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# Modified guanidine polymers: Synthesis and antimicrobial mechanism revealed by AFM

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ABSTRACT

Condensation polymerization was used in the preparation of modified guanidine polymers. The polymers were composed of hexamethylene diamine and guanidine hydrochloride with subsequent crosslinking of epichlorohydrin. The two-step synthesis method increased the molecular weight of guanidine polymers, and also enhanced the charge density of the polymer due to the formation of the four-membered rings containing quaternary ammonium groups. This was found to improve antimicrobial activity. The molecular structure of the prepared guanidine polymer was characterized by 2D heteronuclear correlation NMR spectroscopy (C–H COSY). Results indicated that guanidine polymer was a mixture with various structures. $UV_{260}$ absorption and AFM were applied to reveal the dynamic antimicrobial process of guanidine polymer and morphological change of bacterial cells. Both $UV_{260}$ absorption and AFM images showed that the antimicrobial mechanism of guanidine polymer was to destruct the cell membrane of bacteria and cause the leakage of intracellular components from bacterial cells.



## 1. Introduction

Guanidine polymer is a series of polymers bearing guanidino groups, which are mainly used as an antimicrobial agent. Among various types of guanidine polymers, the one synthesized from hexamethylene and guanidine salt has been the most extensively investigated [1–3]. Due to its high water solubility, wide spectrum antimicrobial activity, excellent biocide efficiency and nontoxicity, guanidine polymer has been used in medical, fiber, textile, plastic, etc. [4–7]. Guanidine polymer can also react with other chemicals or graft to some long chain polymers [5,8] because of the active amino group in its backbone. This method produces more guanidine polymer derivatives, and also broadens their application as an antimicrobial agent.

Much work had been dedicated to reveal the antimicrobial mechanism of guanidine polymer against gram-positive and gram-negative bacteria. It is believed that guanidine polymer can destroy the membrane of bacterial cells and cause leakage of intracellular contents [9,10], therefore, inhibiting the growth of bacteria effectively. These intracellular components show strong absorption at 260 nm, which is easily detected by an UV–vis spectrophotometer [11,12]. By comparing absorbance of bacterial suspension at 260 nm in the presence of biocides, the extent of membrane damage and cytoplasm leakage can be quantified. Though $UV_{260}$ method is a good indicator of cell membrane integrity and has been used for studying some biocides, little work has been conducted for guanidine polymer, especially for monitoring the dynamic antimicrobial process. Atom Force Microscopy (AFM) is another powerful technique for investigating antimicrobial mechanism by visualizing bacterial cells, which was used to reveal the antimicrobial mechanism of some peptides [13,14]. AFM approach provided a vivid and detailed morphology of bacterial cells undergoing various stages or different mechanisms of antimicrobial actions. For guanidine polymers, there are very few reports for visualizing morphology changes at various stages to verify the proposed antimicrobial mechanism so far.

In this work, the modified guanidine polymer was synthesized in two steps: polyhexamethylene guanidine hydrochloride (PHGC) was formed as a prepolymer by condensation; guanidine polymer was crosslinked by prepolymer and epichlorohydrin. From the second step, guanidine polymer not only gained high molecular weight via the crosslinking but also showed high cationic charge density due to the formation of four-membered ring structure containing quaternary ammonium groups. Molecular structure of guanidine polymer was characterized by 2D NMR spectra (C–H COSY). Dynamic antimicrobial process was investigated by

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measuring the $UV_{260}$ absorbance of bacterial suspension with various guanidine polymer concentrations according to the contact time. Morphology of bacterial cells exposed to guanidine polymer was also visualized using AFM to reveal the transformation of cell membrane and antimicrobial mechanism of guanidine polymer.

## 2. Experimental

### 2.1. Materials

Hexamethylene diamine (HMDA), 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) was cultured overnight at 37 °C in LB broth prior to use.

### 2.2. Synthesize and characterization of guanidine polymer

Guanidine polymer was synthesized by a two-step polymerization, and the procedures are detailed as follows: equal molar ratio (1:1) of hexamethylene diamine and guanidine hydrochloride were put into a 500-ml three-neck flask, the mixture was mechanically stirred while reaction temperature was increased step by step, firstly kept at 100 °C for 3 h, then 150 °C for 2 h, and 180 °C for 1 h. The prepolymer was dissolved in distilled and deionized (DD) water at 40% (wt) concentration. Prepolymer solution (50 g) was put into a 250-ml one-neck flask equipped with a magnetic stirrer. EP (9.2 g) (–NH–:EP ≈ 0.2 mol:0.1 mol) was added into the prepolymer solution dropwise at 30 °C within 30 min and kept stirring for 4 h. The reactant was further diluted to 20% mass concentration and the reaction temperature was increased to 60 °C for 6 h, then the reaction was stopped and the modified guanidine polymer was obtained.

