a range of different concentrations. Equal volumes of blood suspension were then added to the polymer solutions and subsequently incubated at 37 °C for 1 h. The mixtures were then subjected to centrifugation (1000 g, 5 min at 4 °C). The supernatant (100 μL) was then pipetted into each well (96-well culture plate), and 4 replicates were repeated for each polymer concentration. The optical density of each well was then recorded at 576 nm using the microplate reader. This approximates to the amount of hemoglobin released and the degree of hemolysis of each polymer. Negative (rRBC suspension without polymer treatment) and positive (rRBC suspension with addition of 0.1% Triton-X) controls were used. The formula below was used to calculate the degree of hemolysis:

$$\text{Hemolysis (\%)} = [(\text{O.D.}_{576\ \text{nm}} \text{ of the treated sample} - \text{O.D.}_{576\ \text{nm}} \text{ of negative control}) / (\text{O.D.}_{576\ \text{nm}} \text{ of positive control} - \text{O.D.}_{576\ \text{nm}} \text{ of negative control})] \times 100\%$$

HC_{50} was taken as polymer concentration at which the polymer causes 50% hemolysis. The experiment was independently repeated three times.

2.8. Water-octanol partition coefficient (log P)

Polymer solutions (500 μL, 125 μg/mL) were prepared in aqueous solution and an equal volume of octanol was then added. The samples were vortexed for 10 min and then allowed to equilibrate overnight in the dark. The samples were then centrifuged at 4000 rpm for 5 min. 100 μL from each phase was transferred to a new tube and diluted 10-fold in methanol. Their corresponding UV–Vis spectra were recorded. The polymer concentration in each phase was determined with reference to calibration curves obtained by measuring the UV–Vis spectra of various concentrations of each polymer in methanol. The partition coefficient was defined as $\log P = \log([C]_{oct}/[C]_{aq})$, where $[C]_{oct}$ and $[C]_{aq}$ are the polymer concentrations in octanol and aqueous phases, respectively. When log P is lower than 0, a polymer is considered to be relatively hydrophilic. Measurements were conducted in triplicates.

2.9. In vitro cytotoxicity study

2.9.1. MTT assay

The MTT assay was performed to evaluate the cytotoxicity of the polymers. HDF cells (10^4, 100 μL) were seeded into each well in 96-well plates. They were then cultured in the standard medium (DMEM supplemented with 10% FBS, 5% penicillin-streptomycin, 2 mM L-glutamine, 4.5 g/L D-glucose and 110 mg/L sodium pyruvate), and incubated at 37 °C, 5% CO_2 overnight. The medium containing polymer at various concentrations (100 μL) was then pipetted into each well to replace the existing culture media. The plates were then subjected to incubation at 37 °C in 5% CO_2 for 2 min. After treatment, the solutions within each well was removed, and subsequently replaced by 100 μL of fresh medium and 20 μL of MTT solution (5 mg/mL). After incubating for 4 h at

37 °C in 5% CO_2, the medium within each well was then replaced with dimethyl sulfoxide (150 μL). Using a microplate reader, absorbance was then measured at 550 nm, with the absorbance at 690 nm taken as a reference. Cell viability was expressed as a percentage of absorbance of the control cells without any treatment. The experiment was independently repeated three times.

2.9.2. LDH (lactate dehydrogenase) assay

The LDH assay (Promega, U.S.A.) was performed to quantitatively measure the amount of LDH enzyme released by cells with damaged or lysed membrane as an indicator of cytotoxicity. HDF cells (10^4 cells per well in 96-well culture plates) were treated with polymer **1** or chlorhexidine digluconate at various concentrations for 2 min or 1 h (8 replicates for each concentration), and the supernatant (50 μL) was then transferred into the wells of a new plate. Freshly prepared LDH assay solution (50 μL) was added, and the plate was incubated for 30 min at room temperature according to the manufacturer's protocol. This was followed by the addition of 50 μL of Stop solution. The optical density at 490 nm of each well was then recorded using the microplate reader. This approximately corresponds to the amount of LDH released and the cytotoxicity of each polymer concentration. Negative (cells without polymer treatment) and positive (cells completely lysed in 1 × lysis solution) controls were used. The formula below was used to calculate the cytotoxicity of the polymer and chlorhexidine,

$$\text{Cytotoxicity}\,(\%) = [(\text{O.D.}_{490\ \text{nm}}\ \text{of the treated sample} - \text{O.D.}_{490\ \text{nm}}\ \text{of negative control})\ /(\text{O.D.}_{490\ \text{nm}}\ \text{of positive control} - \text{O.D.}_{490\ \text{nm}}\ \text{of negative control})] \times 100\%$$

2.10. Animal studies

The animal study protocols were approved by the Institutional Animal Care and Use Committee of Biological Resource Centre, Agency for Science, Technology and Research (A*STAR), Singapore.

2.10.1. Animal skin toxicity evaluation

In vivo dermal compatibility studies were performed with C57BL/6 mice (8 weeks old, 18–22 g). The mice were randomly grouped (5 in each group). Before the test, fur in the dorsal area (about 1.5 cm × 1.5 cm) of each mouse was removed. The mice were then treated with 6 topical solutions: water solution (control), benzalkonium chloride (cationic, control, 0.198% w/v), chlorhexidine (neutral, control, 4% w/v in Bactishield handwash), betadine (control) and polymer **1** (200 and 500 μg/mL). 0.198% w/v was used for benzalkonium chloride as it is the effective concentration of benzalkonium chloride in Dettol handwash. The samples (200 μL) were administered topically and uniformly on the shaved area of each mouse. After exposure for 2 min, the treated skin area was rinsed with water. The mice were treated twice daily for 5 days total. The mice were sacrificed and the treated skin tissues were collected for histological examination. This involved the fixation of samples in 4% neutral buffered formalin followed by paraffin, and subsequent sectioning and staining with hematoxylin (nucleus, blue) and eosin (cytoplasm, purple) under standard protocols.

2.10.2. In vivo toxicity studies

LD_{50} was determined using female Balb/c mice (8 weeks old, 18–22 g). The Up-and-Down-Procedure described in OECD Guidelines for the Testing of Chemicals (OECD 425) was used to determine LD_{50}. Polymer **1** was dissolved in water and administered to mice orally at various doses (i.e., 1000, 1250, 1500, 1750 and 2000 mg/kg, 0.2 mL). Mortality was monitored for 14 days post treatment, and the maximum likelihood method was used to estimate LD_{50}.

