ACS APPLIED MATERIALS & INTERFACES

RESEARCH ARTICLE

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Synthesis of Modified Guanidine-Based Polymers and their Antimicrobial Activities Revealed by AFM and CLSM

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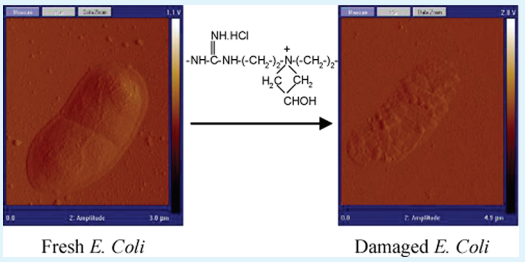
Supporting Information

ABSTRACT: Modified guanidine-based polymers with chain extension were synthesized by condensation and cross-linking polymerizations in an attempt to increase molecular weight and charge density of the antimicrobial polymers. The antimicrobial activity and the corresponding mechanisms were investigated by several approaches. The results indicated that the antimicrobial activities of the modified guanidine-based polymer, based on the minimum inhibition concentration (MIC) against *E.coli*, varied with alkyl monomer ratios. UV absorption at 260 nm further quantified the amount of intracellular components leaked into bacteria suspension. The UV absorption measurements were also used to monitor inhibition processes dynamically. It was found that the modified guanidine-based polymer inhibited the growth of bacteria by causing membrane compromised and intracellular leaked. Dual fluorescent dyes were used to stain all bacteria including the dead ones, which enabled us to utilize CLSM to visualize the viability of bacteria in the presence of various modified guanidine-based polymers without causing any damage. The morphologies of bacteria untreated and treated with modified guanidine-based polymer were observed using an atomic force microscope (AFM), which further demonstrated the damage of *E.coli* membrane and the leakage of intracellular component induced by the modified guanidine-based polymers.



KEYWORDS: antimicrobial polymer, polycondensation, UV absorption, atomic force microscopy (AFM), morphology

1. INTRODUCTION

Antimicrobial polymer is a range of agents with bacteria or fungi inhibition ability, which has been used widely in plastic,[1,2] textile,[3−5] water purification,[6] and so on. Most of antimicrobial polymers bear cationic groups along polymer chains, thus facilitating the adsorption of the polymer onto bacteria surfaces and further inhibiting the growth of bacteria via various mechanisms.[7−11] As one of the cationic antimicrobial polymers, the guanidine-based polymer has attracted substantial interests due to its wide spectrum antimicrobial activity, excellent biocide efficiency and nontoxicity.[12−14] Guanidine polymer is usually synthesized by polycondensation between guanidinium and diamine, and often reckoned as an oligomer because of its low molecular weight (as low as 1000).[15] The chain cross-linking would occur if prolonging reaction time and increasing reaction temperature. Low molecular weight constrains the application of guanidine polymer as antimicrobial agent in some fields, such as papermaking and textile[11,16] owing to poor retention. Guanidine polymer inhibits bacterial growth by attacking them through electrostatic attraction between cationic guanidio groups and anionic groups on the cell surface of bacteria. After attaching to bacteria cells, guanidine polymer induces bacterial membrane collapsed and intracellular components leaked thereafter.[17,18] Charge density, molecular weight and molecular structure of guanidine polymer would influence adsorption on bacterial surface and inhibition efficiency. Guanidine polymer can also react with other chemicals through its reactive amino groups.[19]

Antimicrobial ability of chemicals can be interpreted by the minimum inhibition concentration (MIC) to Gram-positive and Gram-negative bacteria, which is the lowest chemical concentration to inhibit the growth or propagation of bacteria after incubation. Confocal laser scanning microscopy (CLSM) has been used to investigate biological samples alive by staining them with various specific fluorescent dyes.[20−22] Some dyes can readily go through the compromised membrane of dead cell while not the integrated membrane of alive cell, whereas some dyes can stain both alive and dead bacteria simultaneously. With the synergic effects of dual fluorescent dyes, dead cell can be easily distinguished from alive ones by collecting bacteria images at various specific excitation wavelengths. Antimicrobial mechanism can be investigated by several techniques to provide insight of interactions between antimicrobial agents and bacteria. UV is an appropriate approach to quantify the intracellular leakage because cytoplasm leaked into bacteria suspension has strong absorption at 260 nm.[23−25] UV absorption can be measured with prolonging contact time at various antimicrobial polymer concentrations to dynamically monitor the inhibition growth of

Received: January 24, 2011
Accepted: April 14, 2011
Published: April 14, 2011

bacteria. Atomic force microscopy (AFM) is another powerful tool to investigate antimicrobial mechanism by revealing morphology transformation of bacteria under antimicrobial chemicals.[26−30] AFM can provide high-resolution images of biological samples with detailed topography and has been used widely to study the mechanisms of action of antimicrobial peptides on bacteria.