### 2.3. Characterization of modified guanidine polymer

Charge density of modified guanidine polymer was determined by colloidal titration with PVSK solution (0.5 mN) using a Particle Charge Detector (Mütek PCD 03; Herrsching, Germany).

Molecular weight (MW) and MW distribution ($D$) were measured by GPC (pump: Waters 600E System Controller; detector: Waters 410 Differential Refractometer) equipped with Ultrahydrogel 250 and 500 columns. The measurements were conducted in 0.05 M sodium sulfate aqueous solution at 40 °C with the flow rate of 0.7 ml/min. Pullulan samples from P-5 to P-1600, MW ranging from $5.8 \times 10^3$ to $1.66 \times 10^6$, and β-cyclodextrin (MW = 1135) were used as standard samples for calibration. Each sample solution (0.2% wt) was filtered with a 0.45 μm Nylon Cameo filter-syringe prior to the test.

Molecular structure was characterized mainly by NMR. A small amount of modified guanidine polymer solution was air dried and then dissolved in $D_2O$. $^1H$ NMR, $^{13}C$ NMR and C–H heteronuclear correlation spectroscopies (C–H COSY) of guanidine polymers were conducted using a Varian Unity 400 spectrometer (300 MHz).

### 2.4. UV absorption of E. coli suspension

Cytoplasmic constituents of bacterial cells have the specific UV absorbance at 260 nm. If bacterial membrane is compromised with the effect of guanidine polymer, dynamic antimicrobial process can be monitored by measuring the amount of cytoplasm released from bacterial cells. The specific experiments were conducted as follows: centrifuged *E. coli* suspension ($10^7$ CFU/ml) at 5000 rpm for 1 min (IEC Micromax Centrifuge), washed it with PBS twice and re-dispersed the bacterial pellet with PBS to get *E. coli* suspension ($OD_{260} \approx 0.2$–$0.3$) for UV experiment. Prepared the control sample and the sample containing antimicrobial polymer simultaneously, mixed the samples for 30 s and measured $OD_{260}$ for both control samples and treated samples separately at same time intervals (Genesys 10 UV–Vis spectrophotometers) with an empty quartz cell as reference. Results of antimicrobial effects were expressed by $OD_{260}$ ratio (treated sample/control sample). The lower the ratio, the higher the antimicrobial efficiency.

### 2.5. AFM

*E. coli* cells in LB broth was separated by centrifuging bacterial suspension ($10^8$ CFU/ml) at 5000 rpm for 1 min, washed with PBS twice and re-dispersed in DD water (referred to as fresh *E. coli*). Treated *E. coli* suspensions were prepared by mixing fresh *E. coli* with guanidine polymer solution to reach some concentration, then shaken for 30 s. Fresh *E. coli* and treated *E. coli* were dropped on 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 of guanidine polymer

In this work, guanidine prepolymer was firstly synthesized by condensation from hexamethylene diamine and guanidine hydrochloride, then epichlorohydrin was introduced to crosslink the prepolymer to produce a high molecular weight guanidine polymer. The reaction scheme is shown in Fig. 1, and the letterings are assigned to various carbons and protons. Modified guanidine polymer was easy to crosslink via the reaction between methylenechloride and imine groups in molecules (see Fig. 1(A)). The number-average molecular weights (Mn) of prepolymer and modified guanidine polymer were 1040 ($D = 1.2$) and 3600 ($D = 1.3$), respectively. The crosslinking increased the molecular weight of modified guanidine polymer compared to prepolymer and similar polymers reported elsewhere [9]. Without the crosslinking or chain extension, the molecular weight of guanidine polymer tends to be in a relatively low range due to the high viscosity of polymer in bulk condensation polymerization which inhibits the further growth of polymer chains. Meanwhile, a side reaction leading to the formation of four-membered 3-hydroxyazetidinium (AZR) rings occurred in the crosslinking process. Quaternary ammonium groups were created in the side reaction associated with the formation of the AZR structure (Fig. 1(B)), resulting in the increasing of charge density of the modified guanidine polymer. Base on the results of colloidal titration, the as-synthesized guanidine polymer was a cationic polymer with the charge density of 4.80 meq/g which was higher than that of prepolymer (3.58 meq/g). Due to the high reactivity of AZR groups, part of those groups were likely consumed further by the formation of the crosslinkage between the prepolymer chain. From an antimicrobial function point of view, the formation of AZR groups would not deteriorate antimicrobial activity as it is well known that the quaternary ammonium groups are also of antimicrobial activity to some extent. Of course, the formation of AZR groups would not enhance the molecular weight of polymer as high as the crosslinking, thus potentially broadening the MW distribution.