2.10.3. In vivo bactericidal activity

A mouse model was established using a previously reported method [42] to study the bactericidal activity of polymer **1**. C57BL/6 mice (8 weeks old, 18–22 g) were employed for this study. The mice were randomly grouped (5 in each group). Mice were anesthetized using an *i.p.* injection with pentobarbital (50 mg/kg). Once the mice were fully anesthetized, the hair of the dorsal area was clipped and the depilatory cream was introduced to completely shave off the dorsal area. The test area (2 cm^2) on the back of each mouse was disinfected with cotton soaked in ethanol, and allowed to dry for 30 min. Suspension of clinically isolated *P. aeruginosa* 25844 (10 μL, 5×10^8 CFU/mL) was applied to the dorsal area, and then dried for 30 min. Polymer **1** (500 μL, 500 μg/mL) and chlorhexidine (500 μL, 4% w/v in Bactishield handwash) were administered topically on the bacteria-contaminated sites. After 2 min of treatment, the full-thickness skin of the application site was removed under anaesthesia using sterile forceps and scissors, and placed in 5 mL of MHB in order to neutralize the bactericidal activity of the test compounds. The skin of untreated mice in the control group was recovered using the same protocol. The MHB was plated on agar plates. CFUs were subsequently counted after 24 h of incubation.

2.11. Statistics

The results are expressed as mean ± standard deviation. Standard deviation is indicated by the error bars. Student's *t*-test was used to determine significance among the small groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

The strategy for the synthesis of cost-effective yet efficacious macromolecular antimicrobials disclosed herein employs a polyaddition polymerization from commercially available monomers without the need for a catalyst (Scheme 1). The wide range of commercially available α,ω-tetramethyldiamines allows for polymers with a variety of hydrophobicities to be synthesized, an important parameter that is known to influence the antimicrobial activity of a polymer. Dimethylformamide (DMF) was used as the polymerization solvent as it dissolves the reactants at elevated temperatures. Since we have shown that the antimicrobial activity and molecular weights of polymers are independent of each other, we employed conditions that would suppress polymerization by diffusion limited mechanisms (i.e., polymer precipitation) to attain only modest molecular weights, which should aid in the solubility of the polymer in aqueous media. In a representative case, equimolar amounts of *p*-xylylene dichloride and bis[2-(*N*,*N*-dimethylamino)ethyl] ether were dissolved in DMF, slowly combined under nitrogen, and stirred at 85 °C overnight to obtain a precipitate. The precipitate was filtered and washed with diethyl ether to afford polymer **4** as a hygroscopic solid with number averaged molecular weight (M_n) = 7930 Da and polydispersity index ($Đ$) = 2.6, as determined by gel permeation chromatography (GPC) (Fig. 1a). The ^{1}H NMR spectrum of polymer **4** in D_2O exhibited signals that are consistent with the formation of quaternary ammonium centers, characterized by downfield shifts of the *N*-methyl (from 2.15 to 3.10 ppm) and *N*-methylene (from 2.48 to 4.07 ppm) proton signals of the bis[2-(*N*,*N*-dimethylamino)ethyl] ether segment of the polymer relative to that of the starting material (Fig. 1b). With this methodology, we synthesized a library of aryl groups-containing antimicrobial polymers (Scheme 1). As shown in Table 1, under these reaction conditions, the weight averaged molecular weights

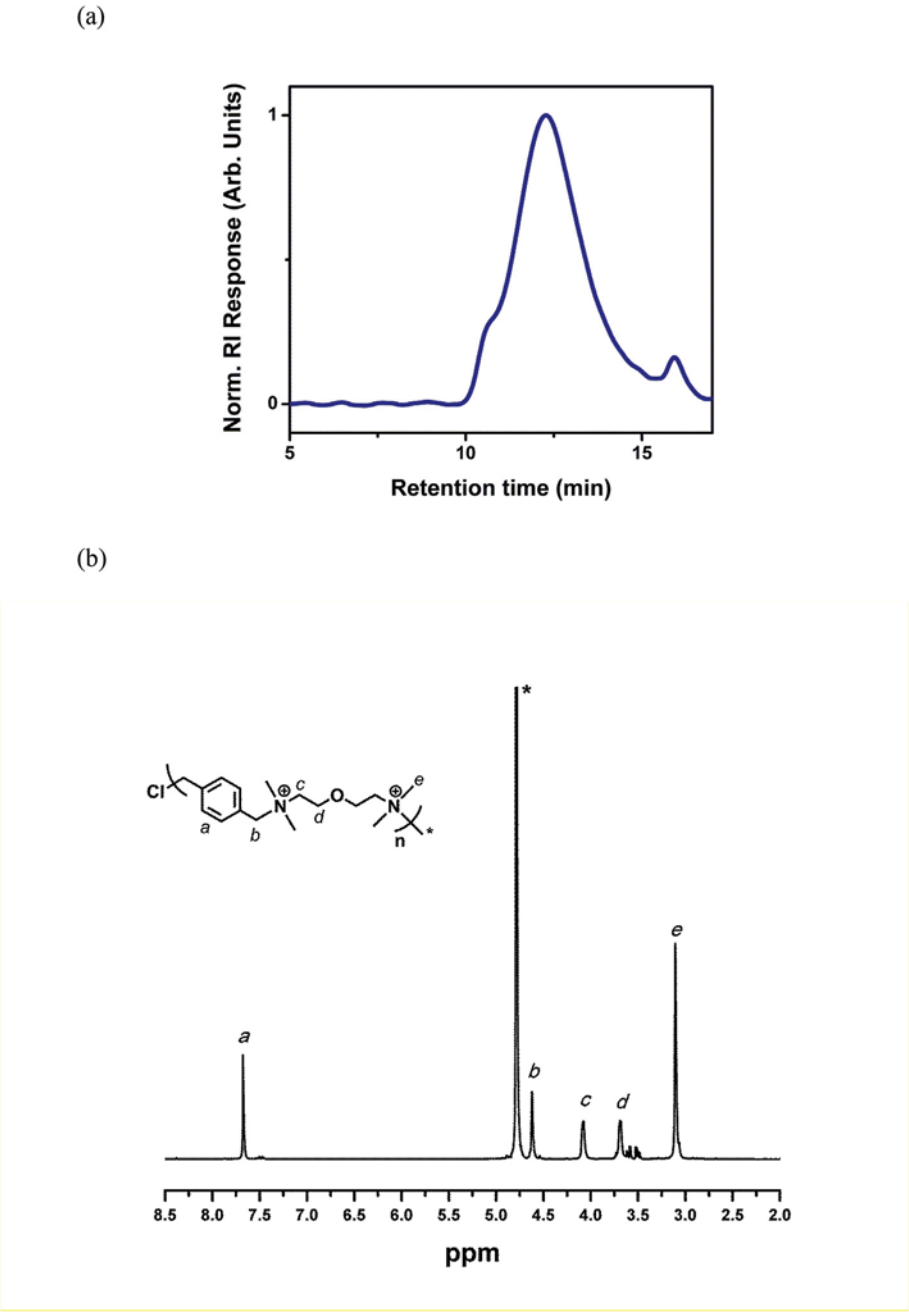


Fig. 1. Physical characterizations of polymer **4**. (a) GPC trace (mobile phase 54/23/23 (v/v/v%) water/methanol/acetic acid and 0.5 M sodium acetate); (b) ^{1}H NMR spectrum (D_2O). * = H_2O.

