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Cationic polymeric *N*-halamines bind onto biofilms and inactivate adherent bacteria

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ABSTRACT

A series of amine-based cationic polymeric *N*-halamine precursors, poly(2,2,6,6-tetramethyl-4-piperidyl methacrylate-co-trimethyl-2-methacryloxyethylammonium chloride)(PMPQ), were synthesized by copolymerizing 2,2,6,6-tetramethyl-4-piperidyl methacrylate (TMPM) with trimethyl-2-methacryloxyethylammonium chloride (TMAC) at different molar ratios (TMPM:TMAC = 10:90,30:70,50:50,70:30, and 90:10). After chlorine bleach treatment, the TMPM moieties in the new copolymers were transformed into amine-based *N*-halamines (Cl-PMPQ). The chemical structures of the samples were characterized with ^{1}H NMR, FT-IR, and UV spectra, and the molecular weights were determined by dynamic light scattering (DLS). With lower than 70 mol% of the original TMPM content, the resulting Cl-PMPQ copolymers were soluble in water, and demonstrated potent antibacterial functions against *Escherichia coli* (*E. coli*, a representative Gram-negative bacteria) and *Staphylococcus epidermidis* (*S. epidermidis*, a representative Gram-positive bacteria). *E. coli* and *S. epidermidis* were allowed to form biofilms on glass slides. Zeta potential analyses demonstrated that the Cl-PMPQ copolymers rapidly adsorbed onto the preexisting biofilms, and bacterial culturing studies confirmed that the bound Cl-PMPQ provided a total kill of the adherent bacteria in the biofilms. The kinetics of the Cl-PMPQ binding onto the preexisting biofilms were studied with UV analyses. The data fitted well to the bimodal model. The binding kinetic parameters of Cl-PMPQ onto the bacterial biofilms were thus determined.

1. Introduction

Formation of unwanted biofilms on solid surface is a long-lasting concern, causing serious industrial, environmental, medical, and hygienic problems [1–7]. Biofilms usually start with only a few microorganisms. In order to adhere to the solid, planktonic microorganisms accumulate on the surfaces, produce polysaccharide matrix acting as “slimes”, and multiply and accumulate in multilayered cell clusters, leading to the formation of biofilms. Protected by the polymeric matrix, microorganisms living in the biofilms can be up to 1000 times more tolerant to disinfectants than the corresponding free-floating bacteria [1–7].

Various biocides, including hypochlorite salts, hydrogen peroxides, quaternary ammonium compounds, silver salts, alcohols, iodine, *etc*, are commonly used as disinfectants to clean high-risk, high-touch surfaces [8,9]. However, these disinfects normally do not bind onto preexisting biofilms, leading to a short residence time on the biofilm matrix and weak disinfecting effect against adherent bacteria living in the biofilms.

This study designed and synthesized cationic polymeric *N*-halamines as a new class of *biofilm-binding* disinfectants to kill bacteria in preexisting biofilms. *N*-halamines are compounds containing one or more nitrogen-halogen covalent bonds, which are formed by halogenation of imide, amide or amine groups. The antimicrobial properties of *N*-halamines are due to the oxidative halogen, which can react with appropriate biological receptors such as thiol groups of amino acids in bacteria. This reaction can impede the metabolism of cells such as respiration, especially the processes related to proteins, resulting in the termination of the bacteria [9–15]. The disinfecting efficacy of *N*-halamines is similar to that of hypochlorite bleach, but they are more stable, less corrosive, non-toxic, and generate much less disinfection byproducts than bleach [16]. *N*-halamines can be divided into three classes: imide-based, amide-based, and amine-based *N*-halamines. Their stability is affected by the strength of the nitrogen-halogen covalent bond, with the order of: amine-based > amide-based > imide-based *N*-halamines. On the other hand, their antimicrobial potency is in

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the reversed order: imide-based > amide-based > amine-based *N*-halamines [15,16].

Aiming at providing long-term disinfecting activities, this study focused on amine-based polymeric *N*-halamines produced by the polymerization of 2,2,6,6-tetramethyl-4-piperidyl methacrylate (TMPM), followed by chlorine bleach treatment to generate chlorinated poly (2,2,6,6-tetramethyl-4-piperidyl methacrylate) (Cl-PTMPM) [17]. However, Cl-PTMPM is insoluble in water, limiting its potential application as disinfectants. Thus, we copolymerized TMPM with a quaternary ammonium monomer, trimethyl-2-methacryloxyethylammonium chloride (TMAC), and chlorinated the resulting copolymer to produce Cl-PMPQ. The function of TMAC in Cl-PMPQ is two folds: (1) it increases the solubility of Cl-PMPQ in water, making them easier to use [16]; (2) since biofilm matrix are normally negatively charged, the positively charged TMAC moieties in Cl-PMPQ can act as "binders" through electrostatic interaction with biofilms, leading to long residence time of the *N*-halamines on bacterial biofilms, which is expected to result in potent antibacterial effects against adherent bacteria. To test this hypothesis, we designed and synthesized a series of Cl-PMPQ copolymers with different compositions of TMPM and TMAC. The relationships between the structures of Cl-PMPQ and their antimicrobial activities were investigated. The binding kinetics of Cl-PMPQ onto preexisting biofilms were studied, and the effects of adsorbed Cl-PMPQ on adherent bacteria in the biofilms were evaluated.