2D NMR spectrum was used to investigate the molecular structure of guanidine polymer because it not only gave the chemical shift of proton and carbon atoms but also revealed the correlation between them. C–H COSY spectra of guanidine polymer, shown in Fig. 2, indicated that the chemical environment of methane protons A and B was very similar. Influenced by long range

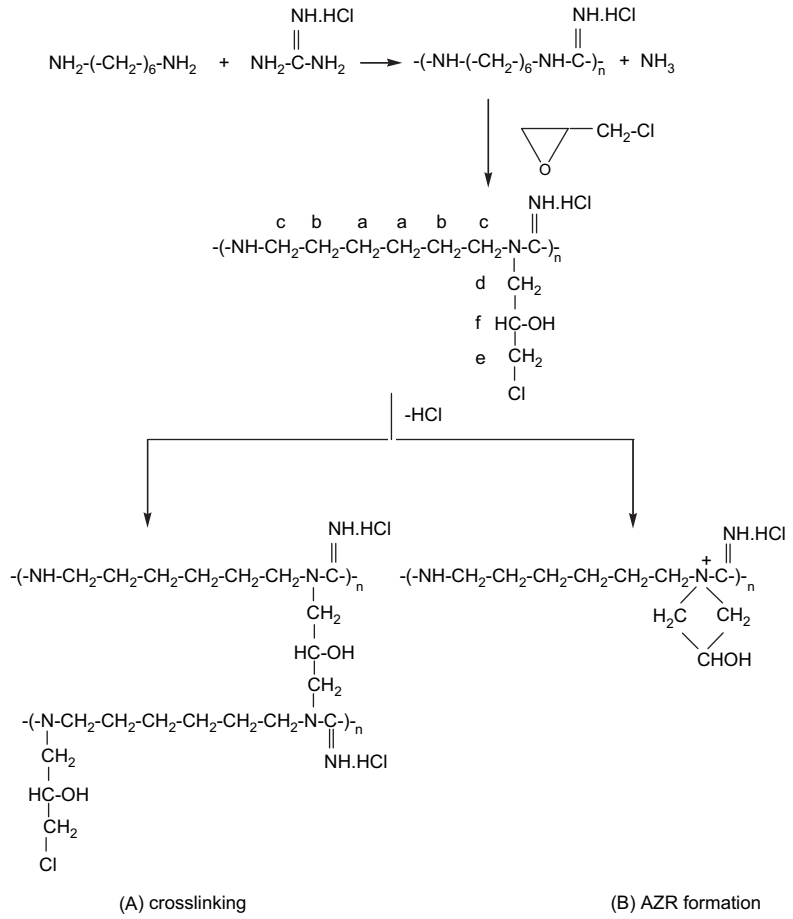


**Fig. 1.** Scheme of the synthesis of modified guanidine polymer.

correlation of amino group, chemical shift of proton B was downfield to 1.599 ppm, whereas chemical shift of proton A was 1.378 ppm. This assignment for protons A and B was different from those reported previously (i.e., 1.378 ppm peak was assigned to the proton of C–NH–C in guanidino and the proton of $CH_3$ for PHGS and PHBGS [2]). Proton of 1.378 ppm had strong correlation with carbon