In this work, the modified guanidine-based polymer was synthesized by a two-step method consisting of condensation and cross-linking reactions. In addition to the polycondensation of diamines and guanidine salt, the cross-linking proceeded between imines group in condensation prepolymer and epichlorohydrin to further increase molecular weight and charge density of modified guanidine-based polymer. Antimicrobial activity of modified guanidine-based polymer was quantified by MIC method and investigated using CLSM. Moreover, the antimicrobial mechanism was revealed by monitoring UV absorption of bacteria suspension dynamically and visualizing the bacteria morphology with AFM at various guanidine polymer concentrations.

2. EXPERIMENTAL SECTION

2.1. Materials. Hexamethylene diamine (HMDA), Diethylenetriamine (DETA), Guanidine hydrochloride (GH) and Epichlorohydrin (EP) were purchased from Sigma-Aldrich and all reagents were used as received without further purification. *Escherichia coli* (*E. coli*) (ATCC11229) were obtained from the University of Guelph Canada; and the *E. coli* was cultured overnight at 37 °C in LB Broth prior to use.

2.2. Synthesis and Characterization of Modified Guanidine-Based Polymer. Modified guanidine-based polymer was synthesized by a two-step polymerization. Prepolymer was obtained by polycondensation from amine and guanidine hydrochloride first. The polyhexamethylene guanidine hydrochloride (PHGC) was synthesized using HMDA alone as an amino compound; whereas the polyhexamethylene diethylenetriamine guanidine hydrochloride (PHDGC) was prepared using a mixture of HMDA and DETA with the molar ratio fixed at 6:4. The prepolymers were then reacted with epichlorohydrin (E), leading to the modified guanidine-based polymer, i.e., E-PHGC and E-PHDGC. The detailed procedures are as follows: equal molar ratio of chemicals containing guanidine groups and amino groups were added into a 500 mL three-neck flask, the reagents were stirred mechanically and reaction temperature was increased step by step, first kept at 100 °C for 3 h, then 150 °C for 2 h, 180 °C for 1−2 h until the stirrer could not work for the high viscosity of prepolymer. Modified guanidine-based polymer was obtained further by cross-linking prepolymer solution (50 g, 40 wt %) with amount of EP (−NH−:EP = 0.2 mol:0.1 mol). EP was first added into prepolymer solution dropwise at 30 °C within 30 min and kept stirring for 4 h. The reactant was further diluted to 20% (wt) and temperature was increased to 60 °C for 6 h; then the reaction was stopped and the modified guanidine-based polymer was obtained. Deionized water was used as a solvent for the prepolymer.

2.3. Characterization of Modified Guanidine-Based Polymer. GPC (Pump: Waters 600E System Controller; Detector: Waters 410 Differential Refractometer; Colums: Ultrahydrogel 250 and 500) was used to measure molecular weight of modified guanidine-based polymer. Charge density of cationic polymer was determined by colloidal titration with potassium polyvinyl sulfate (PVSK) using a Mütek PCD-03(BTG, German). NMR (Varian Unity 400 spectrometer, American) was used to characterize the molecular structure of modified guanidine-based polymer and D_2O was used as a solvent.

2.4. Minimum Inhibition Concentration (MIC). A serial dilution method was used to determine the minimum inhibition concentration (MIC) of prepolymer and modified guanidine-based polymer against *E. coli* (ATCC11229). Fresh cultured *E. coli* was diluted with LB broth to 10^6CFU/ml which was reckoned by an OD_{600}-Con. standard curve. Polymers dissolved in sterile DD water were serially diluted in LB broth, the same amount of *E. coli* were put into polymer-broth solution. Both seeded tubes and growth controls were incubated at 37 °C for 18 h. MIC was the lowest concentration inhibiting visible growth of bacteria compared with control samples.

