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Synthesis and Antimicrobial Properties of a Guanidine-Based Oligomer Grafted with a Reactive Cationic Surfactant through Michael Addition

Bin Wang,[1,2] Bailing Liu,[1] Gang Peng,[1,2] Xu Meng,[1,2] Zhengwu Jiang,[1,2] Hualin Chen[1]

[1]Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Chengdu 610041, China

[2]Graduate University of Chinese Academy of Sciences, Beijing 100049, China

Correspondence to: B. Liu (E-mail: blliuchem@hotmail.com)

ABSTRACT: A guanidine-based oligomer grafted with a reactive cationic surfactant was designed and synthesized through Michael addition in an attempt to combine its antibacterial and emulsification properties. This was also an excellent and efficient strategy for preparing more kinds of guanidine derivatives. Fourier transform infrared spectroscopy, ^{1}H-NMR, and ^{13}C-NMR showed that the guanidine-based oligomer grafted with reactive cationic surfactant was synthesized successfully. The antimicrobial activity and antimicrobial mechanism were investigated with several approaches. The antimicrobial activity results indicated that the introduction of a cationic surfactant into the guanidine-based oligomer effectively raised the antimicrobial activity and showed a synergistic effect. The UV absorption at 260 nm was used to detect the dynamic antimicrobial process of the modified guanidine oligomer. Further, the results of scanning electron microscopy and atomic force microscopy implied that the antimicrobial mechanism of the modified guanidine oligomer changed the permeability of the cell membrane of the bacteria and caused the leakage of intracellular components of the *Escherichia coli* cells. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

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INTRODUCTION

Because bacterial cells generally exhibit a surface with a negative charge, they are often stabilized by divalent cations, such as Mg^{2+} and Ca^{2+},[1,2] present in the external environment. To rapidly capture and kill bacteria, many antimicrobial agents usually contain cationic groups to take advantage of the high binding affinity for these negatively charged bacterial cells. Excellent cationic antibacterial agents include quaternary ammonium compound (QACs), amphoteric surfactants, and guanidine derivatives.[1] QACs and amphoteric surfactants have been used as biocides for a long time.[3,4] However, it has been observed that serious bacterial resistance is usually caused by a large number of QACs after their use for a period of time.[1,5,6] Another disadvantage of QACs is their relatively narrow antimicrobial spectrum, as they are mainly bactericidal and are more effective against Gram-positive than Gram-negative bacteria.[7]

Guanidine derivatives, especially guanidine oligomers, have been considered as broad-spectrum antimicrobial agents. Furthermore, because of their high-water solubility and nontoxicity, guanidine oligomers have been widely used for many years as antiseptics in medicine,[8,9] in textiles,[10,11] in the food[12] and paper industries,[13,14] in water treatment,[15] in anti-infectious surgery, and so on.[16] Guanidine moieties are a ubiquitous group in natural products,[17] and they play a key role in many biological activities. Chlorhexidine is the most frequently used antimicrobial agent of the guanidine class; it is widely used as an oral antimicrobial and antiplaque agent.[18,19] Although small-molecule guanidine compounds have excellent antibacterial properties and biological activity, they also have some drawbacks, such as poor stability and easy volatility. Among guanidine derivatives with antimicrobial activity, polymeric guanidine and biguanidine salts have gained importance as antiseptics because of their thermal stability and excellent antibacterial activity.[20] Polymeric guanidine and biguanidine are effective in controlling bacteria and fungi. Their minimum inhibitory concentration (MIC) is not larger than 200 μg/mL. Nevertheless, the molecular weight of polymeric guanidine is still low, about 1000–3000.[21] The most effective method for increasing the molecular weight and enhancing the antibacterial activity of polymers, to the best of our knowledge, is graft polymerization or copolymerization.[22] Poly(2-aminoethylmethacrylate hydrochloride) is obtained by free-radical polymerization; then, polymer-analogous guanidinylation poly(2-guanidinoethylmethacrylate) is obtained with cyanamide. With a content of 60 mol % guanidine-functionalized repeat units, the MIC was lowered by nearly 50% compared to that of poly(2-aminoethylmethacrylate hydrochloride).[23] Modified poly(hexamethylene guanidine

hydrochloride) (PHGC) oligomer, which was modified via a reaction with glycidyl methacrylate, was used as a comonomer to synthesize acrylonitrile copolymer; it showed excellent antimicrobial activity against *Staphylococcus aureus*.[22] Ashraf et al.[24] integrated the antimicrobial properties of polyhexamethylbiguanide with that of silver nanoparticles, and the antimicrobial activity of these hybrid silver nanoparticles was much higher than that listed in previous reports.

