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Synthesis and characterization of novel antimicrobial polymers containing pendent triclosan moieties

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ABSTRACT

Novel antimicrobial copolymers were produced by first converting the commodity biocide, triclosan (TCS), to an epoxy-functional derivative, 2-((5-chloro-2-(2,4-dichlorophenoxy)phenoxy) methyl)oxirane (ETCS), and then reacting ETCS with polyethylenimine (PEI). While neither ETCS or PEI showed high antimicrobial activity toward either the Gram-positive bacterium, *Staphylococcus epidermidis*, or the Gram-negative bacterium, *Escherichia coli*, some the copolymers showed very high activity toward both bacteria. Antimicrobial activity for these copolymers was found to be highly dependent on both the molecular weight of the PEI utilized and the concentration of pendent groups derived from ETCS. In general, decreasing PEI molecular weight and increasing TCS pendent group concentration increased antimicrobial activity. Surface tension measurements showed that the molecular parameters affecting antimicrobial activity also affected surface activity in a similar fashion. Thus, it was speculated that the mechanism of antimicrobial activity associated with these copolymers involves interaction of the copolymers with the bacterial cell wall. A comparison of the antimicrobial activity of the most effective copolymers to TCS showed that the copolymers were more effective toward *E. coli* than pure TCS when compared using an equivalent TCS content (i.e. TCS pendent group content for the copolymers). This characteristic coupled with the fact that the TCS-containing copolymers are highly aqueous soluble liquids as opposed to a crystalline solid of limited solubility may afford utility of these copolymers for a variety of applications.

1. Introduction

Antimicrobial materials and biocides are compounds that kill or prevent the growth of pathogenic and other unwanted microorganisms [1]. This broad group of chemicals is used extensively in the fields of medicine [2], water treatment [3], personal-care [4], marine vessels [5], and architecture [6]. 2,4,4′-Trichloro-2′-hydroxydiphenyl ether, typically referred to as triclosan (TCS), is a broad-spectrum antimicrobial agent commonly used in personal-care products that was initially developed by the Ciba-Geigy Company in the 1960s [7]. TCS has been formulated into hand soaps, surgical scrubs, shower gels, underarm deodorants, toothpastes, hand lotion, and mouthwashes; incorporated into fabrics and plastics such as children's toys, surgical drapes, cutting boards and toothbrush handles; and even infused into concrete for floors [8]. Extensive investigations have been done on the toxicity of TCS and it has been found to be nontoxic orally as well as showing no mutagenic, carcinogenic, or teratogenic properties [9,10].

There is some discrepancy as to the mode of action of TCS, but it is generally accepted that it is bactericidal with multiple mechanisms of action at higher levels and, at lower levels, inhibits a specific site in the fatty acid biosynthetic pathway [11]. A specific targeted pathway at lower concentrations causes much concern because it allows for bacteria to develop resistant strains when exposed to sublethal concentrations of TCS [8,12,13]. This is particularly concerning considering the prevalence of TCS use.

Several groups have studied different techniques to control the release and biocidal nature of TCS by incorporating the molecule into polymer compositions. All of the recent studies have involved the incorporation of TCS into polymer compositions by either attaching TCS directly to a polymer chain using a hydrolytically-labile functional group or by physically blending it with polymers [14,15]. For these previously studied polymeric compositions, antimicrobial properties were obtained through the release of TCS molecules.

The authors have been interested in generating surface coatings that provide antimicrobial activity without leaching toxic

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components into the environment [16–21]. Most approaches for producing non-leaching, contact-active antimicrobial surfaces involve the generation of bound cationic functional groups at the surface [22]. These bound cationic groups can effectively disrupt the integrity of the bacterial cell wall through ionic interactions leading to cell death. The authors have previously demonstrated that tethering relatively high levels of TCS to a siloxane coating matrix using a non-hydrolyzable linking group provides antimicrobial activity without leaching toxic compounds from the coating [17,19,20]. This result suggested that, at relatively high concentrations, the TCS molecule may have a cell membrane disruption affect. During the course of conducting research focused on the development of contact-active, non-leaching antimicrobial surface coatings, it was discovered that polymers derived from the reaction of polyethylenimine (PEI) and 2-((5-chloro-2-(2,4-dichlorophenoxy)phenoxy) methyl)oxirane (epoxy triclosan) were biocidal. This document describes the synthesis, physical properties, and antimicrobial properties of these novel antimicrobial polymers.

2. Experimental

2.1. Materials

TCS was used as received from Alfa Aesar. PEIs of varying molecular weight (M_n = 423 g/mole, M_n = 600 g/mole, M_n = 10,000 g/mole), epichlorohydrin, potassium hydroxide, isopropanol, toluene, magnesium sulfate, and hexanes were used a received from Sigma–Aldrich Chemical. ChromAR® chromatography grade chloroform was used as received from Mallinckrodt Chemicals. Marine broth (MB) was prepared according to the manufacturer's (Becton Dickinson Labware) specifications.