2.5. UV Absorption of *E. coli* Suspension. *E. coli* suspension (OD_{260} = 0.2−0.3) was obtained by centrifuging *E. coli* (1×10^7 CFU/mL) at 5000 rpm for 1 min, then washing twice and redispersing with PBS. After mixed with antimicrobial polymer for 30s, the OD_{260} values for both *E. coli*-treated samples and controls were measured using a Genesys 10 UV−vis spectrophotometer at the same time intervals. Each experiment was repeated for 3 times. Results of antimicrobial effects were expressed by OD_{260} ratio (treated sample/control sample).

2.6. Confocal Laser Scanning Microscopy (CLSM). A working solution containing two dyes was prepared to stain *E. coli* in this work. Solution A (5 mg fluorescein isothiocyanate (FITC) per mL of absolute ethanol) and solution B (1 mg of propidium iodide per mL of PBS) were prepared fresh daily. The working solution was obtained by mixing 50 μL of solution A and 40 μL of solution B with 1.9 mL of PBS. *E. coli* was stained by adding 3 μL of the working solution and desired amount of modified guanidine-based polymer into 1 mL bacteria suspension. The suspension was then mixed thoroughly and incubated at room temperature in the dark for 25−30 min. The stained *E. coli* was put between a slide and a coverslip, and observed under CLSM (Leica SP2 confocal microscope, German). Images were collected at an excitation wavelength of 488 nm first, to minimize photobleaching of the FITC, followed by collecting images of dead cells using an excitation wavelength of 560 nm. All cells including alive and dead are stained green by FITC and only dead cells are stained red.

2.7. Atomic Force Microscopy (AFM). *E. coli* suspension (1×10^8 CFU/mL) was first centrifuged at 5000 rpm for 1 min, then washed twice with PBS and redispersed in DD water. Modified guanidine-based polymers were added into *E. coli* suspension at various concentrations and shaken for 30s. Untreated and treated *E. coli* were spread onto Silicon wafer (Universitywafer, South Boston) and air-dried in a vacuum desiccator. AFM images were obtained with a Nanoscope IIIa (Veeco Instruments) in tapping mode using a silicon probe (NP-S20, Veeco Instruments) with settings of 512 pixels/line and 1 Hz scan rate.

3. RESULTS AND DISCUSSION

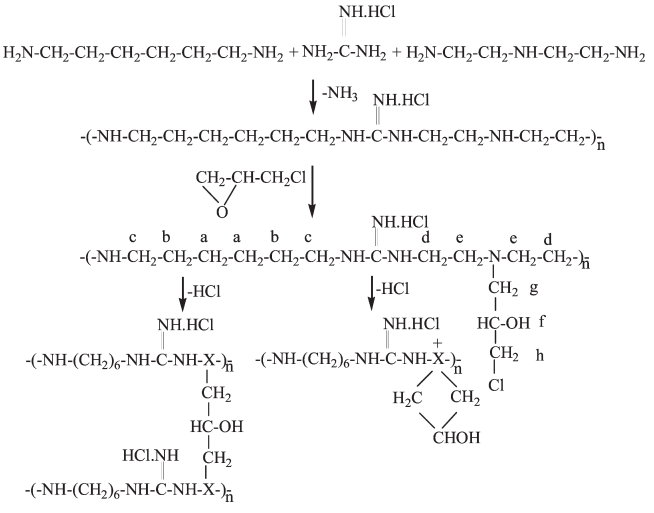
3.1. Synthesis and Characterization of Guanidine Polymer. In this work, the modified guanidine-based polymer was synthesized by a two-step method in an attempt to achieve high molecular weight, i.e., the obtained guanidine polymer by usual polycondensation was used as prepolymer to further cross-link with epichlorohydrin. Epoxy groups of epichlorohydrin is easy to react with imine groups in guanidine prepolymer, and the resulting prepolymer might cross-link or form four-membered rings via the secondary amine of DETA and HMDA. DETA not only provided more reactive sites but also imparted the polymer additional function due to the four-member ring (azetidinium ring) resulting from its reacting with EP. Scheme 1 presents the typical reaction scheme of modified guanidine-based polymer with DETA as a comonomer.