(M_w) of the polymers remained below 10000 Da, with the exception of polymer **4** due to its exceptional solubility in DMF. It was noted that the polymers possess a wide molecular weight distribution (Table 1). As hydrophobicity of polymers plays a key role in the antimicrobial function of polymers, water-octanol partition coefficient (log P) of polymers was therefore measured to investigate the impact of overall polymer hydrophobicity on antimicrobial activity (Table 2). As expected, the introduction of positive charges along the polymer backbone rendered these materials water-soluble, although limited water solubility was observed for polymers **2** and **3**, presumably due to their more rigid structure and higher hydrophobicity as compared to polymer **1** or polymer **4** (log P: −1.90, −0.94 and −3.20 for polymers **1**, **2** and **4**, respectively; Log P of polymer **3** was not measured as it is not water soluble).

Assays against clinically relevant microbes: Gram-positive bacteria *S. aureus*, Gram-negative bacteria *E. coli* and *P. aeruginosa,* and fungi *C. Albicans* were performed to ascertain the antimicrobial activity of the polymers. The broth microdilution method was used to determine the MICs against these microbes, which are the lowest polymer concentrations at which microbial growth was completely inhibited after treatment. As shown in Table 2, polymers **1** and **4** demonstrated more potent antimicrobial activity, with extremely low MICs of 3.9–7.8 μg/mL, than polymer **2**. It is noteworthy that polymers **1** and **4** exhibited high potency towards Gram-negative bacteria and inhibited the growth of *E. coli* and *P. aeruginosa* at 7.8 and 3.9 μg/mL, respectively. This result is of great importance, as

Table 1
Molecular weights of polymers.

Polymer	M_w (GPC)	M_n (GPC)	M_w/M_n
1	6767	3506	1.93
2	– [a]	–	–
3	– [a]	–	–
4	20600	7930	2.60
1a	7617	3559	2.14
1b	5100	2860	1.78
1c	5500	3680	1.49
4a	5497	2656	2.07
4b	4690	2080	2.25
4c	– [a]	–	–
5	5430	2364	2.29
6	3723	1947	1.91

[a] Insoluble in GPC solvent.

there are limited antibiotics available for treating Gram-negative bacteria. In particular, *P. aeruginosa* are resistant to most antibiotics. The remarkable antimicrobial activity observed for polymers **1** and **4** might be due to the fact that they adopt alternating amphiphilic structures, where the cationic hydrophilic and hydrophobic groups are alternatively distributed in the polymer chain. This conformation is highly dynamic, and offers excellent accessibility of the hydrophobic components upon contact with the negatively charged microbial surface. The hydrophobic residues hence facilitate their insertion into the lipid bilayer and disruption of the cytoplasmic membrane, leading to microbial lysis. Similar findings were reported in our previous study, wherein polycarbonates with randomly distributed hydrophobic repeat units displayed enhanced antimicrobial activity as compared to the corresponding block copolymer analog due to better accessibility of the hydrophobic components [43]. However, the latter self-assembled into stable micelles and shielded its hydrophobic portions within the micellar core, and were thereby excluded from interacting with the bacterial cell membrane, making them ineffective against Gram-negative bacteria. In addition to hydrophobicity, the aromatic functionality may be another critical parameter for the interaction between phospholipid bilayer and polymer during penetration [44,45]. Polymer **2** with stronger hydrophobicity exhibited lower antimicrobial activity as compared to polymers **1** and **4**, likely because polymer **2** aggregated in the bacterial culture medium due to its limited water solubility.

Having observed promising broad-spectrum antimicrobial activity for polymers **1** and **4**, we then sought to further enhance antimicrobial properties by systematically bolstering the hydrophobic content. Thus, *p*-xylylene dichloride was copolymerized with varying ratios of TMEDA and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine as shown in Scheme 1, essentially forming statistical copolymers of similar molecular weights comprising the components of polymers **1** and **2** (Table 1, entries 5–7, polymers **1a**–**c**). Similarly, copolymerization of the dichloride with bis[2-(*N*,*N*-dimethylamino)ethyl] ether and *N*,*N*,*N*′,*N*′-tetramethylbenzidine afforded copolymers resembling combinations of polymers **3** and **4** (Table 1, entries 8–10, polymer **4a**–**c**). As shown in Table 2, we observed enhanced antimicrobial activity for polymer **1a** as compared to polymer **1** due to increased hydrophobicity (log P: −1.90 and −1.71 for polymers **1** and **1a**, respectively). This is because the increased hydrophobicity enhanced the interactions with the lipid domains of microbial membrane, disrupting the membrane more effectively. This finding is consistent with our previous observations that an increased content of hydrophobic monomer led to a reduced MIC [36,46]. However, further increasing the content of *N*,*N*,*N*′,*N*′-tetramethylbenzidine to 40% (polymer **1b)** and 60% (polymer **1c**) did not affect antimicrobial activity (log P: −1.68 and −1.60, respectively). Similar observations were also reported by Kuroda et al., where antimicrobial activity of polymers increased with increased butyl methacrylate composition up to 30%, and further increase of the butyl methacrylate composition did not affect the MIC [21]. This might be attributed to the fact that further increase in hydrophobicity might induce polymer aggregation in the bacterial culture medium, preventing antimicrobial action. Similarly, polymers **4a** and **4b** with increased hydrophobicity (log P: −1.85 and −1.73, respectively) exhibited an increased potency in antimicrobial activity especially against *S. aureus* and *P. aeruginosa* as compared to polymer **4** (log P: −3.20) (Table 2). Furthermore, bis[2-(*N*,*N*-dimethylamino)ethyl] ether was copolymerized with varying ratios of *p*-xylylene dichloride and 4,4'-bis(-chloromethyl)-1,1'-biphenyl as shown in Scheme 1. Polymer **6** with higher hydrophobicity (log P: −0.65) exhibited a lower antimicrobial effect than polymer **5** (log P: −1.35) possibly due to aggregation in the bacterial culture medium. Nonethless, both polymers exhibited strong antimicrobial activity against all the microbes tested.