2.2. Synthesis

Epoxy triclosan (ETCS) was synthesized as follows: 80 g of isopropanol and 19.4 g (0.346 mol) of potassium hydroxide were charged to a 1 L, three-neck, round-bottom flask equipped with a magnetic stir bar and the mixture stirred at room temperature until the potassium hydroxide dissolved. In an 800 mL glass beaker, 100 g (0.346 mol) of TCS was dissolved at room temperature in 250 g of isopropanol using magnetic stirring. The TCS solution was subsequently added to the round-bottom flask containing the potassium hydroxide solution. The flask was then placed in a temperature-controlled silicone oil bath and equipped with a condenser and a 250 mL addition funnel. A thermocouple was placed into the reaction flask and the temperature controller set at 60 °C. Once the temperature had equilibrated, 95.9 g (1.036 mol) of epichlorohydrin was added dropwise to the solution over the course of 5 min using the addition funnel. During the course of the reaction, a precipitate (potassium chloride) was formed. The reaction was allowed to run for 16 h. Upon completion of the reaction, the reaction mixture was transferred to a 1-L, single-neck, round-bottom flask and then placed on a rotary evaporator to remove unreacted epichlorohydrin at reduced pressure. Further purification was done using solvent extraction with water and a 1/1 v/v mixture of hexanes and toluene. The organic phase was washed four times with water and dried over magnesium sulfate. Solvent was removed at reduced pressure on a rotary evaporator and the clear viscous liquid product was collected (yield: 88%).

A PEI copolymer, referred to in this document as 423PEI–5%TCS, was synthesized as follows: 10.0 g (0.294equiv NH) of the 423 g/mole PEI and 5.1 g (0.015 mol) of ETCS were dissolved at room temperature in 90 mL of chloroform using a 250 mL single-neck, round-bottom flask and the mixture heated at 50 °C under a blanket of nitrogen. The reaction was allowed to run for 64 h and reaction progress monitored using proton nuclear magnetic resonance spectroscopy (^{1}H NMR) by observing the disappearance of peaks in the spectrum corresponding to protons associated with the epoxy group of ETCS. Upon completion of the reaction, solvent was removed at reduced pressure using a rotary evaporator. The product, a yellow viscous liquid, was collected and characterized using ^{1}H NMR and differential scanning calorimetry (DSC). All other PEI copolymers containing pendent triclosan moieties were synthesized using the same synthetic procedure with the exception that PEI molecular weight and ETCS concentration was varied. In addition, reaction time was adjusted based on reaction monitoring results to ensure complete reaction. Table 1 lists the details of each synthesis conducted.

2.3. Instrumentation

NMR spectra were obtained using a JEOL ECA400 400 MHz NMR equipped with a 24-place autosampler carousel. Samples were measured in deuterated chloroform using 16 scans for proton spectra, pulse width of 14.003 μs, acquisition time of 2.18 s, pulse angle of 45°, attenuation of 6 dB, pulse time of 7.0015 μs, receiver gain of 28, relaxation delay of 4 s, and repetition time of 6.18 s. Carbon spectra were obtained in deuterated chloroform using 1000 scans, pulse width of 10.309 μs, acquisition time of 1.04 s, pulse angle of 30°, attenuation of 9 dB, pulse time of 3.4363 μs, receiver gain of 58, relaxation delay of 2 s, and repetition time of 3.04 s. In addition, carbon spectra were run with decoupling and NOE activated at an NOE time of 2 s.

High performance liquid chromatograpy (HPLC) was conducted using an Agilent 1100 Series HPLC equipped with an Agilent 1100 autosampler and diode array detector. The HPLC column was a Zorbax Eclipse XDB-C18 running a mobile phase of 30.0% water and 70.0% acetonitrile at 40 °C with a column flow rate of 1.8 mL/min. 20 μL sample aliquots were injected using aspiration/dispense rate settings of 200 μL/min. The detected signal of the diode array detector was 280 nm. Data analysis was done using ChemStation for LC 3D Systems supplied by Agilent Technologies.

DSC thermograms were obtained using a TA Instruments Q1000 DSC. 7.12–18.5 mg of polymer were dispensed into hermetic aluminum pans and sealed using the TA instruments DSC Blue Sample Press. The temperature profile consisted of equilibrating the samples at −90 °C for 2 min, heating from −90 °C to 90 °C at 10 °C/min, cooling from 90 °C to −90 °C at 10 °C/min, and finally heating from −90 °C to 90 °C at 10 °C/min. Glass transition temperatures (T_gs) were taken as the inflection point of the last heating regime with the assistance of TA Instruments Universal Analysis 2000 software.

2.4. Surface tension

Surface tension measurements were performed by drop shape analysis on a pendant drop produced with a Contact Angle/Surface

Table 1
Compositions of reaction mixtures used for PEI copolymer synthesis.

Copolymer acronym	PEI M_n (g/mole)	Wt. PEI (g)	Wt. ETCS (g)
423PEI–5%TCS	423	10.0	5.1
423PEI–10%TCS	423	7.5	7.6
423PEI–20%TCS	423	5.0	10.2
600PEI–5%TCS	600	10.0	5.1
600PEI–10%TCS	600	7.5	7.6
600PEI–20%TCS	600	5.0	10.2
10,000PEI–5%TCS	10,000	10.0	5.1
10,000PEI–10%TCS	10,000	7.5	7.6
10,000PEI–20%TCS	10,000	5.0	10.2

Tension Analyzer from First Ten Angstroms (FTÅ125). Measurements were carried out at room temperature on polymer solutions of varying concentration (10^{-4}–10% w/v). All glassware was washed in a 1 N NaOH bath and thoroughly rinsed with Millipore water before use.