For the reaction shown in Scheme 1 with three diamines as monomers, the structure of prepolymer (PHDGC) should be more complicated than the one presented herein because it is difficult to predict the linkage sequence between the three monomers and molecular structure of prepolymer. Even for the

oligoguanidines polycondensed from two monomers, there were three or more major product series in terms of the terminated groups and some minor cyclic structure existed, as reported by Martin et al.[15]

The possibility of linkage between the three monomers in prepolymer chain was supposed to be equal for their similar reaction abilities. According to the reactivity of primary amine and secondary amine, the branched or cyclic structure might exist in the prepolymer to a lesser extent, and it should not be the major molecular structure discussed in this paper. In the process of obtaining the modified guanidine-based polymer (E-PHDGC), there were several various reactive sites of secondary amine groups in prepolymer which could react with EP and only a typical structure is presented in Scheme 1. This structure is similar to the one in polyamideamine-epichlorohydrin (PAE), a widely used wet-strength enhancing polymer for papermaking (e.g., tissues and kitchen towels). The reaction conditions adopted in this work are similar to those used for synthesizing PAE as well. For such reaction, Obokat et al.[31,32] reported that the 3-chloro-2-hydroxypropyl group formed at a low temperature would be mainly converted into a four-membered azetidinium ring (AZR) when the temperature was increased to 60−65 °C within 2 h. Meanwhile the intermolecular cross-linkage would be induced to further increase the molecular weight of the polymer. The formation of AZR groups and their consumption to form the cross-linkage would reach the equilibrium state after the reaction proceeded for 8−10 h.[31] Although the AZR group will be gradually deteriorated during storage,[33] it is fairly stable under acidic conditions at room temperature, but can react with various nucleophiles at elevated temperatures to form various ring-opening structures.[34] The azetidinium ring in the polymer is the reactive group that could potentially increase the wet-strength of paper.[35,36] Generally, the polymers resulting from the current reaction systems possess the complicated structures which are difficult to be characterized precisely. Therefore, the structures and reactions shown in Scheme 1 only represent the key repeat units or typical reactions occurring in the current systems, which were supported by 2D NMR spectra to some extent. The molecular structure of the guanidine polymer modified with DETA appeared to be much more complicated than the one without DETA; and both were the mixtures with various structures. The 2D NMR spectra of prepolymer and polymer as well as the corresponding analysis are available in the Supporting Information for reference. The main focus of the current work was to reveal the antimicrobial performance of as-synthesized polymers.

Scheme 1. Synthesis of Modified Guanidine-Based Polymer (E-PHDGC)[a]



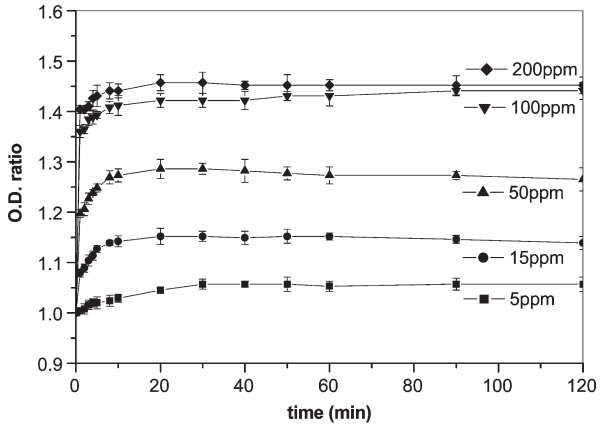
[a] Note: X is −(−CH_2−CH_2−N−CH_2−CH_2−)−, and the side chain (or four-member ring) was connected to −N−.

Table 1 shows the molecular weight, charge density, and minimum inhibition concentration (MIC) values of two types of the prepolymers and the corresponding guanidine-based polymer. Clearly, the MW of modified guanidine-based polymers was increased significantly. Moreover, the presence of DETA increased the charge density from 3.58 to 4.80 meq/g, implying the formation of azetidinium ring carring the quaternary ammonium group.