2. Materials and methods

2.1. Materials

TMPM (2, 2, 6, 6,-tetra-methyl-4-pieridyl methacrylate) was purchased from TCI-Tokyo Chemical Industry. Trimethyl-2-methacryloxyethlammonium chloride (TMAC) and azobisisobutyronitrile (AIBN) were obtained from Sigma-Aldrich. Sodium thiosulfate ($Na_2S_2O_3$) and acetic acid (CH_3COOH) were obtained from Fisher Scientific. Poly (ethylene glycol) was purchased from Aldrich Chemical Company and Acros Organics. Dialysis tube (MWCO, 1000D) was purchased from Spectrum labs, Inc. All the chemicals were used as received. The bacteria, *Staphylococcus epidermidis* (*S. epidermidis*; ATCC 35984) and *Escherichia coli* (*E. coli*, ATCC 15597) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Instruments

Attenuated total reflection infrared (ATR-IR) spectra were obtained on a Nicolet™ iS™10 spectrometer from Thermo Scientific. ^{1}H proton nuclear magnetic resonance (NMR) spectra were generated by a Bruker & Spectrospin Avance DRX 500 spectrometer. A Delsa™Nano HC dynamic light scattering (DLS) particle analyzer from Beckman Coulter was used to calculate the average molecular weight (M.W) and the surface zeta potential of the samples. Ultraviolet (UV) spectra were acquired with a Beckman Coulter DU 520 UV–vis spectrophotometer.

2.3. Methods

2.3.1. Synthesis of poly (2, 2, 6, 6-tetramethyl-4-piperidyl methacrylate-co-trimethyl-2-methacryloxyethylammonium chloride) (PMPQ)

PMPQ was synthesized following a procedure reported by Worley and coworkers [16,18] with slight modifications. Briefly, TMPM and TMAC were copolymerized in 20 mL ethanol with 2.8 wt% azobisisobutyronitrile (AIBN) as initiator at 95 °C for 5 h under nitrogen atmosphere. The molar ratios of TMPM and TMAC were: 0 mmol: 40 mmol (control), 4 mmol: 36 mmol (10:90), 12 mmol: 28 mmol (30:70), 20 mmol: 20 mmol (50:50), 28 mmol: 12 mmol (70:30), and 36 mmol: 4 mmol (90:10). Based on the molar ratios, the resulting polymers were denoted as PolyTMAC, PMPQ1090, PMPQ3070, PMPQ5050, PMPQ7030, and PMPQ9010. When the TMPM starting molar ratio was lower than 70%, the resulting copolymers were soluble in water. Since the objective of this study was to develop biofilm-binding disinfectants, PMPQ9010 (which was not soluble in water) was removed from the following studies.

After evaporation of ethanol, the polymer samples were purified by dialysis against water to remove impurities. After evaporating the majority of water, the resulting samples were dispersed in toluene and the toluene was rotary evaporated at 40 °C to remove residual water in the products. This process was repeated 3 times. The resulting polymers were vacuum dried at 40 °C for 24 h. The yields of PMPQ1090, PMPQ3070, PMPQ5050, and PMPQ7030 were 91.23%, 90.01%, 88.02% and 90.00% respectively. The molecular weights of the copolymers were determined by photon correlation spectroscopy (PCS) generated by dynamic light scattering [19–21].

2.3.2. Chlorination –preparation of Cl-PMPQ polymers

The PMPQ copolymers were chlorinated in household bleach (8.25% sodium hypochlorite) with a 1:30 dilution at room temperature, pH = 4 for 2 h. Afterwards, the samples were dialyzed against water to remove the residual reactants. After evaporating the majority of water, the samples were dispersed in toluene, and toluene was removed by rotary evaporation at 40 °C to remove the residual water. This process was repeated 3 times. The resulting *N*-halamines polymers were denoted as Cl-PMPQ1090, Cl-PMPQ3070, Cl-PMPQ5050, and Cl-PMPQ7030, respectively. The chlorine content in each Cl-PMPQ polymer was determined by iodometric titration, as reported previously [17,22].

2.3.3. Antimicrobial activity of the Cl-PMPQ copolymers

E. coli (Gram-negative bacteria, ATCC 15597) and *S. epidermidis* (Gram-positive bacteria, ATCC 35984) were selected as representative biofilm-forming Gram-negative and Gram-positive bacteria, respectively. The antibacterial activity of the Cl-PMPQ copolymers was evaluated using the agar and broth dilution method to determine minimum inhibitory concentration (MIC), following standard protocols [23]. Each test was repeated 3 times.

2.3.4. Zone of inhibition tests

The zone of inhibition study was performed following the procedure of a previous report [17]. In this test, 1.0 mL of 10^8–10^9 CFU/mL of *S. epidermidis* overlaid on an agar plate. The plate was incubated at 37 °C for 2hr to form a layer of bacteria. Sterile Oxford cups were placed vertically on the agar plate with seeded bacterial layer on the surface and filled with 100 μL 0.01 g/mL Cl-PMPQ5050 and PMPQ5050, respectively. The resulting plate was incubated at 37 °C for 24 h and the inhibition zone around the Oxford cups was recorded (in Fig. S1).

2.3.5. Biofilm-binding behaviors of the Cl-PMPQ copolymers

In the study of biofilm-binding behaviors, a series of glass slides (1×1 cm^2 each) were cleaned with ethanol, and immersed individually in 3 mL broth containing 10^6 CFU/mL of *E. coli* or *S. epidermidis* at 37 °C for 80 h under gentle shaking to allow biofilm formation. Afterwards, the slides were taken out and rinsed gently with sterile PBS 3 times to remove reversibly attached bacterial cells.

The biofilm-containing glass slides were immersed individually in 3 mL sterile PBS containing a certain amount of a specific Cl-PMPQ polymer at 37 °C. Cl-PMPQ concentration in the PBS solution was monitored with UV spectrometer at the absorption of 277 nm during the study period of 24 h, which was used to calculate the binding amount of each Cl-PMPQ on the biofilm at each time point.