2.5. Antimicrobial properties

The antimicrobial properties of the polymers were determined using an adaptation of the standard minimum inhibitory concentration (MIC) test. MIC is typically reported as the lowest concentration of antimicrobial that completely inhibits growth over a set period of incubation relative to a control containing no antimicrobial [23]. For the polymers investigated, the amount of bacterial growth at each polymer concentration was reported. The microorganisms used for the study included the Gram-positive bacterium, *Staphylococcus epidermidis* ATCC 35984, and the Gram-negative bacterium, *Escherichia coli* ATCC 12435. 100 mg of each polymer was dissolved into 10 mL of methanol to generate a 10 mg/mL working solution. 10 mL of the appropriate growth medium, tryptic soy broth (*S. epidermidis*) or Luria–Bertani broth (*E. coli*), was spiked with 200 μL of the 10 mg/mL polymer working solution to achieve a final concentration of 0.2 mg/mL. A 1:1 serial dilution of the 0.2 mg/mL polymer suspension was then prepared and 0.5 mL of bacterial suspension in growth medium (1:1000 of overnight culture) was then added to 0.5 mL of each dilution generating polymer concentrations of 0.78–100 μg/mL. 0.2 mL of each polymer solution (spiked with bacteria) was then added in triplicate to a 96-well plate, placed in a 37 °C incubator for 24 h with shaking, and measured for growth by taking absorbance measurements at 600 nm with a multi-well plate spectrophotometer. 0.2 mL of growth medium and 0.2 mL of growth medium with the appropriate bacterium served as a negative and positive growth control, respectively. The antimicrobial activity for each polymer concentration was reported as a percent reduction in bacterial growth as compared to the positive growth control. Each percent reduction value was reported as the mean of three replicate samples.

3. Results and discussion

A series of PEI copolymers containing pendent TCS moieties were synthesized using the synthetic scheme shown in Fig. 1. TCS (**I**) was first derivatized by reaction with epichlorohydrin (**II**) to produce ETCS (**III**) and ETCS was subsequently reacted with PEI (**IV**) to produce the copolymers of interest (**V**). Fig. 2 displays the ^{1}H NMR spectrum obtained for ETCS. The peaks in the region of the spectrum ranging from 2.6 ppm to 4.3 ppm confirm successful incorporation of the epoxide functionality into the compound. Since trace amounts of TCS in the ETCS may result in false positives with respect to the characterization of antimicrobial activity, HPLC was used to identify trace impurities of TCS in the ETCS sample. The elution time for TCS was determined to be 4.7 min while that for ETCS was 6.8 min. For the ETCS sample, no peak at 4.7 min was discernable indicating the lack of trace amounts of TCS. ETCS was isolated as a liquid; however, crystallization was observed with time at ambient temperature. The melting temperature of the crystals was determined by DSC to be 43 °C.

TCS-containing PEI copolymers were readily synthesized by simply heating solutions of PEI and ETCS at 50 °C. As shown in Fig. 3, reaction progress was easily monitored using ^{1}H NMR by observing the disappearance of protons associated with the epoxy ring of ETCS. The series of TCS-containing PEI copolymers produced possessed variations in the number-average molecular weight (M_n) of the PEI backbone and the concentration of TCS pendent groups. The PEI M_ns, as specified by the manufacturer, were 423, 600, and 10,000 g/mole. TCS pendent group concentration was varied at 5, 10, and 20 mol% based on total PEI repeat units assuming that the nominal chemical structure of the PEIs was $(CH_2CH_2NH)_n$. For this document, the following formula was used to identify the different copolymers produced:

$$x\text{PEI} - y\%\text{TCS}$$

where x is the M_n of the PEI backbone and y is the mole percent of TCS pendent groups based on the total number of PEI repeat units. A

