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Highly dynamic biodegradable micelles capable of lysing Gram-positive and Gram-negative bacterial membrane

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ABSTRACT

The development of biodegradable antimicrobial polymers adds to the toolbox of attractive antimicrobial agents against antibiotic-resistant microbes. To this end, the potential of polycarbonate polymers as such materials were explored. A series of random polycarbonate polymers consisting of monomers MTC-OEt and MTC-CH_2CH_3Cl were designed and synthesized using metal-free organocatalytic ring-opening polymerization. Random polycarbonate polymers self-assembled in solution but appeared highly dynamic; such behaviors are desirable as ready disassembly of polymers at the microbial membrane facilitates membrane disruption. Their activities against clinically relevant Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*E.coli* and *Pseudomonas aeruginosa*) revealed that the hydrophobic-hydrophilic composition balance in polymers are important to render antimicrobial potency. Scanning electron microscopy (SEM) studies indicated microbial cell surface damage after treatment with polymers, and confocal microscopy studies also showed entry of FITC-dextran dye in *Escherichia coli* as a result of membrane disruption. On the other hand, the polymers exhibited minimal toxicity against red blood cells in hemolysis tests. Therefore, these random polycarbonate polymers are promising antimicrobial agents against both Gram-positive and Gram-negative bacteria for various biomedical applications.

1. Introduction

The growing emergence of antibiotic-resistant infections has caused great concern within the healthcare field and provided an impetus for continued antimicrobial development [1]. Host defense peptides and synthetic polymers are two classes of macromolecules currently being studied as effective antimicrobials. These materials are cationic and of amphiphilic structures. They can selectively target and disintegrate bacterial membranes *via* electrostatic interaction and insertion into the membrane lipid domains, hence avoiding potential bacterial resistance [2–4]. Despite their efficacious antimicrobial activity, both peptides and synthetic polymers have seen limited clinical applications because of several inherent problems. For example, antimicrobial peptides (the first host defense for many organisms against environmental parasitic infections) are generally sensitive to enzymatic degradation, suffer from expensive large-scale production, and their pharmacokinetics are inadequately studied [5]. Regardless of promising clinical trial results, no antimicrobial peptide has received FDA approval for general public use [6,7]. On the other hand, a plethora of bio-inspired synthetic polymers have been proposed and are achieving considerable success in overcoming many drawbacks found in using peptides [8]. These polymers often have comparable if not better antimicrobial activities than peptides. Unfortunately, biocompatibility and/or biodegradability have presented significant problems during *in vivo* administration. This issue was recently addressed through the construction of biodegradable and biocompatible amphiphilic triblock polycarbonates that self-assemble into cationic micellar nanoparticles [9]. These materials demonstrated strong antimicrobial activities towards drug-resistant Gram-positive bacteria, however, they were ineffective towards all Gram-negative strains tested.

Considering that Gram-negative pathogens cause more drug-resistant infections compared to Gram-positive ones, their treatment should be considered a top priority. Further exacerbating this problem is that current treatments for Gram-negative infections are terribly inadequate compared to the management of Gram-

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positive infections [10]. Reasons for the discrepancy between the two bacterial types can be identified when examining their respective outermost membrane/wall layers. Gram-positive bacterial cells have a highly cross-linked peptidoglycan cell wall as the outmost layer while Gram-negative cells have an additional lipopolysaccharide (LPS)-containing membrane. This secondary LPS barrier creates added impedance that further complicates association of cationic antimicrobials. The rule of thumb for antimicrobial polymer design is the amphiphilic architecture [8]: the cationic charge/hydrophilic portion of the polymer enables favorable binding with the negatively charged microbial wall or membrane surface; the hydrophobic portion hence facilitates its insertion and destruction of local membrane lipid organization. The aforementioned amphiphilic triblock polycarbonates [9] shielded their hydrophobic portions within the micellar nanoparticle cores, and thus were excluded from readily associating with the outermost LPS barrier. It is this reason we presume that Gram-negative antimicrobial activity was disallowed for those block polymer systems.

Herein, efforts at developing polymeric antimicrobials capable of destroying both Gram-positive and Gram-negative bacteria were presented. In order to provide improved hydrophobic accessibility, a series of random polycarbonate copolymers were synthesized by metal-free organocatalytic ring-opening polymerization of benzyl 2,2-bis(methylol)propionate with 5-methyl-5-(3-chloropropyl) oxycabonyl-1,3-dioxan-2-one (MTC-O(CH_2)$_3$Cl) and 5-methyl-5-ethyloxycabonyl-1,3-dioxan-2-one (MTC-OEt) monomers (Fig. 1, Schemes S1 and S2 in Supplementary Material). The random copolymers were further aminated using trimethylamine to render cationic charges. The cationic composition and molecular weight of random copolymers were systematically varied to study their effects on antimicrobial activities against clinically relevant both Gram-positive and Gram-negative bacteria, and selectivity towards bacteria over mammalian cells (red blood cells). In addition, additional important structural factors such as counter ion, quaternizing agent, and oligosaccharides were also investigated. Moreover, the antibacterial mechanism of random copolymers was explored by confocal and scanning electron microscopy.

