Design and Synthesis of Biodegradable Grafted Cationic Polycarbonates as Broad Spectrum Antimicrobial Agents

Zhan Yuin Ong,1 Daniel J. Coady,2 Jeremy P. K. Tan,1 Yan Li,1 Julian M. W. Chan,2 Yi Yan Yang,1 James L. Hedrick2

1Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669, Singapore
2IBM Almaden Research Center, 650 Harry Road, San Jose, California 95120
Correspondence to: Y. Y. Yang (E-mail: yyyang@ibn.a-star.edu.sg) or J. L. Hedrick (E-mail: hedrick@us.ibm.com)

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INTRODUCTION The emergence of antibiotic-resistant “superbugs” coupled with the scarcity of new small molecule antibiotics coming through the drug development pipeline have necessitated the discovery and identification of alternative classes of antimicrobial agents.1 In recent years, polymeric biocides have drawn significant attention as a class of antimicrobial agents with a novel mechanism of action that could potentially overcome antibiotics resistance.2–5 These polymers are often amphiphilic in nature, containing cationic functionalities that interact electrostatically with anionic microbial membranes, and hydrophobic groups that insert into the lipid bilayers to cause membrane disintegration. In contrast to small molecule antibiotics that target a specific biosynthetic pathway, the rapid and extensive membrane-lytic action of antimicrobial polymers greatly reduces the likelihood of resistance development. To date, numerous antimicrobial polymers such as poly(4-vinyl-N-alkylpyridinium bromide),6 cationic poly(methacrylate) derivatives,7 amine- or ammonium-terminated carbosilane dendrimers,8 and cationic polyurethanes9 have been synthesized and were shown to be active against various pathogens. However, poor selectivities and non-degradability of the polymer backbone have raised serious bioaccumulation- and toxicity-related concerns for in vivo applications. Therefore, there is a strong impetus to develop bio-friendly polymeric biocides as therapeutic agents for a wider range of antimicrobial applications.

Aliphatic polycarbonates have recently emerged as a highly attractive and versatile class of biomaterials due to their inherent biodegradability, low cytotoxicity, and tunable materials properties.10–12 Recently, we reported the synthesis of the first biodegradable linear cationic triblock polycarbonates by metal-free organocatalytic ring-opening polymerization. These polymers were highly active against Gram-positive Bacillus subtilis, Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA) and Enterococcus faecalis, as well as the fungus Cryptococcus neoformans.13 Notably, it was demonstrated that the biodegradable polymers induced minimal hemolysis at minimum inhibitory concentrations (MICs), and did not significantly alter the electrolyte balance nor liver and kidney functions at 48 h following intravenous injection in mice. In a subsequent study, a series of highly dynamic random cationic polycarbonates were designed and found to have a wider spectrum of antimicrobial activities, which now extends to Gram-negative microbes such as Escherichia coli and Pseudomonas aeruginosa, than the previously reported block copolymers.14 The improved antimicrobial activities of the random copolymers against Gram-negative bacteria were attributed to the ease of micellar disassembly at the bacterial surface, which exposes the hydrophobic moieties required for membrane insertion and disruption. The results of these studies demonstrate that the rationally designed cationic polycarbonates possess good antimicrobial selectivities and hold great potential as therapeutic agents in the fight against antibiotic-resistant microbes.