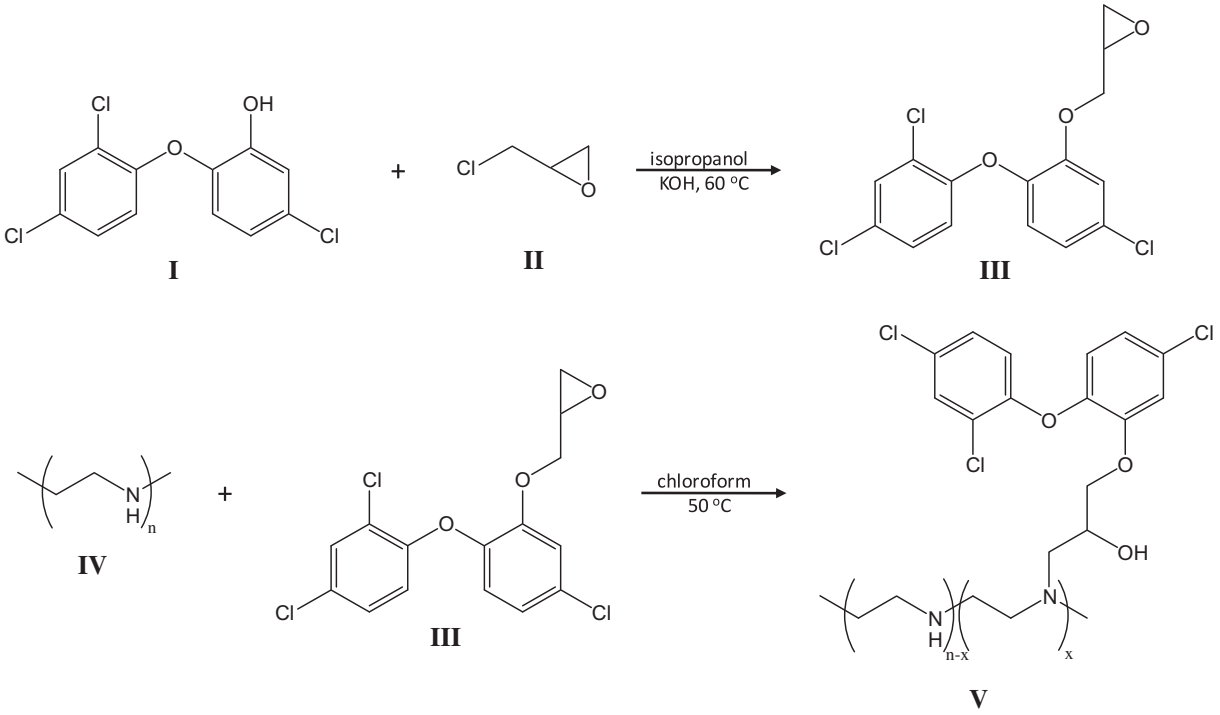

Fig. 1. The synthesis of ETCS (**III**) from epichlorohydrin (**II**) and triclosan (**I**), and the subsequent synthesis of PEI–TCS (**V**) from PEI (**IV**) and ETCS (**III**).

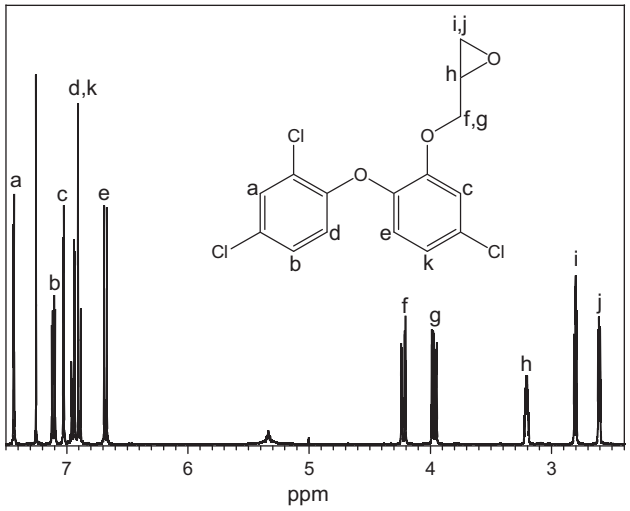

Fig. 2. ^{1}H NMR spectrum for ETCS with peak assignments. *This peak corresponds to residual $CHCl_3$ present in the $CDCl_3$ used as the solvent.

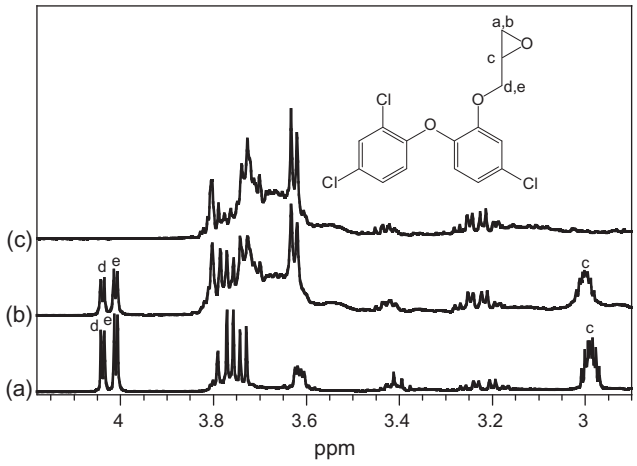

Fig. 3. ^{1}H NMR spectra as a function of reaction time for the synthesis of a PEI–TCS copolymer: (a) 0 h; (b) 4 h; and (c) 28 h. These spectra correspond to a reaction involving the 423 g/mole PEI.

description of the nine different copolymers produced is provided in Table 1.

As shown in Fig. 4, incorporation of TCS moieties as pendent groups to the PEI backbone sharply increased glass transition temperature (T_g). T_g increased with TCS pendent group concentration for each of the PEIs used for the study. The variation in T_g for the parent PEIs can be attributed to the variation in molecular weight. Polymer end-groups possess more conformational freedom than internal segments resulting in an overall increase in material free volume with decreasing molecular weight. The magnitude of the increase in T_g with TCS pendent group concentration was quite high. This result can be understood by considering the bulkiness and relatively high rigidity of the TCS structure, the relatively short length of the linking group between the TCS moieties and the PEI backbone, and the ability of the secondary hydroxyl present in the linking group between TCS moieties and the PEI backbone to undergo hydrogen bonding.

3.1. Antimicrobial properties

Prior to determining the antimicrobial activity of the TCS-containing PEI copolymers, the antimicrobial activity of the precursors

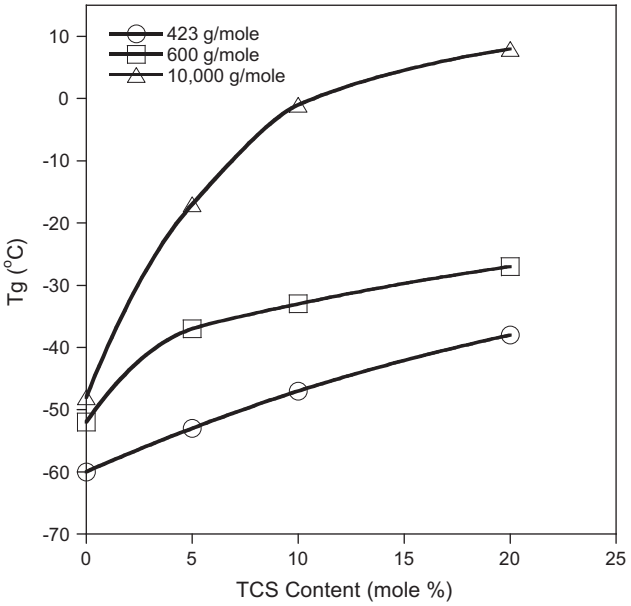

Fig. 4. T_g as a function of TCS pendent group concentration.