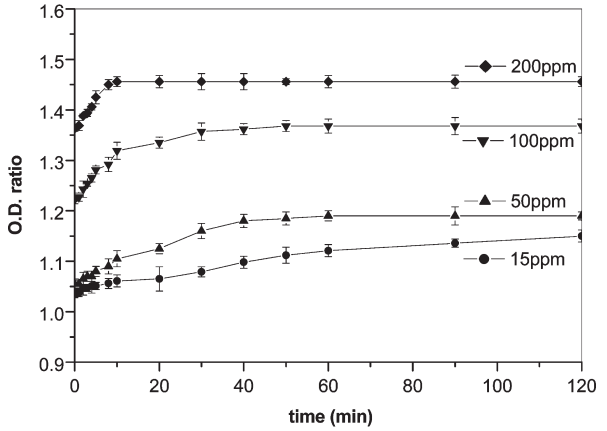
3.2. Antimicrobial Effects of Modified Guanidine-Based Polymer. The values of the minimum inhibition concentration (MIC) of prepolymer and modified guanidine-based polymer with various monomer ratios are shown in Table 1. Guanidio group contents were the same for both modified guanidine-based polymers obtained in this work, but one with DETA and another without. MIC values of E-PHDGC and E-PHGC were 31.2 ppm and 7.8 ppm, respectively. Though guanidio group was generally thought to be the key functional group for inhibiting bacteria, the antimicrobial activity was found to be sensitive to the amino group ratios. MIC of E-PHDGC was much higher than that of E-PHGC, suggesting that the addition of DETA decreased the antimicrobial effects of modified guanidine-based polymer. Both molecular chain length and stiffness of DETA are less than those of HMDA. Modified guanidine-based polymer with high cationic charge density can absorb and attach to anionic bacteria cell membrane readily via electrostatic association; whereas long and rigid molecular chains are easy to perforate into cell membrane due to hydrophobic interaction, thus facilitating the leakage of contents of bacterial. With the equal guanidio group content, modified guanidine-based polymer without DETA could inhibit the growth of bacteria more efficiently. Comparing the MIC of prepolymer and final product, it was found that the cross-linking hardly affected the antimicrobial activity. The presence of DETA in the modified guanidine-based polymer does not improve the antimicrobial activity of the polymer but leads to the formation of azetidinium ring which plays a critical role in enhancing wet-strength of paper as addressed previously. Cationic modified guanidine-based polymer is feasible to attach to bacteria cell

Table 1. Characterization of Modified Guanidine-Based Polymers

	E-PHDGC (HMDA:DETA = 60:40)		E-PHGC (HMDA:DETA = 100:0)	
monomer ratio	prepolymer	modified polymer	prepolymer	modified polymer
MW	1800 ± 100	3700 ± 200	1040 ± 100	3600 ± 200
charge density (meq/g)	3.58 ± 0.11	4.80 ± 0.14	5.37 ± 0.16	4.82 ± 0.14
MIC (ppm)	31.2	31.2	7.8	7.8



(A) E-PHGC



(B) E-PHDGC

Figure 1. Dynamic UV absorption of *E. coli* with different guanidine polymer concentration.

membrane bearing some anionic charges. The phospholipid bilayer of bacteria cell can be redistributed through electrostatic interaction with positively charged guanidine-based polymer.[1] Bacteria cell membrane became damaged exposed to the cationic polymer; and the inner content of cell released gradually, small ions initially followed by large organic molecules. It was known that the large organic intracellular components had a strong absorbance at 260 nm,[16,17] so that the release of cytoplasm was an appropriate indication for membrane integrity of bacteria cells and antimicrobial activity of cationic polymer. UV absorbance of *E. coli* suspension at 260 nm with modified guanidine-based polymer at various concentrations was measured at same time intervals; and the antimicrobial process interpreted by OD_{260} ratio, which was dynamically monitored, is shown in Figure.1. For both modified guanidine-based polymers, OD_{260} ratio increased rapidly at an initial period and reached plateau finally after various contact times at different polymer concentrations, shorter for higher concentration. It only took 10 min or less for E-PHGC to inhibit most of bacteria and to reach the maximum OD_{260} ratio when the polymer concentration was 15 ppm or higher. OD_{260} ratio for the concentration below MIC only reached 1.05−1.10 eventually; whereas the ratio was higher than 1.15 for the concentration above MIC, suggesting that most of bacteria cells were inhibited due to the leakage of cytoplasm.

