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Block copolymer mixtures as antimicrobial hydrogels for biofilm eradication

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ABSTRACT

Current antimicrobial strategies have mostly been developed to manage infections due to planktonic cells. However, microbes in their nature state will tend to exist by attaching to and growing on living and inanimate surfaces that result in the formation of biofilms. Conventional therapies for treating biofilm-related infections are likely to be insufficient due to the lower susceptibility of microbes that are embedded in the biofilm matrix. In this study, we report the development of biodegradable hydrogels from vitamin E-functionalized polycarbonates for antimicrobial applications. These hydrogels were formed by incorporating positively-charged polycarbonates containing propyl and benzyl side chains with vitamin E moiety into physically cross-linked networks of "ABA"-type polycarbonate and poly(-ethylene glycol) triblock copolymers. Investigations of the mechanical properties of the hydrogels showed that the G′ values ranged from 1400 to 1600 Pa and the presence of cationic polycarbonate did not affect the stiffness of the hydrogels. Shear-thinning behavior was observed as the hydrogels displayed high viscosity at low shear rates that dramatically decreased as the shear rate increased. *In vitro* antimicrobial studies revealed that the more hydrophobic VE/BnCl(1:30)-loaded hydrogels generally exhibited better antimicrobial/antifungal effects compared to the VE/PrBr(1:30) counterpart as lower minimum biocidal concentrations (MBC) were observed in *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative) and *Candida albicans* (fungus) (156.2, 312.5, 312.5 mg/L for VE/BnCl(1:30) and 312.5, 2500 and 625 mg/L for VE/PrBr(1:30) respectively). Similar trends were observed for the treatment of biofilms where VE/BnCl(1:30)-loaded hydrogels displayed better efficiency with regards to eradication of biomass and reduction of microbe viability of the biofilms. Furthermore, a high degree of synergistic antimicrobial effects was also observed through the co-delivery of antimicrobial polycarbonates with a conventionally-used antifungal agent, fluconazole. These hydrogels also displayed excellent compatibility with human dermal fibroblasts with cell viability >80% after treatment with hydrogels loaded with cationic polymers and/or fluconazole at minimum biocidal concentrations (MBC).

1. Introduction

In the on-going battle against microbial infections, the risks of acquiring drug resistance has not ceased despite a considerable number of strategies that have been devised for new cellular targets. Bearing this in mind, there is an increasing need for more effective antimicrobial formulations that can be applied broadly in a variety of biomedical-associated contexts. Antimicrobial hydrogels, which are heavily hydrated networks of polymers possessing the ability to eliminate infectious microbes, are being exploited in many pharmaceutical applications, including medication, disinfectants, sanitizers and personal care products [1]. Ideally, these materials should exert antimicrobial actions effectively, eliminating and preventing the recurrence of both planktonic as well as biofilm organisms. This is essentially important for limiting the emergence of resistant subpopulation since antimicrobial resistance has become a major global healthcare concern.

The development of an effective antimicrobial hydrogel requires the understanding of the different growth behavior and treatment susceptibility between planktonic cells and those embedded within biofilms. The formation of biofilms is initiated by the deposition of planktonic cells onto living tissue or an inanimate surface, such as those of medical devices. These cells adhere and anchor themselves to the surfaces *via* the production of exopolymers. As proliferation

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occurs, microcolonies appear and the thickening of polymer matrix around the microcolonies results in the growth of the biofilm [2,3]. Biofilm formation has been recognized as one of the leading causes of a significant amount of human infections [4,5]. Pathogens that are commonly associated with biofilm-induced chronic infections include *Staphylococcus aureus* in chronic rhinosinusitis [6], enteropathogenic *Escherichia coli* in recurrent urethritis [7,8] and *Candida albicans* in candiasis [9].

Microbes that grow in biofilms have been known to exhibit dramatically higher resistance against antimicrobial agents compared to free-floating cells [10–12]. Several factors contribute to the intrinsically lower susceptibility to antimicrobials, including restricted penetration of antimicrobials into a biofilm due to limited diffusion within the cell polymer matrix, decreased growth rate which minimizes the uptake of biocides into the cells, as well as the expression of possible resistance genes [13,14]. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antimicrobials to biofilm-associated microbes vary widely depending on the strains and drugs used, and in some case, it may be up to 1000-fold higher compared with planktonic bacteria [2]. For instance, Stoodley et al. reported that biofilm-associated *S. aureus* required 3.5 times higher than the MBC of oxacillin to provide a 3.0-log reduction in bacterial counts [15], and Ceri et al. reported that *E. coli* required >500 times the MIC of ampicillin for a 3.0-log reduction in bacteria growth [16].

With the emergence of antibiotic-resistant 'superbugs', the development of newer, more potent antibiotics with antimicrobial actions that are different from the conventional small molecule drugs is crucial [17]. Polymeric biocides are a class of materials that are engineered as synthetic mimics of naturally occurring host-defense antimicrobial peptides. These amphiphilic, cationic polymers are able to selectively target and bind to microbes by electrostatic interactions and disintegrate the bacterial membranes by insertion into the membrane lipid bilayer [18,19]. Such membrane disruption mechanism causes rupture and lysis of the microbes, thereby decreasing the potential of resistance development.