used to produce the TCS-containing PEI copolymers was determined and compared to that of pure triclosan. As shown in Fig. 5, the PEIs and ETCS showed little to no biological activity toward the two microorganisms used in this study, *E. coli* and *S. epidermidis*, while pure triclosan showed a substantial decrease in growth over the same concentration range. The effectiveness of triclosan toward *S. epidermidis* and *E. coli* was consistent with expectations based on previous results [7]. Considering that, at low concentrations, triclosan is believed to inhibit a specific bacterial target within the fatty acid biosynthetic pathway, it was not surprising that altering the chemical structure of triclosan by capping the hydroxy group with epichlorohydrin to produce ETCS inhibited antimicrobial effectiveness.

In aqueous media at essentially neutral pH, PEI is a polycation [24] and, thus, would be expected to interact with the negatively charged components at the outer surface of the cell envelope. It has been shown that binding of PEI to the bacterial cell wall through electrostatic interactions can increase the permeability of the cell wall enabling the permeation of antibiotics into the interior of the cell that would normally be unable to penetrate the cell wall [25]. Similar to the results shown in Fig. 5, Helander et al. [26] found that PEI had no bactericidal effect when tested toward the Gram-negative bacteria, *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. This result was not surprising considering the plethora of research that has demonstrated that cationic antimicrobial agents require a minimum degree of lipophilicity to compromise the integrity of the cell envelope to the extent that cell death occurs [27–29]. According to the generally accepted mechanism of antimicrobial action for cationic disinfectants, once the cationic compound has adsorbed onto the surface of the bacterial cell through electrostatic interactions between the positively charged quaternary nitrogen and the head groups of acidic -phospholipids, the lipophilic portion of the compound penetrates into the hydrophobic core of the cell wall, resulting in a loss of the integrity of the cell wall and osmoregulatory capability and subsequent cell death.

Figs. 6 and 7 display the antimicrobial properties of the TCS-containing polymers toward *S. epidermidis* and *E. coli*, respectively. From these figures, it can be easily seen that both copolymer molecular weight and TCS moiety concentration strongly affected antimicrobial activity. Copolymers based on the lowest molecular

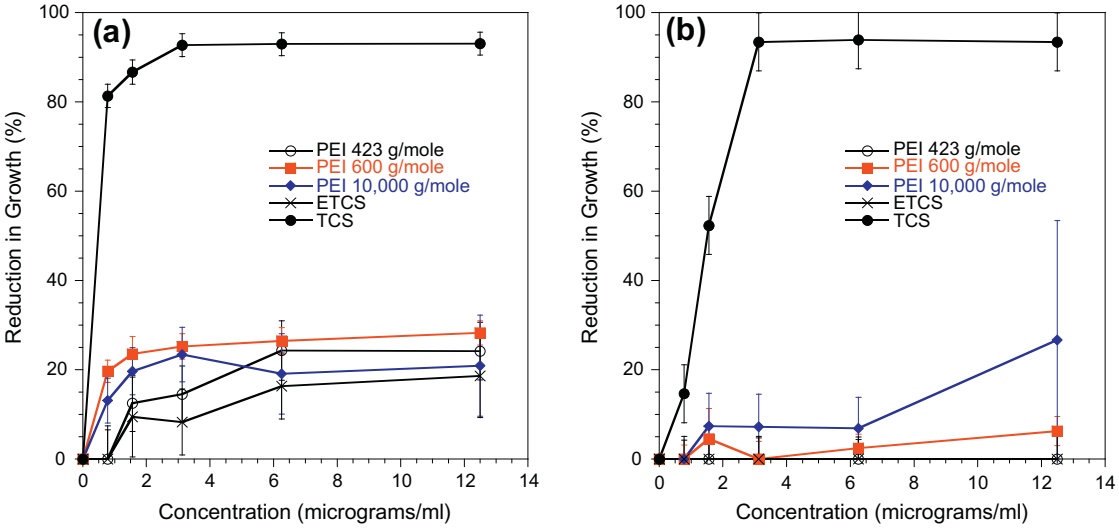

Fig. 5. Antimicrobial activity of the precursor materials used in the study and TCS toward *S. epidermidis* (a) and *E. coli* (b).