The antimicrobial activity of triclosan and chlorhexidine digluconate, which are widely used in personal care products, was compared with the polyionenes under the same testing conditions. Both of them exhibited a lower antimicrobial effect especially against Gram-negative bacteria than the polymers (Table 2). Compared to other polymers, polymer **1** displayed excellent antimicrobial potency and the highest selectivity (HC_{50}/MIC: > 732, where MIC was defined as the average MIC of polymers against the various microbes) (Table 2), and was therefore selected for further studies.

Table 2
MICs and hemolytic activity (HC_{50}) of polymers.

Polymer	MIC (μg/mL)				HC_{50} (μg/mL)	Log P	Selectivity[a] (HC_{50}/MIC)
	S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*			
1	3.9	7.8	7.8	7.8	>5000	−1.90	>732
2	7.8	31.3	250	31.3	188	−0.94	2.3
4	3.9	3.9	3.9	3.9	>1000	−3.20	>256
1a	1.95	3.9	3.9	3.9	1000	−1.71	293
1b	1.95	3.9	3.9	3.9	500	−1.68	146
1c	1.95	3.9	3.9	3.9	500	−1.60	146
4a	1.95	3.9	1.95	7.8	>1000	−1.85	>256
4b	1.95	3.9	1.95	7.8	>1000	−1.73	>256
5	3.9	3.9	7.8	3.9	800	−1.35	164
6	3.9	7.8	7.8	7.8	200	−0.65	29
Chlorhexidine digluconate	N.T	15.6	31.3	3.9	250		15
Triclosan	N.T	N.T	>500	N.T	N.T		

N.T. denotes not tested.
[a] Selectivity is calculated as HC_{50}/MIC, where MIC was defined as the average MIC of polymers against various microbes.

To assess the activity of polymers under physiological conditions, the MIC values of polymer **1** were measured in the presence of 10% FBS. The MICs against *E. coli* and *P. aeruginosa* slightly increased to 15.6 μg/mL, indicating that polymer **1** retained its potency even in the presence of serum. This is of importance for its application under physiological conditions as peptides and antibiotics were reported to have reduced antimicrobial activity in the presence of serum due to association with serum proteins [47].

To determine the microbicidal properties of the polymers, colony counting assays were performed. Bacterial samples (*S. aureus* and *E. coli*) treated with polymer **1** at MIC and 2 × MIC for 18 h showed that the bacteria were completely eradicated (no colonies were observed), indicating a microbicidal mechanism. For clinical applications, the disinfecting agents should exert potency in a rapid bactericidal manner. The killing kinetics of the polymers was further evaluated on *S. aureus* and *E. coli* using polymer **1** as an example. As illustrated in Fig. 2a, the polymer caused a greater than 2-log reduction in the number of viable *S. aureus* colonies (>99% killing efficiency) within 2 min, and eradicated all the bacterial cells in 10 min at 4 × MIC (i.e. 15.6 μg/mL). At 31.3 μg/mL, a killing efficiency of ~100% was obtained in 15 s. In the case of *E. coli*, the killing efficiency was almost 100% with concentrations of 15.6 μg/mL and 31.3 μg/mL (Fig. 2b) at 2 min. For *P. aeruginosa*, which is notoriously difficult to kill, the killing efficiency was 99.5% (>2-log reduction of the initial inoculum) and ~100% with a concentration of 100 mg/L at 10 min and 20 min, respectively (Fig. 2c). Notably, *P. aeruginosa* were completely killed by polymer **1** in less than 2 min at a higher concentration of 200 μg/mL (Fig. 2c). To the best of our knowledge, polymer **1** possesses the fastest biocidal kinetics over a broad spectrum of microbes compared to the numerous antimicrobial peptides and polymers published in the literature thus far.

The antimicrobial activity of polymers was further investigated using clinically isolated multidrug-resistant bacteria and fungi. As seen in Table 3, polymer **1** effectively inhibited the growth of all microbes with low MIC values of 1.95–31.3 μg/mL, demonstrating strong and broad-spectrum antimicrobial activity against multidrug-resistant bacteria *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, as well as fungi *C. albicans* and *C. neoformans*. It is noted that treatment of clinically isolated *E. coli* and *P. aeruginosa* with polymer **1** showed slightly higher MIC value as compared to that in the ATCC strain (15.6 *vs* 7.8 μg/mL). This was possibly due to the fact that the phospholipid composition of cell membranes differed with different strains.

To assess the potential emergence of bacterial resistance after repeated use of cationic polymers, bacteria were exposed multiple times to polymer **1** at a sub-lethal concentration (1/8 of MIC). The conventional antibiotic ciprofloxacin was used as the control. Multiple treatments with ciprofloxacin induced drug resistance at passage 4, as evidenced from the doubling of the MIC value ($MIC_4/MIC_0 = 2$, $MIC_0 = 3.9$ μg/mL). This was followed by a 4-fold increase in MIC value at passage 7 (Fig. 3), indicating the emergence of resistant strains. There were a number of studies reporting chlorhexidine-resistant *S. aureus* isolated from patients, and chlorhexidine-resistant methicillin-resistant *S. aureus* (MRSA) [48,49]. More recently, reduced levels of microbial susceptibility to chlorhexidine were reported [50]. Notably, repeated treatments with polymer **1** did not induce any drug resistance during the course of study over 10 passages.

To elucidate the antimicrobial mechanism of the polymers, we investigated the membrane permeability changes before and after incubation with polymer **1** for 2 h at 2 × MIC and 4 × MIC using propidium iodide (PI) and FITC-labeled dextran dye (100 kDa). It was observed that there was no fluorescence in the untreated *S. aureus*, suggesting that the bacterial membrane was intact