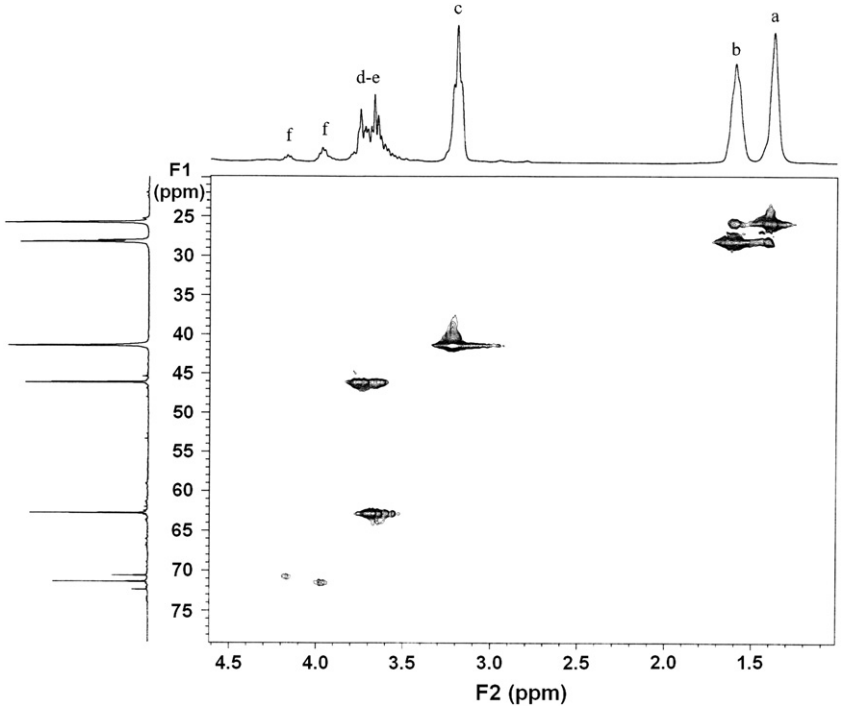


**Fig. 2.** C–H COSY (2D NMR) spectrum of modified guanidine polymer.

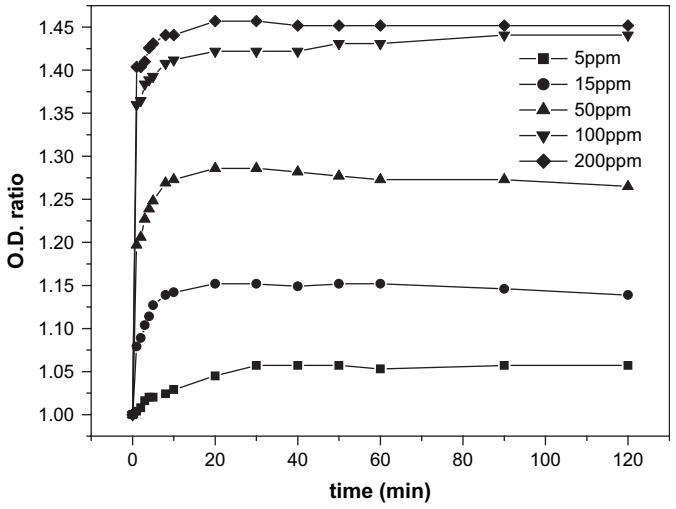


**Fig. 3.** Dynamic UV absorption of *E. coli* in the presence of modified guanidine polymers with various concentrations.

A (25.75 ppm) and weak correlation with carbon B (28.22 ppm), so it should connect with carbon A directly and have some coupling effects with carbon B. Chemical shift of proton C was assigned to 3.195 ppm further downfield by directly connecting with electronegative amino group and the corresponding carbon C was 41.45 ppm. Protons D and E were located at 3.55–3.79 ppm with splitting peaks and overlapped area because there were several structures formed in crosslinking process, i.e., protons with same carbon could show different chemical shifts. Carbons D and E were assigned to 62.84 ppm and 46.17 ppm respectively according to results for similar structure [15]. Peaks at both 3.97 ppm and 4.17 ppm were assigned to proton F for their connecting to the same carbon F (71.52 ppm). In terms of C–H COSY spectra, guanidine polymer was believed to be a mixture with various structures.