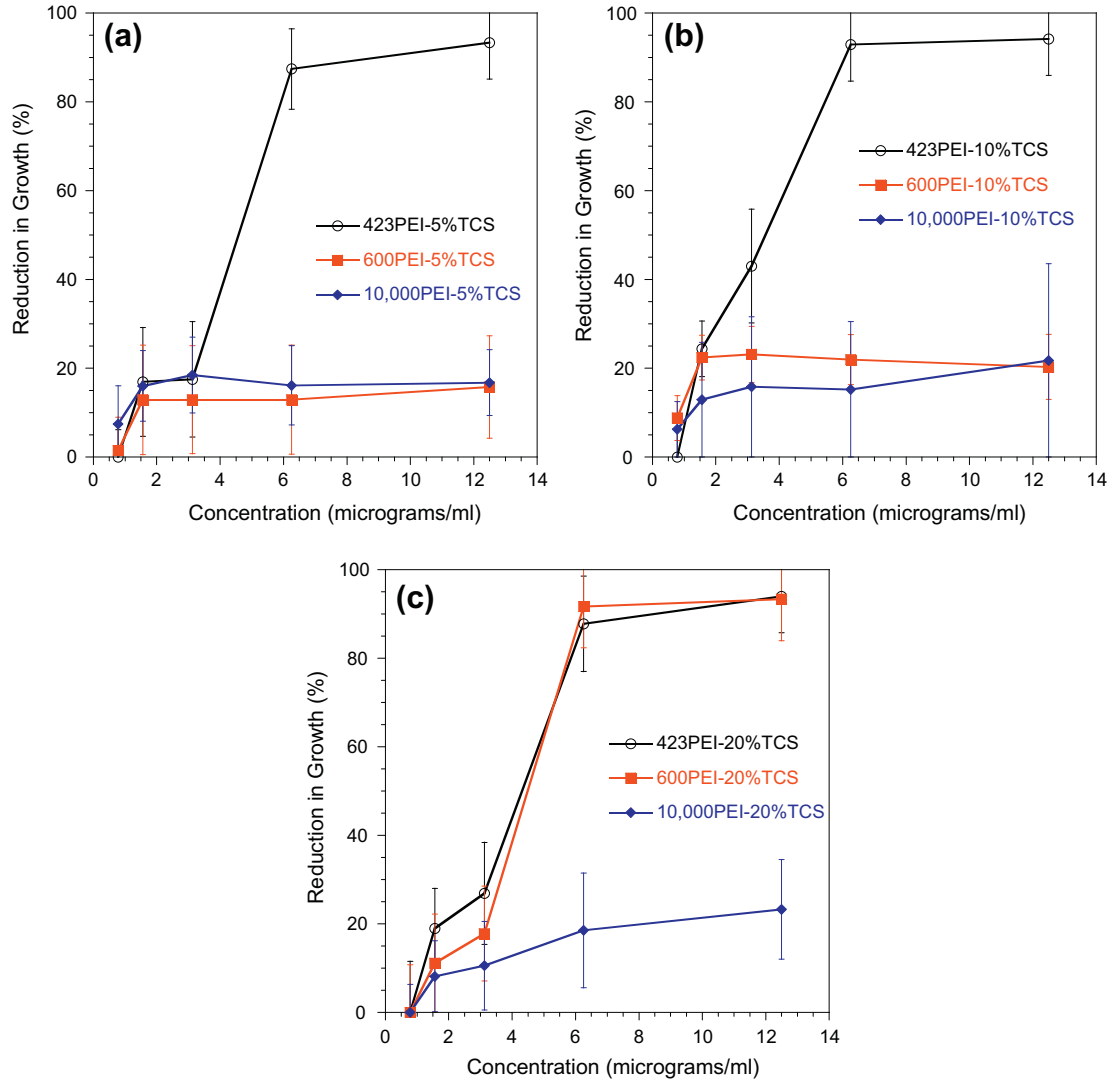

Fig. 6. Inhibitory response of the PEI–TCS copolymers toward *S. epidermidis*. PEI–TCS copolymers containing 5 (a), 10 (b) and 20 (c) mole percent pendent TCS moieties.

weight PEI were very effective at inhibiting growth of both *S. epidermidis* and *E. coli*, while copolymers based on the highest molecular weight PEI were ineffective. For the copolymers based on the medium molecular weight PEI (i.e. 600 g/mole), those containing the low and medium levels of TCS pendent groups (i.e. 600PEI–5%TCS and 600PEI–10%TCS) showed no antimicrobial activity, while the copolymer based on the highest concentration of pendent TCS groups, 600PEI–20%TCS, showed high antimicrobial activity toward both bacteria. While the mechanism of antimicrobial activity associated with these PEI–TCS copolymers is unknown, the observation that antimicrobial activity varied with polymer molecular weight and copolymer composition suggested that adsorption and diffusion characteristics of the copolymers with respect to the bacterial cell wall were important factors.

Stark variations in antimicrobial activity with molecular weight have been previously observed by several others involved in the study of antimicrobial polymers. For example, Ikeda et al. found that biological activity of in-chain quaternary ammonium salts toward *Staphylococcus aureus* depended strongly on molecular weight with higher molecular weight polymers providing increased activity [30]. Kanazawa et al. also observed increased antimicrobial efficacy toward *S. aureus* with increasing molecular weight for poly[tributyl(4-vinylbenzyl)phosphonium chloride] cationic polymers that ranged in molecular weight from 16,000 to 94,000 g/mole [31]. Cationic poly(vinyl pyridine) was found to be bactericidal at 160,000 g/mole but not at 60,000 g/mole [32], and N-alkylated PEI showed excellent activity at 25,000 g/mole but not at 2000 g/mole [33]. Chen and coworkers utilized quaternary ammonium-functionalized poly(propylene imine) dendrimers of very narrow molecular weight distribution and controlled molecular weight to study the influence of molecular size and charge density on antimicrobial activity and found a parabolic dependence of molecular weight on antimicrobial activity [34]. Considering the mechanism of antimicrobial action by cationic materials, this complex relationship between molecular weight and antimicrobial activity is most likely due to the interplay between charge density and diffusivity. With increasing polycation molecular weight, the process of adsorption onto the bacterial cell surface would be expected to be enhanced due to a higher charge density, while the process of diffusion into the interior of the cell wall would be expected to be inhibited.