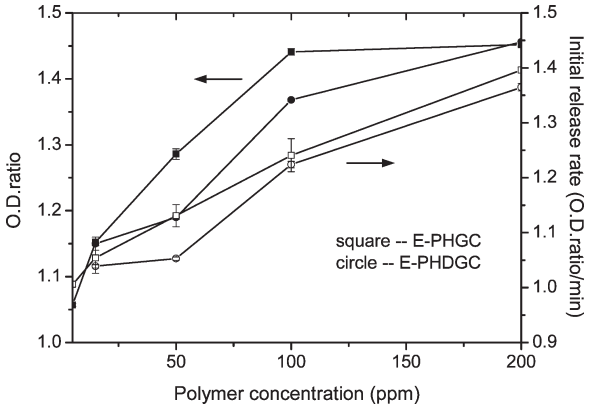
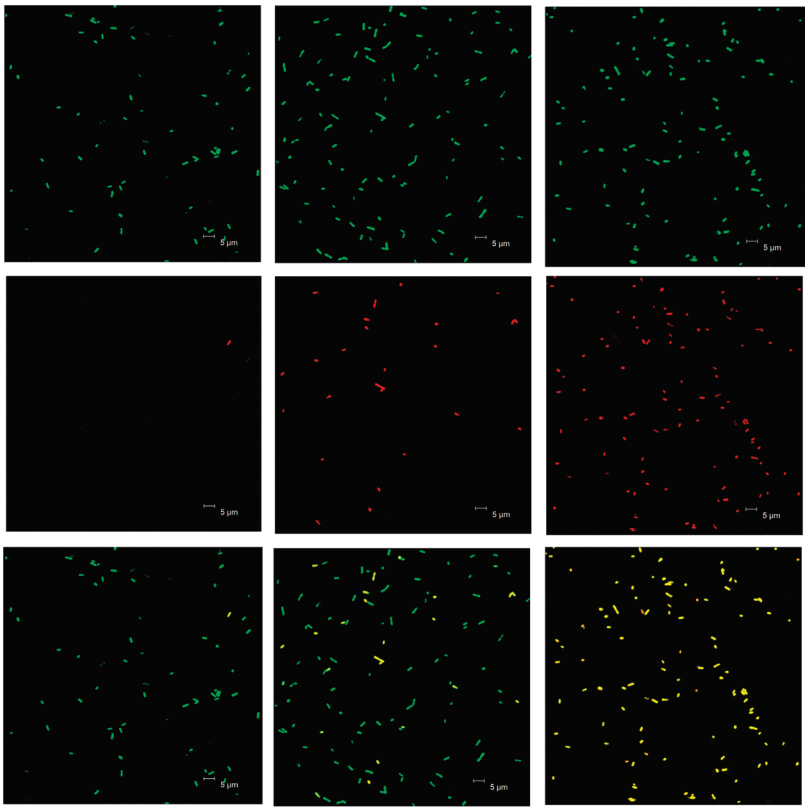


Figure 2. Relationship between the final O.D. ratio and the initial release rate at various polymer concentrations.

Increasing the polymer concentration to 200 ppm increased the OD_{260} ratio up to 1.45 for both polymers.

The bacterial membrane serves as a structural component which may become compromised when exposed to antimicrobial agents, therefore the release of intracellular components that have strong UV absorption at 260 nm is a good indicator of membrane integrity.[23] This is a well-received approach for determining membrane integrity, which was adopted in the current research.[23,24] The release rate of cytoplasm as a function of the polymer concentration is shown in Figure.2. Clearly, the higher the modified guanidine-based polymer dosages, the faster the releasing of cytoplasm for both polymers. At the same polymer concentration, the initial release rate of the intracellular components or cytoplasm triggered with E-PHGC was higher than that with E-PHDGC, which indicated the better antimicrobial activity of E-PHGC. Though the initial release rate of intracellular components provided insight into the release kinetics, and it is still difficult to establish a release kinetics model only based on the UV absorption results. The final O.D. ratio (plateau value in Figure 1) is also polymer-dosage dependent or proportional to the polymer concentration as shown in Figure 2. The plateau curve suggested the release of cytoplasm reached the equilibrium under the specific polymer concentrations. The polymer at a high concentration leading to a high O.D. ratio at plateau also implied the more complete disruption of the cell membrane. Between two modified guanidine-based polymers, E-PHDGC showed relatively lower antimicrobial activity than E-PHGC according to OD_{260} ratios, which is consistent with the MIC results. Overall, the rate and amount of cytoplasm leakage were varied by the polymer composition and concentration. In other words, the antimicrobial activity of modified guanidine-based polymer could be tailored or controllable by adjusting monomer ratios for targeted applications.