In the design of polymeric biocides, graft and dendritic systems offer advantages over linear systems in terms of having higher cationic charge densities and the greater ease of tuning the hydrophobic/hydrophilic balance for optimal polymer solubility and antimicrobial activity.5,15,16 However, the main challenge in the synthesis of branched and graft polymers lies in the poor control of molecular weights, giving rise to heterogeneous polymer systems with biological effects that
are difficult to delineate. In our efforts to investigate structure-activity relationships and to expand on the current arsenal of biodegradable polycarbonate-based biocides, we designed and synthesized a new class of narrowly-dispersed cationic graft polycarbonates using metal-free polymerization. Specifically, functional carbonate monomers were organocatalytically ring-opened to give a linear aliphatic polycarbonate with pendant alcohols that can serve as initiators for the subsequent grafting of polymer brushes composed of trimethylene carbonate (TMC), \( \varepsilon \)-lactide (LAC), methacryloxytrimethylene carbonate-3-chloropropyl ester (MTC-OPrCl), and methacryloxytrimethylene carbonate-benzyl chloride ester (MTC-OBnCl) monomers. Subsequent quaternization of the copolymers with the bis-tertiary amine tetramethylethylenediamine (TMEDA) affords quaternary and tertiary amines for interaction with anionic microbial membranes. The hydrophobic composition and molecular architecture of the cationic graft copolymers were systematically varied to investigate their antimicrobial activities against clinically relevant Gram-positive \( S. \) aureus, Gram-negative \( E. \) coli, and yeast \( C. \) albicans as well as their cytotoxicities in comparison to a linear control polymer. In addition, the microbial membrane-disrupting activities of the cationic graft polycarbonates were studied using dye leakage experiments, confocal-laser scanning microscopy, and field-emission scanning electron microscopy.

**EXPERIMENTAL**

**General**

All monomers were synthesized using a previously reported synthetic procedure.\(^{17} \) The general synthetic protocol involved combination of MTC-OC\(_6\)F\(_5\) (1 eq.), respective alcohol (1 eq.), and proton sponge (10 mol %) in THF [Scheme 1(a)]. The products were readily purified using column chromatography. Ring-opening polymerization of the functionalized cyclic carbonates was conducted using a previously reported procedure.\(^{18} \) The polymer backbone was synthesized by random copolymerization of MTC-OBn (40 eq.) and MTC-OEt (68 eq.) using 2,2-bis(methyl) propionic acid benzyl ester (Bn-BisMPA) as the initiator. The material (\( M_n = 11.1 \) kDa/PDI = 1.08) was deprotected via hydrogenolysis to produce a polymer with pendant hydroxyl group initiators [Polyol—Scheme 1(b)]. Using the Polyol pendant alcohols to initiate similar ROPs, each respective style of graft arms was installed, allowing for the subsequent functional activation as previously reported.\(^{19,20} \)

**Materials**

10 \( \times \) phosphate-buffered saline (PBS) was purchased from 1st Base (Singapore) and diluted to the intended concentration before use. Tryptic soy broth (TSB) and yeast mold broth (YMB) powders were purchased from BD Diagnostics (Singapore) and used according to the manufacturer’s instructions. *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 25922) and yeast *Candida albicans* (ATCC No. 10231) were obtained from ATCC (USA) and reconstituted according to the recommended protocols.

Phospholipids 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DOPG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained as dry powders from Avanti Polar Lipids, Inc.

**Polymer Synthesis**

**Polyol**

In a nitrogen-filled glovebox, a vial was charged with Bn-BisMPA (0.011 g, 0.050 mmol), MTC-OEt (0.650 g, 3.45 mmol), MTC-OBn (0.650 g, 2.02 mmol), \( N'-(3,5\)-bis(trifluoromethyl)phenyl\)-\( N\)-cychohexylthiourea (0.050 g, 0.13 mmol), DCM (2.5 mL), and a stir bar. The reaction was initiated by the addition of \( (\sim)\)-sparteine (0.0360 g, 0.153 mmol). Upon full monomer conversion the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration yielding a white polymer. Without additional purification the material was redissolved in THF/methanol (5/1, 30 mL) followed by addition of Pd/C (0.2 g, 10%). The reaction vessel was charged with hydrogen gas (40 psi) and reacted for 16 h. All volatiles were evaporated, yielding 1.12 g (98%) of the deprotected polymer (\( M_n = 11.6 \) kDa; PDI = 1.09).

**Polymer A (Poly(ethyl carbonate-g-lactide)-b-poly(benzyl carbonate))**

In a nitrogen-filled glovebox, a vial was charged with polyol (0.050 g, 0.0042 mmol), \( \varepsilon \)-lactide (0.12 g, 0.83 mmol), \( N'-(3,5\)-bis(trifluoromethyl)phenyl\)-\( N\)-cychohexylthiourea (0.030 g, 0.081 mmol), DCM (0.75 mL), and a stir bar. The reaction was initiated by the addition of \( (\sim)\)-sparteine (0.0360 g, 0.153 mmol). After 1 h MTC-OBnCl (0.750 g, 2.49 mmol) in DCM (1 mL) was added to the reaction mixture. Upon full monomer conversion the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration, yielding 0.86 g (93%) of the graft polymer (\( M_n = 29.0 \) kDa; PDI = 1.10) as a white solid.