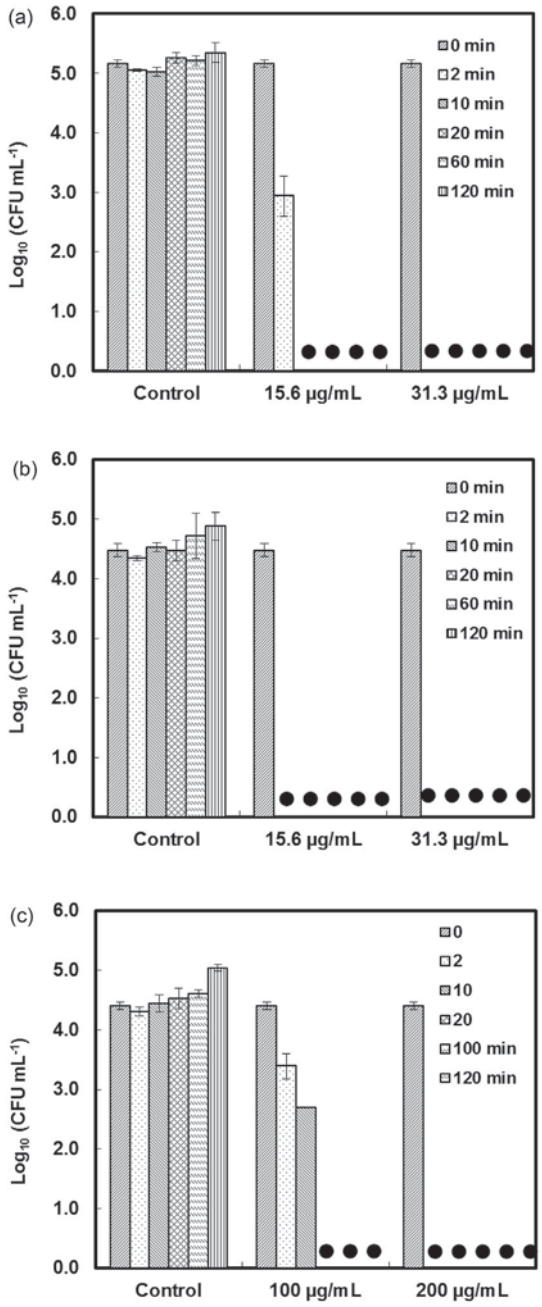


Fig. 2. Killing kinetics of polymer **1** at 15.6 μg/mL and 31.3 μg/mL on *S. aureus* (a), 15.6 μg/mL and 31.3 μg/mL on *E. coli* (b), 100 μg/mL and 200 μg/mL on *P. aeruginosa* (c). Initial bacterial loading: 1.5 × 10^5 CFU/mL (• denotes no colonies were observed).

(Fig. 4). In sharp contrast, cells treated with polymer **1** showed red fluorescence at both concentrations, indicating that PI had intercalated with DNA upon entering the membrane-disintegrated cells via passive diffusion. Disintegration of bacterial cell membranes was also observed by uptake of FITC-labeled dextran. These observations clearly revealed that the antimicrobial property of polymer **1** was derived from the disruption of the bacterial membrane. As the size of FITC-labeled dextran particles in solution was ~13 nm or larger, the size of the pores induced by polymer **1** on the membrane of *S. aureus* was likely to be ~13 nm or larger. This finding is in agreement with the membrane lytic mechanism of various synthetic antimicrobial polymers reported in the literature [19]. In addition, after treatment at 4 × MIC, the intensity of

Table 3
Antimicrobial activity of polymer **1** against clinically isolated microbes.

	MIC (μg/mL)
S. aureus 34698	3.9
MRSA34712	3.9
MRSA34689	7.8
E. coli 34723	15.6
E. coli 34729	15.6
P. aeruginosa 25844	15.6
A. baumannii 34727	3.9
A. baumannii 34716	15.6
K. pneumoniae 34693	31.3
K. pneumoniae 34694	15.6
C. albicans 34092	7.8
C. neoformans 34709	1.95

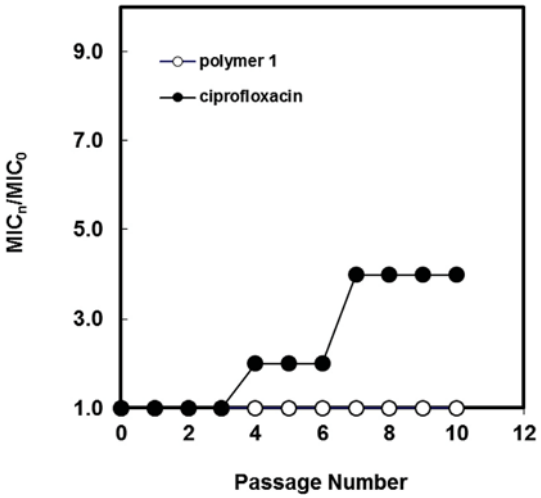


Fig. 3. Changes in MIC of antimicrobial agents against *E. coli* 25922 upon multiple sub-lethal dose exposures.

fluorescence was greater, indicating that polymer **1** demonstrates membrane disintegration in a dose-dependent manner. In the case of *P. aeruginosa*, strong red fluorescence for PI was seen for the treated cells, whereas weak green fluorescence for FITC-labeled dextran was observed (Fig. 4). The results revealed that polymer **1** caused a greater extent of membrane disruption towards *S. aureus* as compared to *P. aeruginosa*. This correlated well with the varying antimicrobial activity of polymer **1** towards *S. aureus* as compared to *P. aeruginosa* (Table 2, Fig. 2). It is expected that the physical disruption of bacterial cell membranes by polymer **1** will provide advantages for various medical applications due to the reduced probability of developing drug resistance.

Hemolysis has been commonly used as a preliminary assessment of selectivity of antimicrobial agents over mammalian cells. The hemolytic behavior of the polymers towards rat red blood cells (rRBCs) was evaluated at various concentrations. As shown in Table 2 and Fig. 5a, negligible or no hemolysis was observed for all polymers at their respective MIC values. Notably, polymer **1** showed negligible hemolytic activity even up to 5000 μg/mL (the highest concentration tested, Fig. S1), demonstrating its excellent selectivity toward a broad range of pathogenic microbes over mammalian cells. This also indicates that polymer **1** binds weakly to the RBCs membrane, and there was no polymer permeation into RBCs membrane. Hydrophobic/hydrophilic balance of an antimicrobial polymer is a pivotal structural determinant of how the polymer interacts with cellular membranes, and as a result impacts selectivity of microbes over mammalian cells. The log P values of the polymers were thus used to better understand the impact of overall polymer hydrophobicity on its biological activity. The hemolytic activity (HC_{50} values) increased with an increase in the hydrophobicity of cationic polymers. For instance, the log P value of polymer **2** bearing aromatic substituents of higher hydrophobicity was significantly higher than that of polymer **1** (log P: −0.94 and −1.90, respectively). As such, polymer **2** was far more hemolytic (HC_{50} = 188 μg/mL) than polymer **1** ($HC_{50} \gg$ 5000 μg/mL). Polymer **4** containing bis[2-(*N*,*N*-dimethylamino)ethyl] ether substituents of lower hydrophobicity (log P = −3.2) when compared to polymer **1** caused 20% hemolysis at 1000 μg/mL because of its higher molecular weight. Compared to polymer **1**, polymers **1a**–**c** of greater hydrophobicity (log P: −1.71, −1.68 and −1.60, respectively) were more hemolytic (HC_{50}: 1000, 500 and 500 μg/mL, respectively). Similarly, hemolytic activity of polymers **4**–**6** increased with increasing hydrophobicity (log P: −3.2, −1.35 and −0.65, respectively). This observation is consistent with previous reports that increased hydrophobicity enhances hemolytic activity due to strong interaction between the polymer and the lipid region of the cellular membrane [21]. The results suggest that by rationally designing cationic group structure, it is possible to yield polymers with remarkable selectivity towards microbial cells and low toxicity to mammalian cells. For potential application as topical antimicrobials and antiseptics, the cytotoxicity of polymer **1** was evaluated in human dermal fibroblasts (HDFs). Even with 500 μg/mL of polymer **1**, the cell viability was more than 80% after 2 min of treatment (Fig. 5b). The cytotoxicity of polymer **1** was further investigated by determining the amount of LDH enzyme released by HDFs with damaged membranes (Fig. 5c and d). Interestingly, the LDH release (cytotoxicity) in HDFs exposed to polymer **1** for 2 min was less than 2% at concentrations of 4–500 μg/mL (Fig. 5c). However, HDFs exposed to chlorhexidine digluconate for 2 min demonstrated significantly higher LDH release than polymer **1** when the concentration was above 125 μg/mL. Even when the treatment time with polymer **1** increased to 1 h, LDH release in HDFs was still less than 10% at concentrations up to 500 μg/mL (Fig. 5d). In sharp contrast, increasing the concentration of chlorhexidine digluconate and its exposure time drastically increased its cytotoxicity (e.g. more than 80% at a concentration of 125 μg/mL for 1-h treatment) (Fig. 5c and d). Taken together, the results indicated that polymer **1** exhibited excellent biocompatibility with HDFs.