In addition to the extensive interest in guanidine compounds for their biological applications, they are also a focus of attention for technical uses, such as in molecular recognition,[25] sensors,[26] and catalysts.[27]

Because of the active amino groups [—NHC(=NH)NH— and NH_2] in their molecular skeletons, guanidine oligomers can react with other compounds.[10,28,29] These reactions can be used in an excellent strategy for the synthesis of more guanidine oligomer derivatives by Michael addition. The Michael addition of a nucleophile to electron-deficient alkenes is one of the most important reactions in organic chemistry.[30] The significant features of this reaction are its (1) mild reaction conditions, (2) operational simplicity, (3) lack of toxic and expensive reagents, (4) short reaction time, and (5) high product yields.[31] The Michael addition reaction is generally needed to add an alkali as a catalyst; in this study, thanks to its alkaline properties,[32] the guanidine oligomer could be considered as both the reactant and catalyst in the reaction.

The purpose of this study was to design and synthesize a novel modified guanidine-based oligomer by a Michael addition reaction that could enhance the antimicrobial activity and extend the antimicrobial spectrum of nonylphenol polyoxyethylene (10) ether maleate acyloxy ethyl trimethyl ammonium chloride (OPMAC) and to investigate the antibacterial mechanism of the modified guanidine oligomer. This modified guanidine oligomer was prepared by the grafting of the macromolecular quaternary ammonium salt with unsaturated double bonds onto the molecular skeleton of PHGC through a Michael reaction. The molecular structure of the obtained modified oligomer was characterized by Fourier transform infrared (FTIR) spectroscopy, ^{1}H-NMR, and ^{13}H-NMR spectra. An antibacterial effect was observed through the MIC and antibacterial rate. Moreover, the antimicrobial mechanism was revealed by dynamical detection of the UV absorption of the bacteria suspension and through visualization of the bacterial morphology with scanning electron microscopy (SEM) and atomic force microscopy (AFM) images.

EXPERIMENTAL

Materials

PHGC, with a weight-average molecular weight (M_w) around 1800, was purchased from Shanghai Hipoly Industry Co., Ltd. (Shanghai, China). OPMAC (Scheme 1) was purchased from Henan Titaning Chemical Technology Co., Ltd. (Henan, China). All of the reagents were used as received without further purification. *Escherichia coli* (ATCC25922) was kindly provided by the Institute of Sichuan Antibiotic Industry, State Pharmaceutical Administration of China.

Synthesis and Characterization of the Modified Guanidine Oligomer

The modified guanidine oligomer was synthesized by the grafting of OPMAC, a macromolecular quaternary ammonium salt with unsaturated double bonds, onto the molecular skeleton of PHGC through a Michael reaction (Scheme 1). The PHGC was dissolved in water at a concentration of 25 wt % and was put into a 250-mL, three-necked flask equipped with mechanical stirrer with OPMAC in a molecular ratio of 1:0.1, that is, PHGC [—NH(C=NH)NH—]/OPMAC = 1:0.1. The reaction was conducted at 80°C for 8 h; then, the crude modified guanidine-based oligomer was harvested by complete removal of the water. The crude product was purified via repeated precipitation/dissolution in acetone/ethanol under stirring three times. After it was dried *in vacuo* at room temperature for 12 h, the purified product was obtained for further analysis.

Characterization of the Modified Guanidine Oligomer

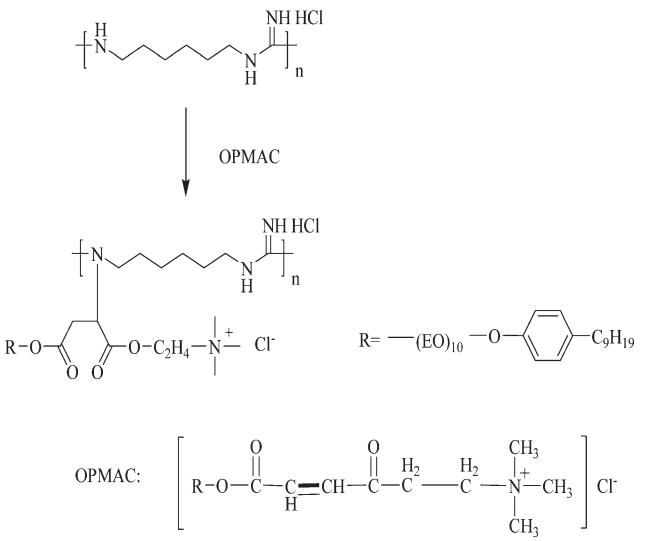
An LVDV-C viscometer (Brookfield) was used to measure the viscosity of the modified guanidine oligomer at 25 ± 0.1°C. The FTIR spectrum of the oligomer was recorded by a Nicolet MX-1E Fourier transform infrared spectrometer (Nicolet, Japan), and the sample was prepared as a potassium bromide pellet. NMR (AM 300-MHz NMR instrument, Bruker) was used to characterize its molecular structure with hexadeuterated dimethyl sulfoxide as the solvent.