**Polymer B (Poly(ethyl carbonate-g-trimethylene carbonate)-b-poly(benzyl carbonate))**

In a nitrogen-filled glovebox, a vial was charged with polyol (0.050 g, 0.0042 mmol), trimethylene carbonate (0.085 g, 0.83 mmol), \( N'-(3,5\)-bis(trifluoromethyl)phenyl\)-\( N\)-cychohexylthiourea (0.030 g, 0.081 mmol), DCM (0.75 mL), and a stir bar. The reaction was initiated by the addition of \( (\sim)\)-sparteine (0.0360 g, 0.153 mmol). After 1 h MTC-OBnCl (0.750 g, 2.49 mmol) in DCM (1 mL) was added to the reaction mixture. Upon full monomer conversion the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration, yielding 0.81 g (92%) of the graft polymer (\( M_n = 22.9 \) kDa; PDI = 1.10) as a white solid.

**Polymer C (Poly(ethyl carbonate-g-benzyl carbonate))**

In a nitrogen-filled glovebox, a vial was charged with polyol (0.050 g, 0.0042 mmol), MTC-OBnCl (0.26 g, 0.83 mmol), \( N'-(3,5\)-bis(trifluoromethyl)phenyl\)-\( N\)-cychohexylthiourea (0.030 g, 0.081 mmol), DCM (0.75 mL), and a stir bar. The reaction was initiated by the addition of \( (\sim)\)-sparteine (0.0360 g, 0.153 mmol). Upon full monomer conversion, the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration, yielding 0.81 g (92%) of the graft polymer (\( M_n = 22.9 \) kDa; PDI = 1.10) as a white solid.
filtration, yielding 0.28 g (90%) of the graft polymer ($M_n = 540.2$ kDa; PDI = 1.10) as a white solid.

**Polymer D (Poly(ethyl carbonate-g-propyl carbonate))**

In a nitrogen-filled glovebox, a vial was charged with polyol (0.050 g, 0.0042 mmol), MTC-OPrCl (0.20 g, 0.83 mmol), $N'$-(3,5-bis(trifluoromethyl)phenyl)-$N$-cyclohexyl-thiourea (0.030 g, 0.081 mmol), DCM (0.75 mL), and a stir bar. The reaction was initiated by the addition of (−)-sparteine (0.0360 g, 0.153 mmol). Upon full monomer conversion, the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration, yielding 0.23 g (92%) of the graft polymer ($M_n = 23.0$ kDa; PDI = 1.23) as a white solid.

**Polymer E (Poly(benzyl carbonate))**

In a nitrogen-filled glovebox, a vial was charged with benzyl alcohol (0.0038 g, 0.035 mmol), MTC-OBnCl (0.310 g, 1.04 mmol), $N'$-(3,5-bis(trifluoromethyl)phenyl)-$N$-cyclohexyl-thiourea (0.030 g,
0.081 mmol), DCM (0.75 mL), and a stir bar. The reaction was initiated by the addition of (−)-sparteine (0.0360 g, 0.153 mmol). Upon full monomer conversion, the reaction mixture was precipitated into 2-propanol and collected via vacuum filtration, yielding 0.29 g (92%) of the polymer (Mn = 4.3 kDa; PDI = 1.31) as a white solid.

**Quaternization Procedure**
Each respective polymer was dissolved in DMF (0.5 M) followed by the addition of TMEDA (15 eq.). The reaction mixture was stirred for 16 h at ambient temperature (−25 °C (polymers a, b, c, and e) or 50 °C (polymer d)) then precipitated into diethyl ether. The quaternized polymer was redissolved in methanol and precipitated a second time in diethyl ether followed by drying under vacuum.