To demonstrate its safety for future clinical applications (e.g. surgical scrubs), polymer **1** was evaluated for dermal toxicity. Mice were randomly grouped (five in each group), and skin treated with six topical solutions twice a day over 5 days. The six treatments were as follows: water (control), polymer **1** (200 and 500 μg/mL), betadine (control), chlorhexidine (neutral, control, 4% w/v in Bactishield handwash) and benzalkonium chloride (cationic, control, 0.198% w/v, a concentration used in Dettol handwash). These samples were evaluated specifically for any compromise in cellular integrity, changes in tissue structure or evidence of inflammation in the epidermis, dermis and subcutaneous levels. As shown in Fig. 6b and c, in the samples treated with polymer **1** (200 and 500 μg/mL), no significant accumulation of inflammatory cells was seen. Mild interstitial oedema (accumulation of fluid in dermis, arrow 2) not amounting to a pathology and mild parakeratosis (thickening of top-most layer of epidermis, arrow 1) were observed. Epidermal cellular vacuolization (intracellular oedema signifying cell death) and acantholysis (separation of adjacent cells due to loss of intercellular adhesions) that signify early cellular damage were not observed. Importantly, the overall structure and cellular integrity of the skin was preserved. In the samples treated with the positive controls, including clinically used betadine (povidone-iodine) and

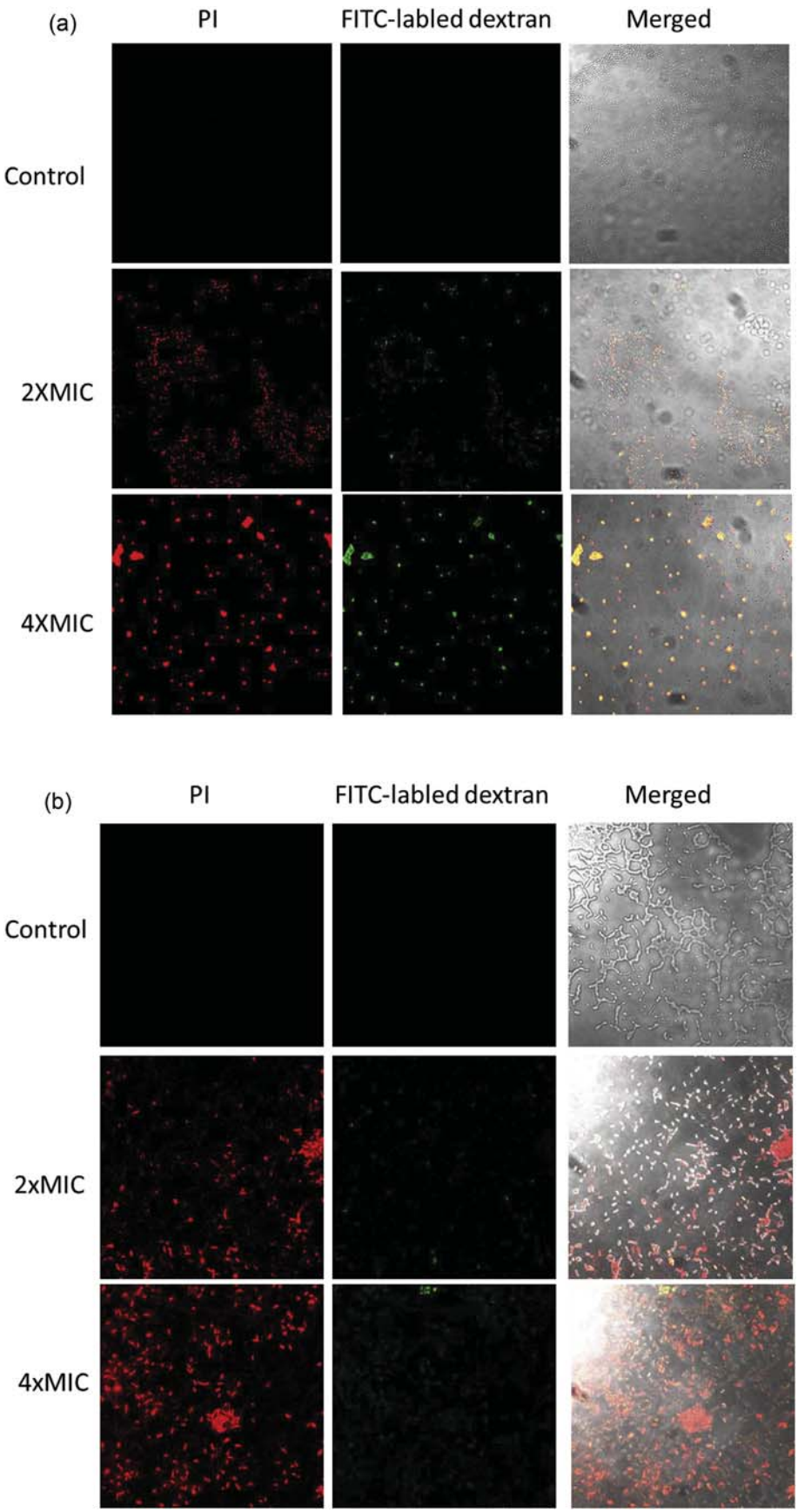


Fig. 4. Confocal microscopic images of (a) *S. aureus* and (b) *P. aeruginosa* incubated with polymer **1** for 2 h at various concentrations (red fluorescence: PI; green fluorescence: FITC-labeled dextran (100 KDa)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