Considering the previous reports on polymeric biocides, it was expected that antimicrobial activity would have increased with increasing molecular weight for the series of PEI–TCS copolymers investigated. The highest molecular weight PEI–TCS copolymer was derived from a 10,000 g/mole PEI. This molecular weight is well

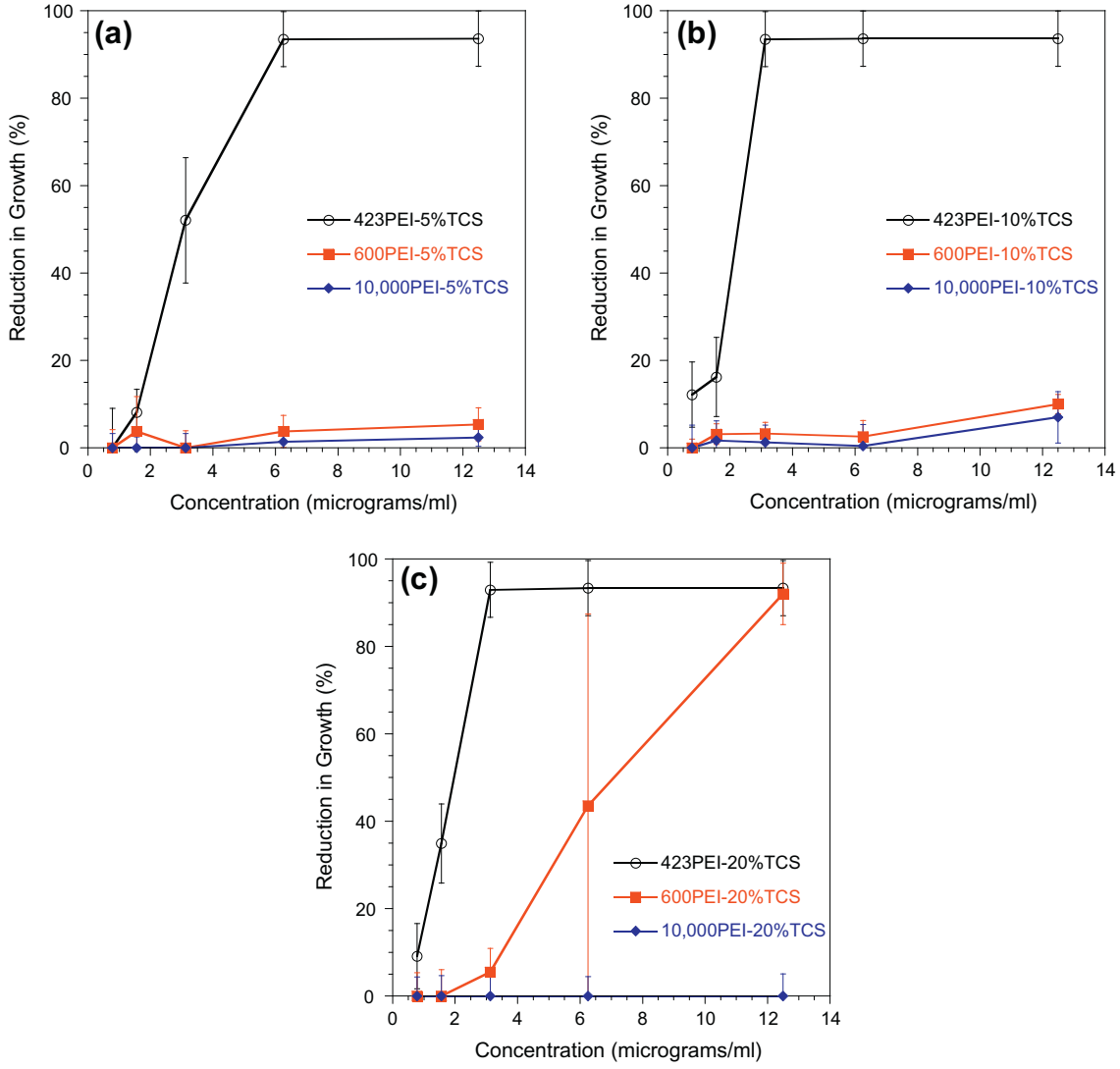

Fig. 7. Inhibitory response of the PEI–TCS copolymers toward *E. coli*. PEI–TCS copolymers containing 5 (a), 10 (b), and 20 (c) mole percent pendent TCS moieties.

below any molecular weights previously reported to be too high to provide effective antimicrobial activity. To obtain a better understanding of this trend in molecular weight, the surface activity of copolymers possessing 5 mol% pendent TCS groups was characterized by measuring surface tension as a function of polymer concentration. As shown in Fig. 8, the two low molecular weight copolymers, 423PEI–5%TCS and 600PEI–5%TCS, were significantly more surface active than the higher molecular weight copolymer, 10,000PEI–5%TCS. The higher surface activity observed for the two lower molecular weight copolymers would be expected to provide enhanced interaction with the bacterial cell wall which most likely contributed to the enhanced antimicrobial activity observed for these copolymers. In addition, the surface activity of 600PEI–20%TCS was measured to understand the influence of TCS content on surface activity. A comparison of the surface tension data obtained for 600PEI–20%TCS to that of 600PEI–5%TCS, as shown in Fig. 8, indicated that increasing TCS pendent group concentration increased surface activity. Thus, the higher antimicrobial activity observed for 600PEI–20%TCS as compared to 600PEI–5%TCS may be partly attributed to the higher surface activity of the former resulting in an enhanced affinity for the bacterial cell wall.