**Antimicrobial Activity and Cytotoxicity Characterizations**
The antimicrobial activities of the cationic graft polycarbonates were investigated against *S. aureus*, *E. coli*, and *C. albicans* using the broth microdilution method. The colony counts of microorganisms remaining after treatment with the polymers were determined by serially diluting the treated samples followed by plating onto LB agar plates. To study the antimicrobial mechanisms of the cationic graft polycarbonates, calcein dye leakage assay, confocal laser scanning microscopy following a double staining procedure with Hoechst 33342 and FITC-dextran (500 kDa) and field emission-scanning electron microscopy (FE-SEM) were performed. The hemolytic activities of the cationic graft polycarbonates, calcein dye leakage mechanisms of the cationic graft polycarbonates, calcein dye leakage assay, and human dermal fibroblasts (HDF) cell line. Full experimental details are available in the Supporting Information.

**RESULTS AND DISCUSSION**
The functional polycarbonates utilized in this study were all prepared from the common starting material MTC-OG6F5 that bears a readily substituted pentafluorophenyl moiety. Using our previously published method,17 a range of different cyclic carbonate monomers can be easily synthesized via nucleophilic substitution by an appropriate alcohol [Scheme 1(a)]. Each monomer is taken up in the polymerization step to produce different segments of the random block copolymer, with each segment having unique pendant side chains. This method, based on an organocatalyzed living ring-opening polymerization, allows for fine control over the polymer structure/composition, molecular weight, dispersity, and three-dimensional (3D) architecture. It also allows for the introduction of side chains that are amenable to postpolymerization modification. In the case here, polymer segments functionalized with benzyl-protected alcohols are introduced in the polymerization step, and subsequently deprotected via Pd-catalyzed hydrogenolysis. The free OH groups serve as initiation sites for “grafting from” polymerization of TMC, LAC, MTC-OBnCl, and MTC-OPrCl monomers, leading to highly complex macromolecular structures. Using gel permeation chromatography, all polymers were found to have narrow unimodal molecular weight distributions with polydispersity indices ranging from ≈1.1 to 1.3. A subsequent amination of the benzyl chloride or propyl chloride functional groups in the polymer side chains using a large excess of TMEDA confers cationicity to the resultant polymers.

In order to investigate the effects of polymer architecture on antimicrobial properties, the minimum inhibitory concentrations (MICs) of the graft and linear polymers were determined against Gram-positive *S. aureus*, Gram-negative *E. coli*, and yeast *C. albicans*. As seen in Table 1, the graft polymers demonstrated broad-spectrum antimicrobial activities against the panel of clinically relevant microorganisms with MICs ranging from 62.5 to 1000 mg L−1. To compare the effects of polymer architectures on antimicrobial properties, a linear cationic homopolymer was synthesized. In general, the higher molecular weight graft Polymers a–c containing MTC-BnCl monomers were found to be more effective than their corresponding linear polymer (Polymer e) at inhibiting microbial growth, as evident from the lower MIC values obtained (Table 1). This result is consistent with various reports in the literature, which suggest that polycations with larger molecular weights, up to a certain limit, have increased adsorption onto negatively charged microbial membranes, resulting in enhanced membrane disruption.22,23

Additionally, it was also found that both the hydrophobic and hydrophilic compositions of the grafted polymer chains influenced antimicrobial activities to varying extents. For instance, Polymer c, which does not contain a hydrophobic block in its side chains displayed the strongest and widest spectrum of activities, with a geometric mean MIC value (calculated as the sum of MIC values divided by the number of microorganisms for which growth is inhibited) of 145.8 mg L−1 as compared with higher values of 229.2 and 531.3

### Table 1 Minimum Inhibitory Concentrations (MICs) and Selectivities ([HCO2/MIC]; in Parentheses) of the Cationic Polycarbonates