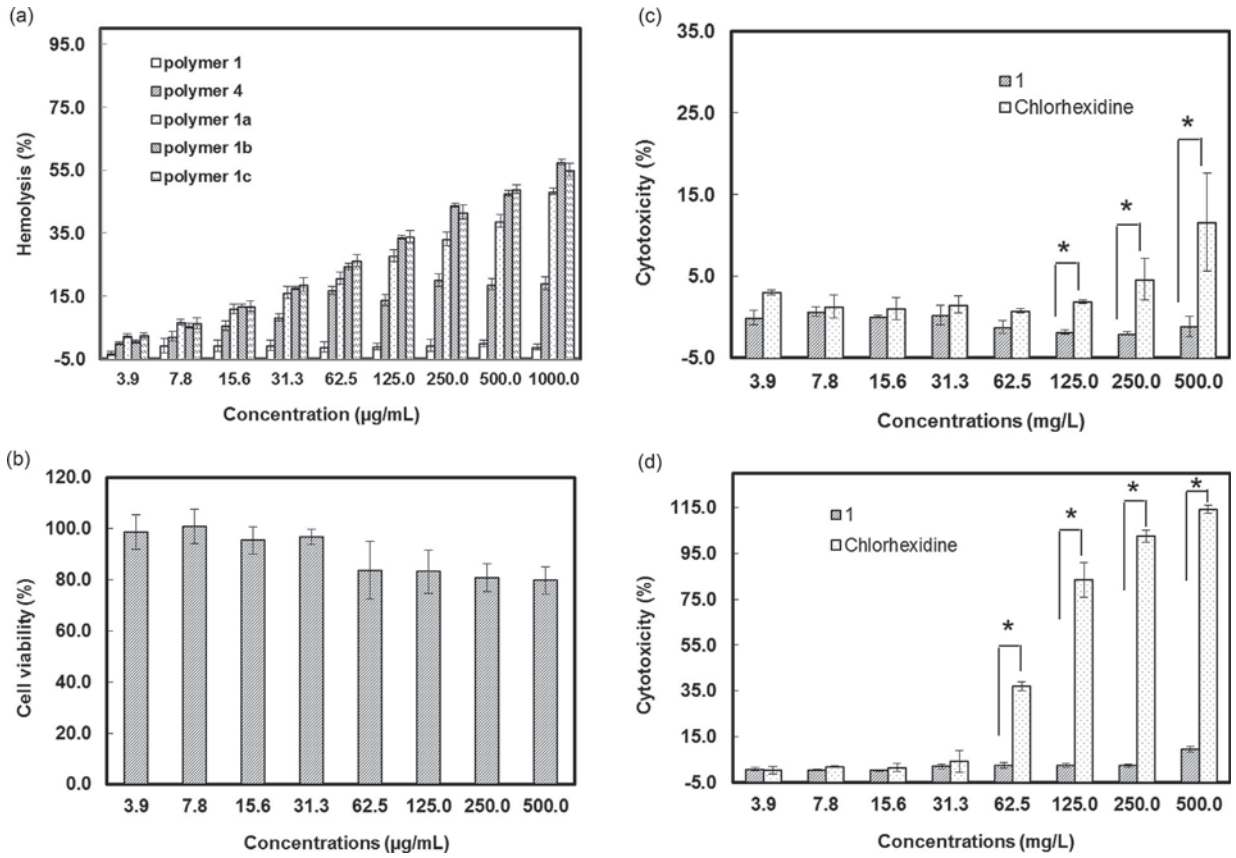


Fig. 5. *In vitro* biocompatibility studies. (a) Hemolytic activity of the polymers with various cationic group structures as a function of polymer concentration. (b) Viability of human dermal fibroblasts after 2 min of treatment with polymer **1** via MTT assay. (c) Cytotoxicity was determined by LDH release after (c) 2 min and (d) 1 h of treatment of HDFs with polymer **1** or chlorhexidine *via* LDH assay. *: $p < 0.05$.

chlorhexidine, however, varying extents of damage to the skin was noted (Fig. 6d–f). There were spongiotic changes (significant intercellular accumulation of fluid in epidermis) associated with inflammatory infiltration in the epidermal to dermal layer (arrow 3), suggesting significant dermal inflammation. In the epidermal layer, there was evidence of acantholysis (intercellular separation and rounding up of cells), denoting an early insult to the skin (arrow 5). Acantholysis surrounding the hair follicular units could also be seen (arrow 6). The findings of acanthosis (thickening of the epidermal layer, arrow 4), parakeratosis and hyperkeratosis (thickening of dead skin, the top-most layer of epidermis) also suggested a cumulative irritation effect to the skin.

The results indicated that topical application of polymer **1** (200 and 500 μg/mL) showed a better skin compatibility profile in mice as compared to the clinically used surgical scrubs betadine and chlorhexidine. Acute oral toxicity (LD_{50}, lethal dose at which half the mice are killed) was studied to further evaluate whether the compound would be safe to handle. LD_{50} of polymer **1** was estimated to be 1871 mg/kg *via* oral administration, indicating that the polymer has low toxicity. Moreover, most surgical scrubs and hand sanitizers in hospitals and clinical environments are alcohol-based, e.g., betadine. Non-alcohol based formulations are desirable due to their non-dehydrating characteristics. They are gentler on skin particularly in the presence of cuts and chapped hands and less expensive. Except for polymers **3**, all other polymers are soluble in water, easy to be formulated into non-alcohol based surgical scrubs and hand sanitizers.

To evaluate the *in vivo* bactericidal activity of polymer **1**, *P. aeruginosa* was introduced to contaminate the normal dorsal skin of mice [42]. The contaminated skin was treated with polymer **1** (500 μg/mL) and the clinically used surgical scrub chlorhexidine (4% w/v in Bactishield handwash); mice without treatment were employed as control. The number of *P. aeruginosa* recovered from the contaminated skin treated with polymer **1** and chlorhexidine handwash for 2 min (2.50 and 3.59 $\log_{10}$ CFU) was found to be significantly reduced as compared to the control (5.25 $\log_{10}$ CFU, $p < 0.0005$, Fig. 7). The treatment with polymer **1** decreased the number of *P. aeruginosa* on the skin more effectively than chlorhexidine handwash ($p < 0.01$, Fig. 7).