### *3.2. Dynamic monitoring of antimicrobial behavior of guanidine polymer*

Minimum inhibitory concentration (MIC) of guanidine polymer against *E. coli* ATCC11229 was 8 ppm, determined by a serial

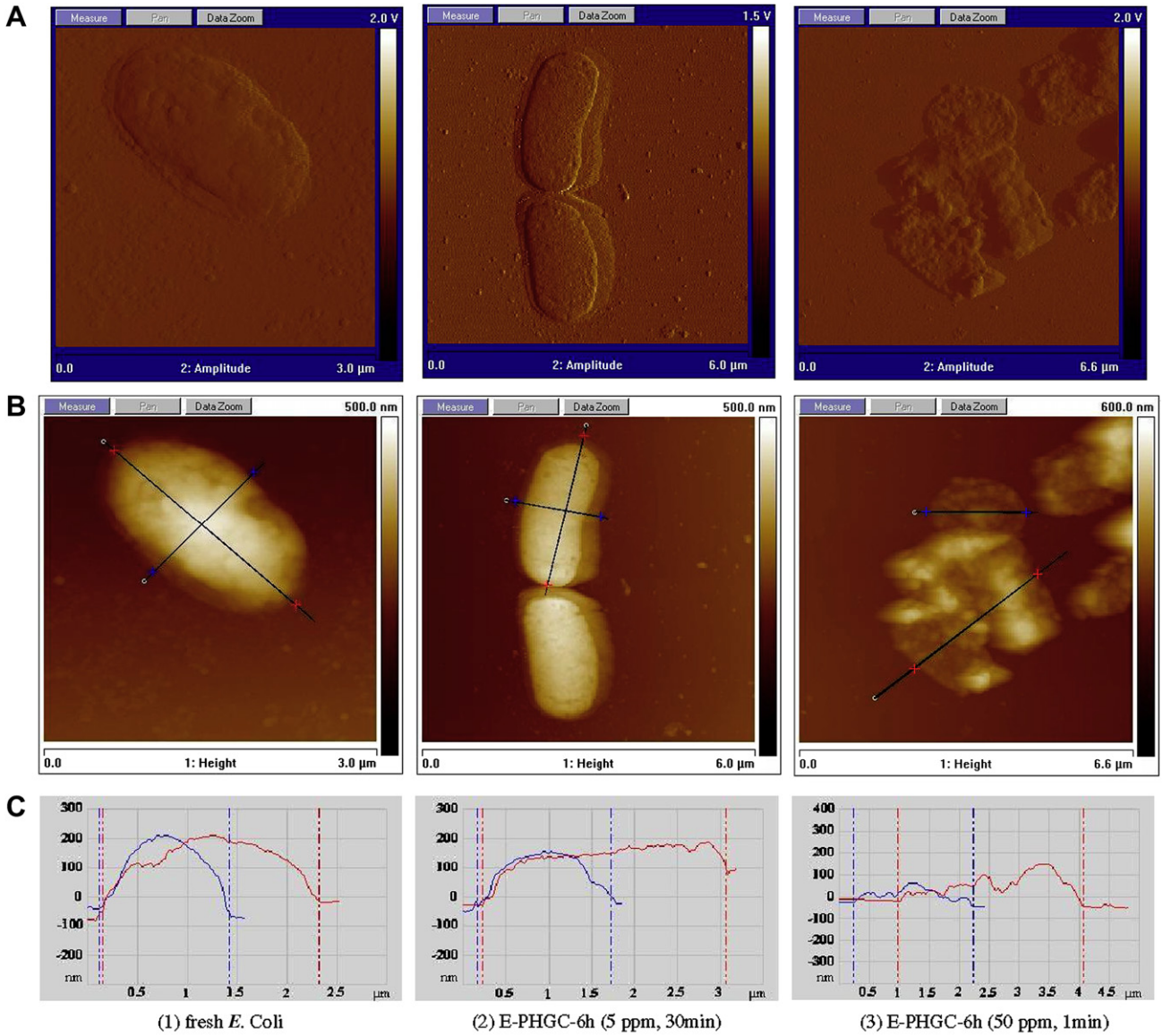


**Fig. 4.** Morphology of *E. coli* treated with modified guanidine polymer with different concentration.

dilution method. Surface of bacterial cells normally bear a small quantity of anionic charges which are easy to be attacked by cationic polymer through electrostatic attraction. Bacterial membrane became compressed when exposed to cationic guanidine polymer solution and intracellular components were released thereafter. It was known that the intracellular components had a strong absorbance at 260 nm [11,12] so that the release of cytoplasm was a good indication for membrane integrity of bacterial cells and antimicrobial activity of cationic polymer.