Determination of the MIC

The MIC was determined by the addition of 0.2 mL of serially diluted antimicrobial agents to 0.8 mL of broth and 1 mL of bacterial suspension (*E. coli*, 10^5 cfu/mL) and incubation at 37°C for 24 h. The final concentration of the antimicrobial agent in the tubes ranged from 128 to 1 ppm. The MIC was interpreted as the lowest concentration that could inhibit the visible growth of bacteria in comparison with the control sample without antimicrobial agent, in which the bacteria would grow without any inhibition.

UV Absorption of *E. coli* Suspension

The cytoplasmic cell membrane undoubtedly is the target for many antibacterial agents. Interactions of bacterial membranes



Scheme 1. Synthesis of modified guanidine oligomer.

with biocides frequently cause fundamental changes in both the membrane structure and function[33] and result in the leakage of intracellular components.[34] The quantity of leakage is a classic indication of the damage to the bacterial membrane in a bacterial suspension treated with a bacteriostatic. The intracellular contents showed strong absorption at 260 nm; this was easily detected by an ultraviolet–visible spectrophotometer.[35] It is a perfect strategy to indicate the cell membrane integrity, and it has been used to study the sterilization kinetics of biocides via the UV Absorption method.

E. coli was cultivated overnight at 37°C and 95% humidity before use (1×10^8 cfu/mL). The culture media containing the required nutrients[36] was used for the cultivation. An *E. coli* suspension (OD_{260nm} = 0.2–0.3, OD_{260nm} represents the optical density of bacterial suspension at 260 nm) was obtained by centrifugation of the *E. coli* culture at 3000 rpm for 5 min; we then washed the suspension twice and redispersed it in double-distilled water. After it was mixed with the modified guanidine oligomer for a certain contact time, the *E. coli* suspension was filtered through a 0.2 μm syringe filter. The OD_{260nm} values of both oligomer-treated samples and the control samples were measured with a U-2010 spectrophotometer (Hitachi, Japan) at same time intervals with an empty quartz cell as the reference. The results of antimicrobial effects were represented by the OD_{260nm} ratio. The higher the OD_{260nm} ratio was, the higher the obtained antimicrobial efficiency was. The OD_{260nm} ratio was calculated by the following equation:

$$OD_{260nm}\ \text{ratio} = OD_{260nm(\text{treated sample})} / OD_{260nm(\text{control sample})}$$

SEM and AFM Analysis

SEM and AFM can provide a visualizing, vivid, and detailed morphology of bacterial cells undergoing various stages or different mechanisms of antibacterial actions. SEM images can show the shrinkage of the protoplast at a low concentration of antimicrobial material;[37] and AFM was used to reveal the antimicrobial mechanism for another powerful technique.[38,39]

SEM Sample Preparation Methods. After it was centrifuged at 3000 rpm for 3 min, the *E. coli* cell collection was then washed twice with phosphate-buffered saline (PBS) and redispersed with PBS solution. Modified guanidine oligomers with various concentrations were added to the *E. coli* suspension, and the mixture was shaken for 30 s. A glutaraldehyde solution (2.5%) was added to *E. coli,* and we treated the *E. coli* suspensions to fix the surface morphology of the cells. Then, the *E. coli* suspension was recentrifuged at 3000 rpm for 3 min, washed with PBS, and redispersed in a PBS solution three times. The *E. coli* cells were dehydrated with ethanol solutions (50, 70, 80, 90, and 100%, respectively) for 15 min and air-dried in a vacuum desiccator. SEM images were obtained with a scanning electron microscope (Leitz-AMR-1000).

AFM Sample Preparation Methods. The *E. coli* suspension (1×10^8 cfu/mL) was first centrifuged at 3000 rpm for 3 min, then washed twice with PBS, and redispersed in PBS. Modified guanidine-based oligomers with various concentrations were added to the *E. coli* suspension, and the mixture was shaken for 30 s. The fresh *E. coli* and treated *E. coli* samples were recentrifuged at 3000 rpm for 10 min, and the centrifugal pellets were washed and redispersed with PBS three times. Then, *E. coli* cells were dropped onto a silicon wafer and air-dried in a vacuum desiccator. The morphological images of *E. coli* were observed via AFM (a Veeco Nanoscope IIIa Multimode instrument).