4. Conclusion

Potent and cost-effective antimicrobial polymers have been successfully synthesized through simple addition polymerization using commercially available, relatively inexpensive starting materials. They have demonstrated strong and broad-spectrum microbicidal activity through membrane-lytic mechanism and ability of mitigating drug resistance. By varying the ratio of hydrophobic-to-hydrophilic groups, polymers with high potency and low cytotoxicity can be easily synthesized. At the optimal compositions, the antimicrobial polymer exhibits rapid killing kinetics over a broad spectrum of microbes. More importantly, the polymer shows superior skin compatibility and *in vivo* bactericidal activity, as compared to the clinically used surgical scrubs betadine and chlorhexidine. Thus, the polymers reported herein may be promising antimicrobial agents, e.g. as antimicrobial surgical scrubs for the prevention of MDR infections.

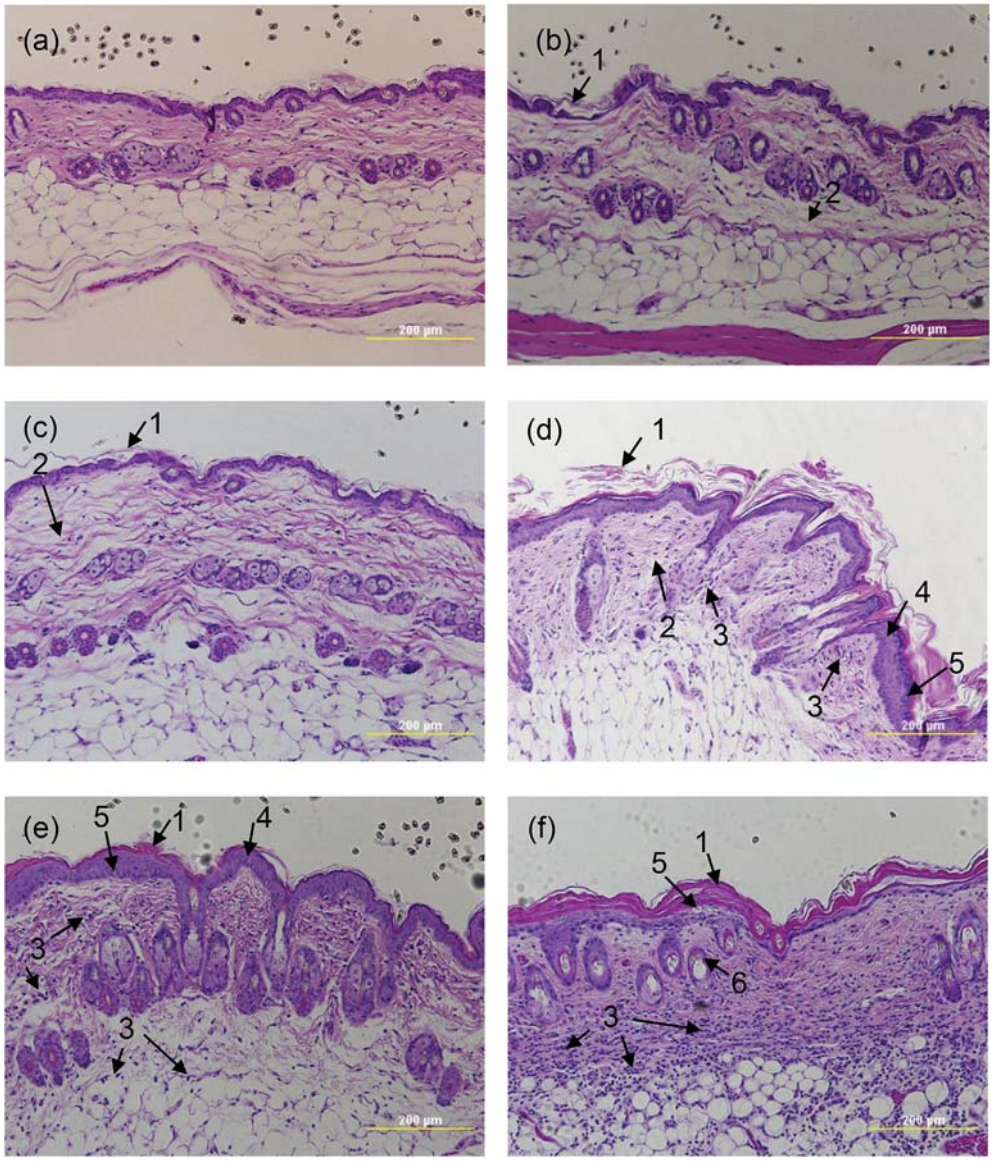


Fig. 6. Histological evaluation of mouse skin before and after topical treatment with various solutions: (a) water, (b) polymer **1** (200 μg/mL), (c) polymer **1** (500 μg/mL), (d) betadine, (e) benzalkonium chloride, and (f) chlorhexidine. Arrow 1 denotes parakeratosis (thickening of top-most layer of epidermis). Arrow 2 denotes interstitial oedema (accumulation of fluid in dermis). Arrow 3 denotes inflammatory infiltration. Arrow 4 denotes acanthosis (significant thickening of the epidermal layer). Arrow 5 denotes acantholysis (intercellular separation and rounding up of cells of epidermal layer). Arrow 6 denotes perifollicular acantholysis (separation of adjacent perifollicular cells).

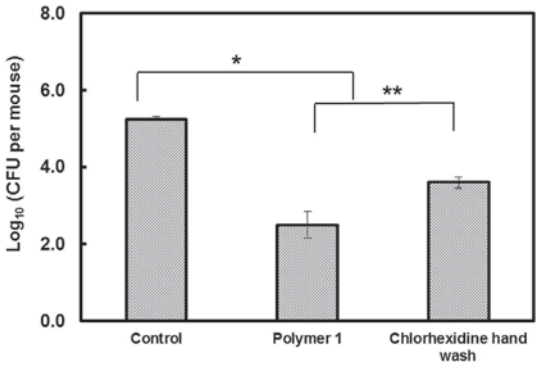


Fig. 7. CFU of *P. aeruginosa* on the contaminated mouse skin after treatment with polymer **1** (500 μg/mL) and chlorhexidine (4% w/v in Bactishield handwash); mice without treatment were used as control. *: $p < 0.0005$; **: $p < 0.01$, polymer **1** *vs.* chlorhexidine handwash. (n = 5).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.02.027.

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