UV absorbance of *E. coli* suspension at 260 nm in the presence of guanidine polymer with various concentrations was measured at same time intervals, and the antimicrobial activity, represented by $OD_{260}$ ratio, was dynamically monitored. The results are presented in Fig. 3. As can be seen, the $OD_{260}$ ratios reached plateau eventually regardless of polymer concentrations. The prolonged contact time unlikely killed more bacteria at certain antimicrobial agent concentration. However, the final $OD_{260}$ ratio was proportional to the guanidine polymer concentration. For the concentration (5 ppm) lower than MIC (8 ppm), OD ratio only reached 1.05, suggesting that few bacteria was disinfected. Whereas for high concentrations ($\geq$50 ppm), OD ratios were higher than 1.27, implying that much cytoplasm was leaked by damaging *E. coli* cells. It was also noted that OD ratios were almost kept same when guanidine polymer concentration was above 100 ppm. Most of the *E. coli* were damaged under 100 ppm and further increasing concentration did not lead to better antimicrobial effect. OD ratio reached the maximum at different contact times for the guanidine polymer of different concentrations, i.e., shorter for higher concentration. When the concentration was 5 ppm, $OD_{260}$ ratio leveled off after 30 min, whereas for the concentrations at 15 ppm and higher, it only took 10 min to reach plateau. Guanidine polymer could damage most of the bacterial cells in a few seconds when its concentration was sufficiently high. This meant that the initial release rate of cytoplasm from *E. coli* cells increased with increasing guanidine polymer concentration, which was agreeable with the findings reported by other researchers [11].

### 3.3. Antimicrobial mechanism of guanidine polymer revealed by AFM

AFM was applied to reveal the morphology of fresh *E. coli* and the *E. coli* treated with modified guanidine polymer. Fig. 4(A) presents the topography of fresh *E. coli* and treated *E. coli* at various guanidine polymer concentrations and contact times, (B) shows the height images, and (C) shows the section images corresponding to lines in (B). For fresh *E. coli*, the surface membrane was structured and integrated, and there were no indentations and grooves on cell surface. Air drying process prior to AFM observation hardly dehydrated the bacterial cells and there were no leaked residues around them. From height and section images, *E. coli* cells showed elliptical with middle high and ends low shape with height difference above 200 nm. After treated with the guanidine polymer, *E. coli* showed different patterns at low and high concentrations. It is of interest that the morphology of *E. coli* could interpret the corresponding UV results. As can be seen from Fig. 4(2A), bacterial cells could maintain the intact shape after 30 min in the guanidine polymer solution when the polymer concentration was lower than MIC. There were no obvious indentations observed on the surface, whereas a small amount of leakage was found around the entire membrane. Therefore, UV absorption of bacterial suspension at 260 nm increased slightly because most of the intracellular components were still kept in cell membrane and only minority was leaked out. Section images showed that height of bacteria decreased to 100–150 nm, and there was a transition line between cell and leaked fluid which was different from the smooth height line shown in Fig. 4(1C). As can be seen in Fig. 4(3A), bacterial cells collapsed and cell membrane was completely destructed after exposed to the polymer solution at a higher concentration (50 ppm) for 1 min, resulting in hardly distinguishing individual bacterial cell. Significant losses of intracellular components from bacterial cells caused the membrane to collapse and the remaining height of bacteria was less than 100 nm. Disintegrated cell membrane made massive amounts of cytoplasm left in bacterial suspension so that OD ratio at a high guanidine polymer concentration became much higher. Comparing AFM images of fresh *E. coli* and treated *E. coli*, it could be concluded that the antimicrobial mechanism of guanidine polymer was to destruct cell membrane of *E. coli* and induce the leakage of intracellular components from bacterial cells.

## 4. Conclusions

Modified guanidine polymers were synthesized by a two-step method to increase molecular weight and charge density of final product. 2D NMR spectra showed that modified guanidine polymer was a mixture with various molecular structures according to correlation between proton and carbon. Modified guanidine polymer was an effective antimicrobial agent with MIC as low as 8 ppm. $UV_{260}$ absorption was applied to monitor bacterial inhibition process with modified guanidine polymer dynamically because intracellular components leaked from bacterial cells have specific absorption at 260 nm. Results showed that the leakage of intracellular components reached the maximum at different contact times for various modified guanidine polymer concentrations and prolonged contact time unlikely killed more bacteria at certain concentration. AFM images showed that bacterial cells could maintain intact shape after contacting with modified guanidine polymer for up to 30 min at a low concentration (5 ppm), whereas the cells collapsed and cell membrane was completely destructed after exposed to a higher concentration of guanidine polymer (50 ppm) for only 1 min.

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## Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.polymer.2008.03.042.

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