To compare the overall effectiveness of the TCS-containing copolymers to pure TCS, Fig. 9 was constructed. In contrast to Figs. 6 and 7, which express concentration on the *x*-axis as polymer concentration, the *x*-axis in Fig. 9 displays concentration as the concentration of TCS-moieties. By expressing concentration in this manner, it can be seen that attaching TCS moieties to the low molecular weight PEI provides greater antimicrobial activity toward *E. coli* than pure TCS. This characteristic coupled with the fact that the TCS-containing copolymers are highly aqueous soluble liquids as opposed to a crystalline solid of limited solubility may afford utility of these copolymers for a variety of applications.

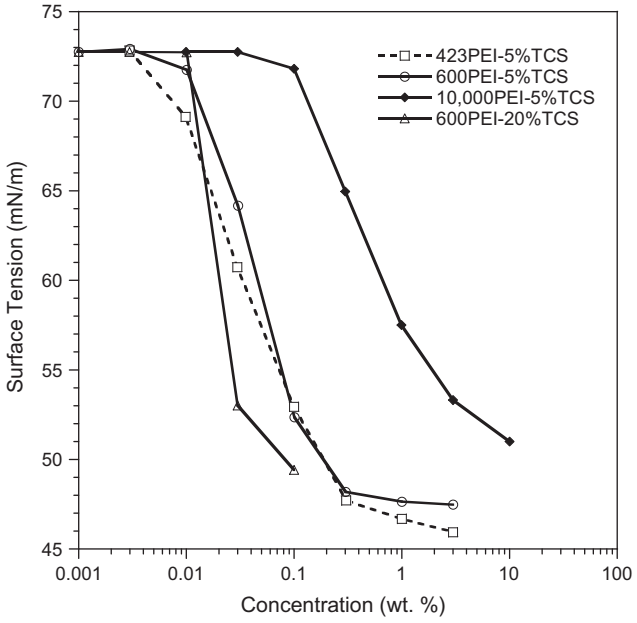

Fig. 8. Surface tension as a function of PEI–TCS copolymer concentration in water.

4. Conclusion

It was discovered that copolymers produced by reacting ETCS with PEI produced antimicrobial polymers. Although the mechanism of antimicrobial activity for these novel polymers is unknown, the influence of molecular weight on antimicrobial activity suggested that the mechanism is highly dependent on the interaction of the polymer with the bacterial cell wall. For most polymeric antimicrobials that function by disrupting the integrity of the cell envelope, such as cationic polymers, a molecular weight dependence on antimicrobial activity is often observed. This molecular weight dependence stems from the interplay between cationic charge density and diffusivity. High molecular weights and high charge densities would be expected to enhance adsorption of a cationic polymer onto the bacterial cell wall, however, these same characteristics would be expected to impede diffusion of the polymer into the interior of the cell wall.

For the PEI–TCS copolymers produced, antimicrobial activity decreased with increasing copolymer molecular weight which was counter-intuitive considering previous work described in the literature for polymeric antimicrobials. Surface tension measurements made as a function of copolymer molecular weight showed that surface activity at a constant TCS pendent group concentration of 5 mol% increased with decreasing molecular weight. Since this trend was similar to the trend found between molecular weight

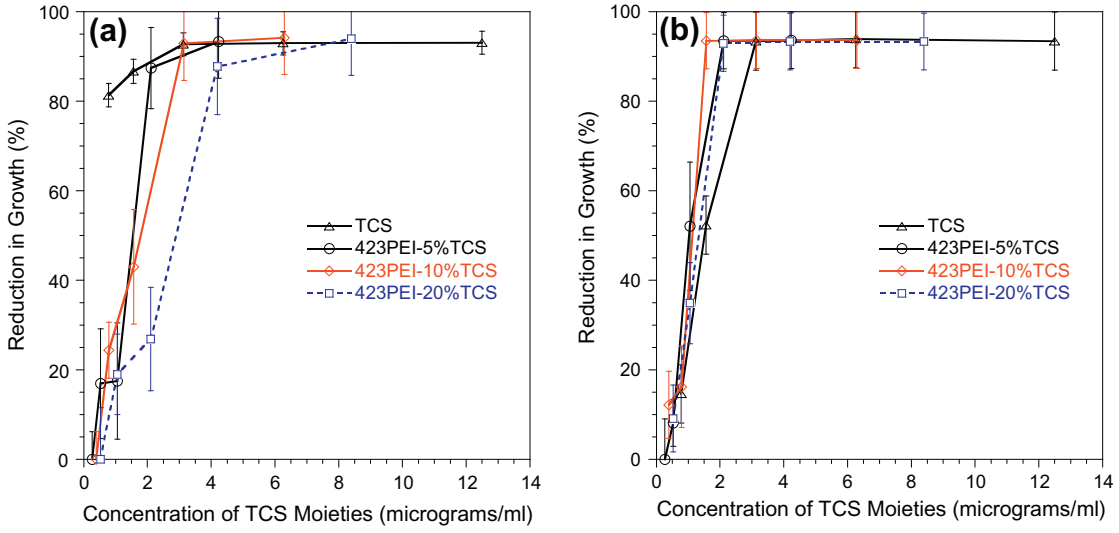

Fig. 9. A comparison of the antimicrobial activity of the copolymers derived from the lowest molecular weight PEI (i.e. 423 g/mole) to TCS. For this comparison, concentration is expressed as the concentration of TCS pendent groups as opposed to copolymer concentration. The two bacteria used were *S. epidermidis* (a) and *E. coli* (b).

and antimicrobial activity, it supports the suggestion that the mechanism of antimicrobial activity involves interaction with the bacterial cell wall.

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