When used *in vivo*, selectively killing of microbes generally comes from enhanced long-range electrostatic interaction between the polymeric biocides and microbes in comparison to mammalian cells [19,20]. The design of these polymers is dependent of several factors that can greatly affect the antimicrobial activity and selectivity, such as the molecular weight, hydrophobic/hydrophilic balance, cationic chemical functionality [21,22]. Chan-Park et al. recently reported the antimicrobial effects of UV-crosslinked chitosan-based hydrogels and found that the degree of quaternization of the polymers played a significant role in influencing their biocidal effects [23]. At a high cationic chitosan concentration (10 wt.%), the hydrogels gave rise to >2.0-log reduction in microbial counts (i.e. >99.0% killing efficiency) for *Pseudomonas aeruginosa*, *E. coli*, *S. aureus* and *Fusarium solani* after 1 h exposure, but >3.0-log reduction (i.e. >99.9% killing efficiency) was seen only in *S. aureus* and *F. solani*. More recently, the same group reported a photopolymerized hydrogel system based on the antimicrobial peptide ε-poly-L-lysine (EPL) grafted to methacrylic acid (MA) [24]. Antimicrobial activities of EPL-MA were retained after polymerization and the hydrogels were able to reduce the counts of *S. aureus* and *P. aeruginosa* by more than 99.9% (3-log reduction) at high EPL-MA concentrations (21–25 wt.%) after 2 h exposure, while the hydrogels were less effective against *C. albicans* and *F. solani* (fungi) (<2-log reduction in the fungal count). We have previously reported physically crosslinked antimicrobial hydrogels prepared based on the stereocomplexation between poly(L-lactide)-PEG-poly(L-lactide) and poly(D-lactide)-cationic polycarbonate-poly(D-lactide) [25]. Physically crosslinked hydrogel is advantageous when used as a topically applied antimicrobial formulation because it can be easily spread onto surfaces of different confirmation. However, these polymers required a high content of hydrophobic poly(lactide) blocks for gelation and lactide blocks that were longer than 1 kDa prevented complete aqueous dissolution. In addition, the polymers that were water soluble required high polymer concentration for gelation (13.2 wt.%) [25].

Herein, we report an antimicrobial hydrogel system based on vitamin E-containing polycarbonate copolymers synthesized by organocatalytic ring-opening polymerization (ROP). These hydrogels contain two parts – first being the 'ABA'-type triblock copolymer, consisting of a hydrophilic PEG middle block flanked on both ends by hydrophobic vitamin E-functionalized polycarbonate blocks and the other is biocidal cationic polycarbonates that possess vitamin E moieties. The hydrogels were formed based on hydrophobic interactions between vitamin E-functionalized polycarbonate blocks, and their mechanical properties were characterized. The incorporation of vitamin E in the cationic polycarbonates could potentially enhance the antimicrobial efficacy as a result of increased hydrophobicity of the polymers [26]. α-Tocopherol was chosen as it is the most biologically active form of vitamin E and extensively studied amongst the other variants in the family. In the body, it functions as an important lipid-soluble antioxidant and assists in the process of wound healing [27,28].

Antimicrobial efficacy of these physically crosslinked hydrogels was investigated against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria as well as *C. albicans* (fungus). The hydrogels were also evaluated for their efficiency in eradicating biofilms through metabolism assessment, biomass removal and scanning electron microscopy. In addition, synergism studies were also performed on the co-delivery of cationic antimicrobial polycarbonates with conventionally-used antifungal agent, fluconazole, on *C. albicans*. The cytotoxicity of the hydrogels loaded with cationic polymers and/or fluconazole at MBCs was evaluated against human dermal fibroblasts.

2. Materials and methods

2.1. Materials

MTC-VE (5-methyl-5-(α-tocopheryl)carboxyl-1,3-dioxan-2-one), MTC-BnCl (5-methyl-5-(4-chloromethyl)benzylcarboxyl-1,3-dioxan-2-one) and MTC-PrBr (5-methyl-5-bromopropylcarboxyl-1,3-dioxan-2-one) and N-(3,5-trifluoromethyl) phenyl-N′-cyclohexylthiourea (TU) were synthesized by adapting a protocol previously reported by Pratt et al. (See the Supplementary Information for full experimental and characterization details) [29]. All reagents were bought from Sigma–Aldrich and used as received unless otherwise mentioned. 1,8-Diazabicyclo[5,4,0] undec-7-ene (DBU) and benzyl alcohol were dried over calcium hydride and vacuum distilled twice before being transferred to the glove box. Methanol and ethanol (ACS grade) were purchased from Tee Hai (Singapore). Glacial acetic acid was obtained from VWR (U.S.A.). Ultra pure (HPLC grade) water was obtained from J.T. Baker (U.S.A.). Tryptic soy broth (TSB) powder and yeast mould broth (YMB) powder were purchased from BD Diagnostics (Singapore) and used to prepare the microbial growth media according to the manufacturer's instructions. *S. aureus* (ATCC No. 29737), *E. coli* (ATCC No. 25922) and *C. albicans* (ATCC No. 10231) were obtained from ATCC (U.S.A), and re-constituted according to the suggested protocols. Fluconazole, formalin solution, crystal violet, menadione and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) were purchased from Sigma, U.S.A.

2.2. Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H NMR spectra of monomers and polymers were recorded using a Bruker Avance 400 spectrometer, and operated at 400 MHz, with the solvent proton signal as the internal reference standard.

2.3. Molecular weight determination by size exclusion chromatography (SEC)

SEC was conducted using THF as the eluent for monitoring the polymer conversion and also for the determination of polystyrene equivalent molecular weights of the polymers. THF-SEC was recorded on a Waters 2695D (Waters Corporation, U.S.A.) Separation Module equipped with an Optilab rEX differential refractometer