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initiator</th>
<th>Hydrophobic Block</th>
<th>Hydrophilic Block</th>
<th><em>S. aureus</em></th>
<th><em>E. coli</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Polyol</td>
<td>LAC (10)</td>
<td>MTC-BnCl (30)</td>
<td>125 (&gt;16)</td>
<td>500 (&gt;4)</td>
<td>62.5 (&gt;32)</td>
</tr>
<tr>
<td>b</td>
<td>Polyol</td>
<td>TMC (10)</td>
<td>MTC-BnCl (30)</td>
<td>&gt;1000 (&gt;2)</td>
<td>&gt;1000</td>
<td>62.5 (&gt;32)</td>
</tr>
<tr>
<td>c</td>
<td>Polyol</td>
<td>None</td>
<td>MTC-BnCl (10)</td>
<td>250 (4.8)</td>
<td>125 (9.6)</td>
<td>62.5 (19.2)</td>
</tr>
<tr>
<td>d</td>
<td>Polyol</td>
<td>None</td>
<td>MTC-PrCl (10)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>1000 (1.4)</td>
</tr>
<tr>
<td>e</td>
<td>BnOH</td>
<td>None</td>
<td>MTC-BnCl (30)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>125 (&gt;8)</td>
</tr>
</tbody>
</table>

MIC [mg L−1], (Selectivity)
mg L\(^{-1}\) of the more hydrophobic lactide or trimethylene carbonate block-containing Polymers a and b, respectively. Polymer a, on the other hand, was marginally superior to Polymer c against Gram-positive S. aureus. It can also be seen that the incorporation of MTC-BnCl monomers in Polymer c conferred better antimicrobial activities as compared with the MTC-PrCl-containing Polymer d despite both graft polymers having similar charge densities (Table 1). These results suggest that the molecular composition of Polymer c afforded the optimal hydrophobic-hydrophilic balance to interact and disrupt anionic microbial membranes.

Next, the mode of antimicrobial action was investigated by determining microbial colony counts after 18 h incubation with various concentrations of the polymers. Representative growth curves as a function of concentration of various polymers are shown in Supporting Information Figure S1. At MICs and above, the polymers effectively reduced colony numbers by more than 3 log reductions (>99.9% killing efficiency) as compared with microorganisms treated with the control comprised of 10% (by volume) PBS in growth medium. These results clearly indicate that the cationic graft polymers inhibited the growth of microorganisms via a bactericidal effect as opposed to the bacteriostatic mechanism exerted by various classes of small molecule antibiotics such as the macrolides, sulfonamides and tetracyclines.

The amphiphilic nature of cationic antimicrobial peptides (AMPs) and polymers are known to permit selective electrostatic interactions and disruption of anionic microbial membranes, leading to leakage of cytoplasmic contents and rapid cell death.\(^2\) To investigate the membrane disruption ability of the graft cationic polymer, we measured the leakage of the fluorescent calcein dye from negatively-charged large unilamellar vesicles (LUVs) composed of 4:1 (by weight) DOPE/DOPG to mimic E. coli cytoplasmic membrane following treatment with Polymer c. From Supporting Information Figure S2, it can be seen that Polymer c induced a dose-and time-dependent leakage of the entrapped calcein dye. In particular, more than 90% dye leakage was observed almost immediately following addition of Polymer c to a final concentration corresponding to its MIC value (125 mg L\(^{-1}\)) against E. coli, indicating the rapid and efficient permeabilization of the E. coli membrane-mimicking LUVs. In order to visualize the permeation of microbial cell membranes by the cationic graft polymer, E. coli was treated with Polymer c for 1 h and incubated with FITC-conjugated dextran (500 kDa molecular weight) and Hoechst dye as fluorescent probes. Hoechst dye is a cell-permeable DNA-binding dye and thus is able to stain DNA in bacteria cells regardless of their cell viability. The macromolecule, FITC-conjugated dextran, on the other hand, is unable to enter intact bacterial cells unless their membrane integrity has been compromised by treatment with Polymer c. As seen in Figure 1(a), green fluorescence due to conjugated FITC was clearly visible within E. coli cells indicating that the cationic graft polymer had effectively perturbed the microbial membranes, forming pores