2. Materials and methods

2.1. Materials

2,2-Bis(hydroxymethyl)propionic acid (bis-MPA) and *N*-(3,5-trifluoromethyl) phenyl-*N*′-cyclohexylthiourea (TU) were prepared according to our previous protocol [11]. TU was dissolved in dry THF, stirred with CaH_2, filtered, and freed of solvent *in vacuo*. Prior to use, 1,8-diazabicyclo [5,4,0]undec-7-ene (DBU) were stirred over CaH_2 and vacuum distilled before being transferred to a glove box. All other chemical reagents were bought from Sigma–Aldrich and used as received unless especially mentioned. Ultra pure (HPLC grade) water was obtained from J.T. Baker (U.S.A.). Phosphate-buffered saline (PBS) was purchased from 1st BASE (Singapore) and diluted to the intended concentrations before use. Trypic soy broth (TSB) powder was purchased from BD Diagnostics (Singapore) and used to prepare the microbial broths according to the manufacturer's instructions. PBS buffer at 10× concentration was purchased from 1st Base (Singapore) and used after dilution to the desired concentration. Ethanol, glutaraldehyde (synthetic grade, 50% in H_2O), FITC-labeled dextran (500 kDa), DMSO, and calcein were purchased from Sigma–Aldrich (Singapore) and used as received. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained as dry powder from Avanti Polar Lipids, Inc. *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 25922), and *Pseudomonas aeruginosa* (ATCC No. 9027) were obtained from ATCC (U.S.A) and

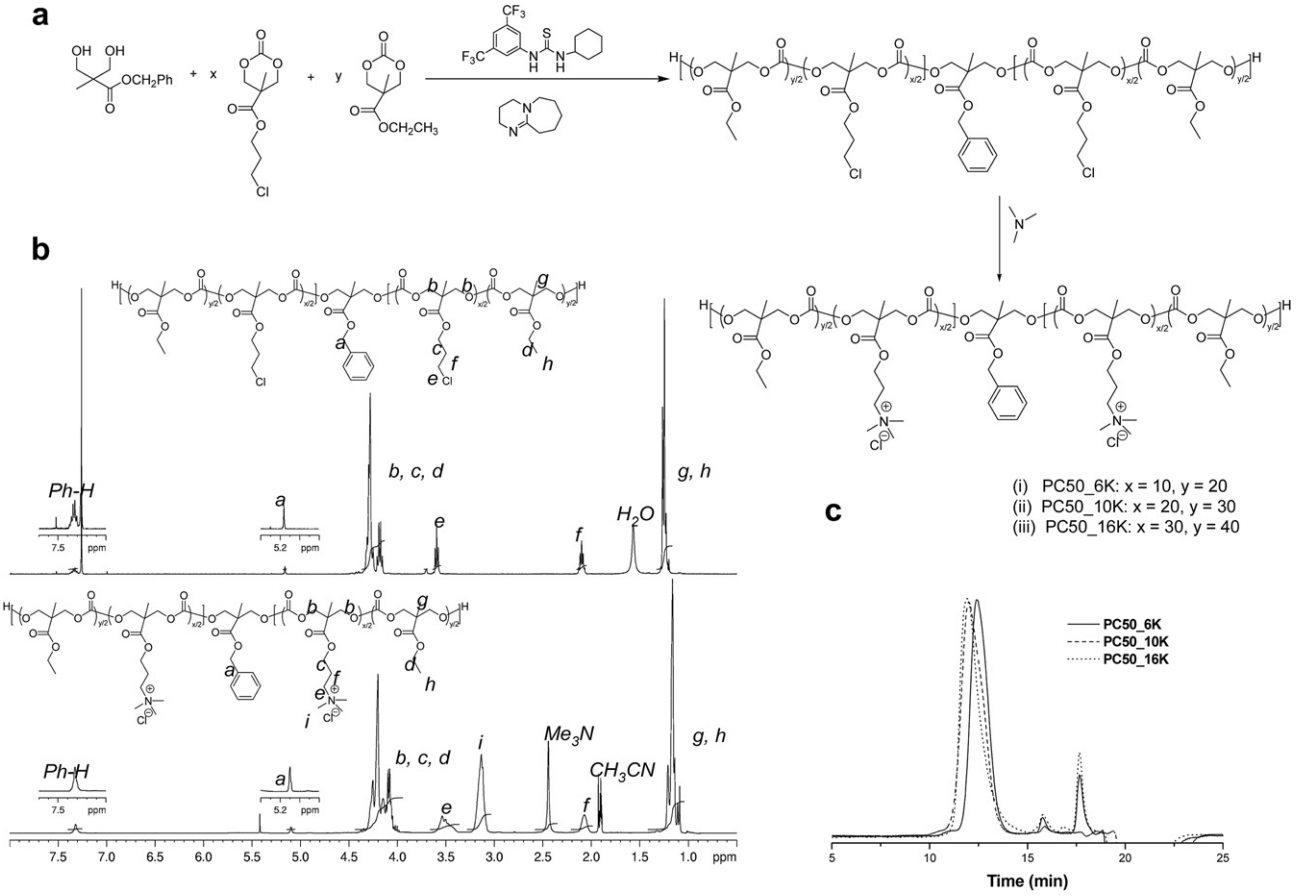

Fig. 1. Antimicrobial random polycarbonate copolymers. (a) Synthetic scheme and structure of random polycarbonate copolymer; (b) ^{1}H NMR of the typical random copolymer, PC50_6K in $CDCl_3$, and its cationic derivative in CD_3CN; (c) GPC diagram of PC50_6K, PC50_10K, PC50_16K.