Confocal laser scanning microscopy (CLSM) was also used for assaying the viability of bacteria in the presence of modified guanidine-based polymers. Among various dyes used as probes to stain bacteria from CLSM, fluorescein isothiocyanate (FITC) and propidium iodide (PI) were proved to be suitable to stain all bacteria and dead bacteria individually.[2] FITC can label all bacteria including alive and dead by forming a covalent bond between dye and protein nonspecifically, whereas PI is a cationic fluorescent dye which is readily go through compromised cell membrane and stain dead bacteria. FITC can be excited at 488 nm and all bacteria fluoresce green, only dead bacteria fluoresce red with PI excited at 560 nm. The images of bacteria were first collected at 488 nm to



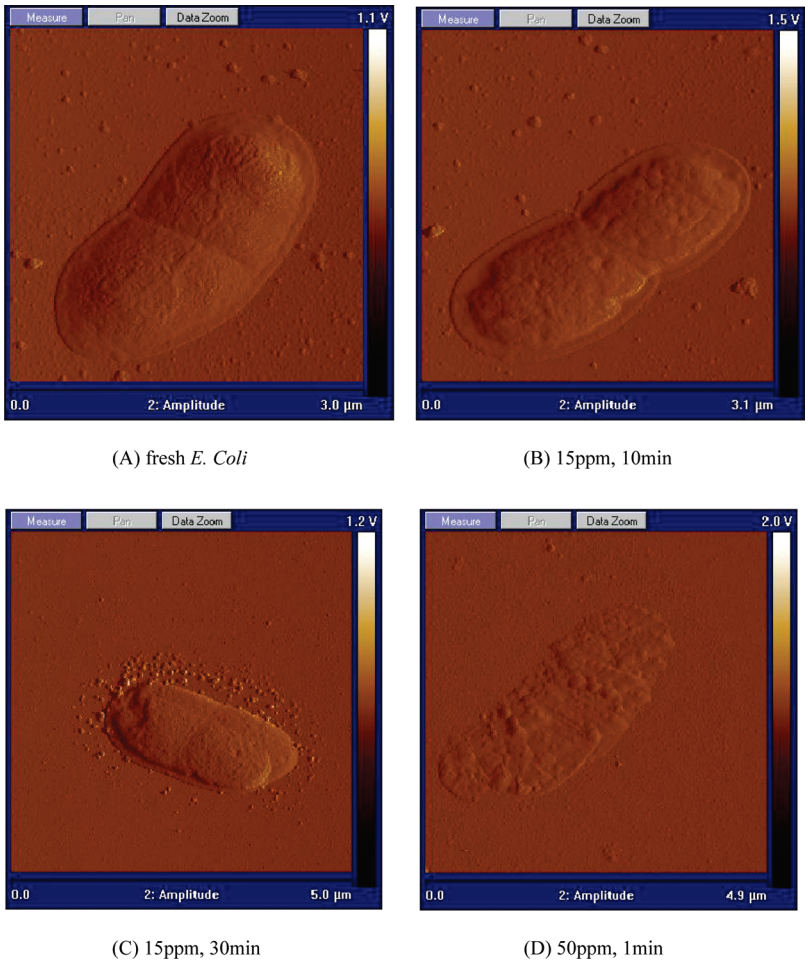
(A) fresh *E. Coli* (B) E-PHGC (5ppm 30min) (C) E-PHDGC (50ppm 1min)

Figure 3. Confocal image of *E. coli* untreated and treated by guanidine polymer. Note: green dots on the top are alive and dead *E. coli*; red dots in the middle are dead *E. coli*; yellow dots on the bottom are dead *E. coli* and green dots are alive ones.

minimize photobleaching of FITC, and then the images of dead bacteria were collected at 560 nm. Comparing the images obtained at 488 nm and 560 nm, dead bacteria cells could be distinguished by red color, and thus representing the antimicrobial performance of modified guanidine-based polymers. Figure 3 shows the images of *E. coli* untreated and treated by modified guanidine-based polymer at various conditions. As can be seen, all bacteria including alive and dead cells were stained green (images on the top), whereas only dead bacteria cells were stained red (images in the middle) and yellow sites in images on the bottom also showed dead cells by merging the images on the top and middle. For fresh *E. coli*, most of bacteria were vigorous and only few bacteria was dead (red). For E-PHGC with high antimicrobial efficiency, a small portion of bacteria was inhibited even after 30 min at the concentration (5 ppm) lower than MIC; for E-PHDGC with lower antimicrobial efficiency, almost no alive bacteria left after only 1 min at the concentration (50 ppm) higher than MIC.