**FIGURE 1** Confocal images of E. coli treated for 1 h with (a) 250 mg L\(^{-1}\) of Polymer c or (b) 10% (by volume) PBS in tryptic soy broth after incubation with FITC-conjugated dextran (500 kDa molecular weight; Intensity average diameter ~129 nm as measured by dynamic light scattering) and Hoechst dye. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
sufficiently large to permit entry of the FITC-conjugated dextran molecules by diffusion. Conversely, no FITC fluorescence was detected in E. coli cells treated with the control comprising 10% v/v of PBS in growth medium [Fig. 1(b)], while blue fluorescence due to Hoechst dye was found to be uniformly distributed throughout all the treated and untreated microbial cells. This result clearly indicates the cellular exclusion of the macromolecular FITC-conjugated dextran by intact microbial membranes. The morphological changes to the bacterial cell surface following treatment with Polymers a and c were further studied using FE-SEM. As shown in Supporting Information Figure S3, treatment of S. aureus and E. coli for 1 h with 250 mg L\(^{-1}\) of Polymer a and c, respectively resulted in obvious membrane roughness and collapse as compared with the relatively smooth and intact surfaces of the respective controls treated with 10% (by volume) PBS in growth medium. The significant membrane damage mediated by the cationic graft polymer after the 1 h incubation is consistent with the detection of FITC-conjugated dextran fluorescent probe in the polymer-treated bacteria cells seen in Figure 1(a).

For therapeutic application of the cationic graft polycarbonates, it is imperative for the polymers to exhibit good selectivities toward microbial membranes with minimal perturbation of higher eukaryotic cell membranes. Thus, we next investigated the activities of the various polymers against mammalian cell membranes using 4% (by volume) rat red blood cells and human dermal fibroblast HDF cells. As seen from Figure 2(a), cationic graft polycarbonates a, b, and c induced minimal hemolysis (<9%) at their MIC values; with high HC\(_{20}\) values (defined as the lowest polymer concentration that induces 20% or more hemolysis) ranging from 1200 to >2000 mg L\(^{-1}\). In this study, HC\(_{20}\) was adopted as a more stringent measure of the hemocompatibility of the polymers as compared with conventional HC\(_{50}\) values cited in the literature. The selectivity indices (SIs) of the polymers were also quantified as the ratio of HC\(_{20}\) to the MIC value and are shown in Table 1. Among the various cationic graft polycarbonates, Polymers a and c demonstrated the best selectivities toward various microorganisms as seen from SIs ranging from >4 to >32. Owing to their stronger antimicrobial activities, Polymers a and c were found to result in stronger selectivities towards S. aureus (SI > 16) and E. coli (SI 9.6), respectively. As such, the effects of Polymers a and c on cell proliferation were further evaluated using an MTT assay. From Figure 2(b), it can be seen that Polymer a demonstrated minimal cytotoxicities to HDF cells with >86.5% cell viability up to the highest MIC value of 500 mg L\(^{-1}\) observed (for E. coli) among the various microorganisms tested. Similarly, Polymer c has an IC\(_{50}\) value of 290 mg L\(^{-1}\), with >85.2% cell viability up to 125 mg L\(^{-1}\), which corresponds to the MIC value for E. coli and is above the MIC value for C. albicans (62.5 mg L\(^{-1}\)). These results thus demonstrate that Polymers a and c with their promising activities and selectivities against S. aureus, E. coli and C. albicans have potential as novel antimicrobial agents to overcome the problem of antibiotics resistance.

CONCLUSIONS

In this study, we have described the synthesis of a novel class of cationic graft polycarbonates by metal-free organocatalytic ring-opening polymerization and demonstrated the influence of polymer architecture on antimicrobial activities. In comparison with a linear control homopolymer, we found that the cationic graft polycarbonates exhibited stronger antimicrobial activities and higher selectivities against clinically relevant Gram-positive S. aureus, Gram-negative E. coli, and yeast C. albicans. The hydrophilic-hydrophobic balance of the cationic graft polymers was also found to be important for antimicrobial activity and selectivity, with polymers containing MTC-BnCl repeat units being superior to the MTC-PrCl-containing polymers. Consistent with previous reports on linear cationic amphiphilic polymers, the graft polymers mediated broad spectrum bactericidal and fungicidal effects through rapid membrane permeabilization and disruption. Taken together, these results suggest that the cationic graft polymers are potentially promising as broad spectrum antimicrobial agents that may help overcome the problem of antibiotics resistance in therapeutic applications.
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REFERENCES AND NOTES