Therefore, the antimicrobial performance of modified guanidine-based polymer was most influenced by polymer concentration and contacting time. Results obtained from CLSM are well correlated to the OD_{260} ratio (shown in Figure 1). CLSM images of bacteria with low E-PHGC concentration (5 ppm) showed some bacteria fluorescing red (dead) after 30 min and its corresponding OD_{260} ratio was only 1.05. The images of bacteria with high E-PHDGC concentration (50 ppm) indicated almost all bacteria were inhibited (red) after 1 min, and its OD_{260} ratio also reached the maximum value of 1.20 after 30 min.

3.3. Antimicrobial Mechanism of Guanidine Polymer Revealed by AFM. AFM was used to reveal morphological change of *E. coli* after treated with modified guanidine-based polymer. Figure.4 presents the morphology of fresh *E. coli* and treated *E. coli* by modified guanidine-based polymer with various concentrations and contacting time. The surface membrane was integrated and structured for fresh *E. coli*, and there were no obvious indentations and grooves on surface of fresh *E. coli*. After treated by modified guanidine-based polymer, *E. coli* showed different patterns corresponding to the different concentrations of the polymer and contacting time. Morphology of *E. coli* exhibited membrane compromised and intracellular component leaked, which interpreted UV and CLSM results clearly. Figure 4B−D compares the morphology of treated *E. coli* with modified guanidine-based polymer. In Figure.4 (B) and (C), bacteria cells treated with E-PHDGC could keep intact shape and cell profile was still legible after 30 min at the concentration (15 ppm) lower than MIC. There were no obvious indentations and grooves observed on the surface, whereas small amount of intracellular components was effused from the entire cell. In this case, cell membrane was not collapsed and most of intracellular components were still kept in bacteria cell, so bacteria was still alive and UV absorption of bacteria suspension increased slightly (OD_{260} = 1.07). Figure 4D shows the morphology of *E. coli* treated with E-PHDGC at concentration (50 ppm) higher than MIC. As can be seen, bacteria cell was damaged severely and cell membrane was collapsed. It is hard to



(A) fresh *E. Coli* (B) 15ppm, 10min

(C) 15ppm, 30min (D) 50ppm, 1min

Figure 4. AFM Morphology of fresh and treated *E. coli* by modified guanidine-based polymer (E-PHDGC).

distinguish the bacterial cell profile and only debris was found, which is consistent with those observed from CLSM and UV tests.

4. CONCLUSION

Modified guanidine-based polymer was synthesized by a two-step polymerization consisting of condensation and cross-linking to obtain the antimicrobial polymer with a higher molecular weight and cationic charge density than prepolymer. The modified guanidine-based polymer was a mixture with various structures for a variety of reactions involved in the both steps. Antimicrobial ability of modified guanidine-based polymer was adjusted by varying alkyl monomer ratios at the same content of guanidio groups. Dynamic UV absorption of bacteria suspension at 260 nm indicated that the modified polymer could inhibit bacteria effectively due to the intracellular leakage induced. Antimicrobial ability interpreted by OD_{260} ratio strongly depended on the polymer concentration in comparison with MIC, higher OD_{260} ratio corresponding to higher concentration for some extent; whereas the OD_{260} ratio reached a maximum value finally regardless of polymer concentration. CLSM could distinguish dead bacteria cells from alive ones by staining bacteria with dual fluorescent dyes. Confocal fluorescent images demonstrated that the antimicrobial performance of modified guanidine-based polymer was mainly influenced by polymer concentration and contacting time. Morphology of bacteria revealed by AFM showed that the bacteria cells could keep intact shape and intracellular components only effused from the entire cell at the low concentration of polymer; whereas at a high concentration (>MIC), bacteria cell was severely damaged to debris and massive amount of intracellular components was leaked. AFM images provided the visualized evidence to elucidate the antimicrobial mechanism, which is consistent with those identified from UV and CLSM characterizations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figures of 2D NMR spectra of prepolymer and modified guanidine-based polymer prepared (E-PHDGC) along with corresponding analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

Financial supports for this work from NSERC SENTINEL-Bioactive Paper Network (Canada), 973 Project (2010CB732201, China), and GDNSF (9451064101002911) are gratefully acknowledged.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on May 25, 2011. Due to a production error, the Abstract graphic was incorrect. The corrected version was reposted on June 22, 2011.