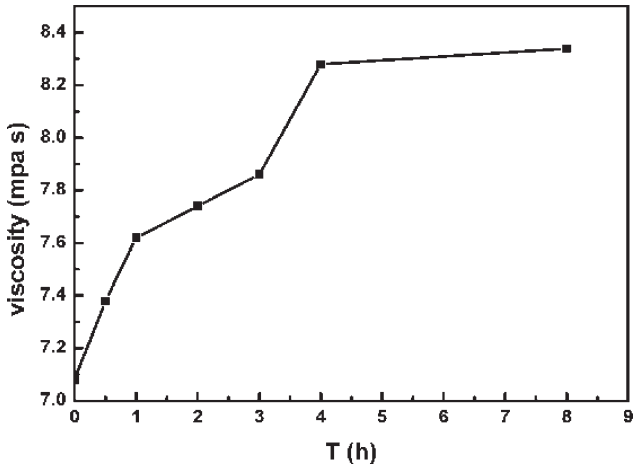


Figure 1. Viscosity of the modified guanidine oligomer versus reaction time.

RESULTS AND DISCUSSION

Synthesis of the Guanidine Oligomer

From an antimicrobial function point of view, quaternary ammonium groups and phenyl groups also have antimicrobial activity to some extent. So, in this study, we designed and synthesized a novel antibacterial oligomeric guanidine salt derivative through a Michael addition reaction to introduce a cationic surfactant containing these antibacterial activity groups onto a oligomer molecular skeleton. Containing unsaturated double bonds, the cationic surfactant OPMAC reacted easily with —NH— in PHGC through mild reaction conditions. As is well known, the Michael addition reaction is generally needed to add an alkali as a catalyst; in this study, thanks to the alkaline properties of guanidine, PHGC could be considered as both the reactant and the catalyst in the reaction.

It is well-known that the viscosity is one of the main factors related to the performance of polymers, such as M_w. The higher the viscosity was, the higher molecular weight of the oligomer was. The influence of the reaction time on the viscosity of the modified guanidine oligomer was investigated with a viscometer (Brookfield). As shown in Figure 1, in the first 4 h, the viscosity of the modified guanidine oligomer increased rapidly with increasing reaction time, that is, increasing molecular weight of the modified oligomer. Subsequently, the viscosity reached a plateau, which indicated that the reaction was complete.

The FTIR spectrum of the modified oligomer is shown in Figure 2. The strong signal in the functional group region at 3330 cm^{-1} was due to N—H stretching vibrations. Benzene derivatives showed absorption for the C—H units at 3174 cm^{-1} and were also in the range from 671 to 870 cm^{-1} (in the fingerprint region). The 2868 and 2933 cm^{-1} peaks were assigned to the

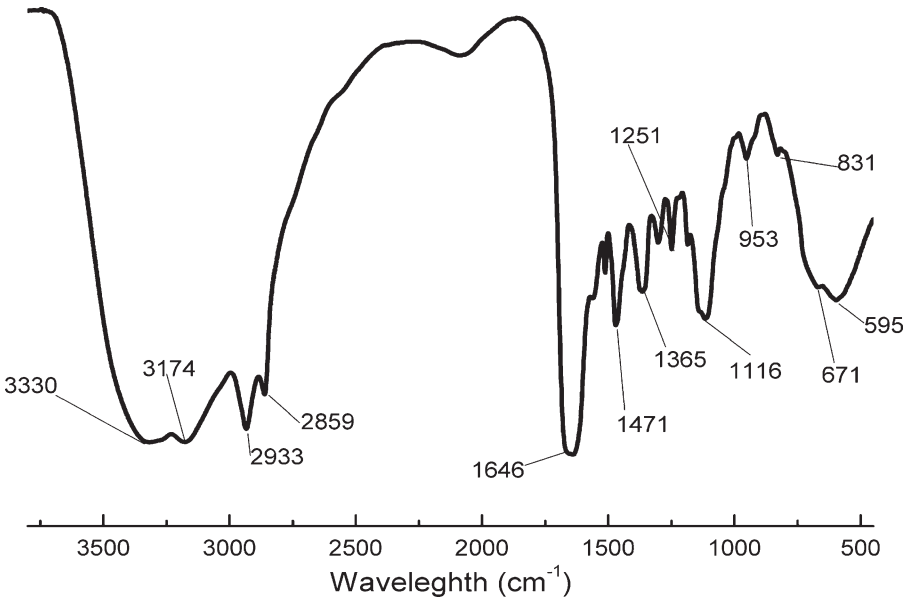


Figure 2. FTIR spectrum of the modified oligomer.

CH_2— symmetric stretching vibration (CH_3-ss) mode, respectively. The strong infrared absorption band of the C═O and conjugated N—C(═N)—N stretching vibrations was observed at 1646 cm^{-1}. Saturated ethers provided a strongly asymmetric C—O—C stretching vibration in the range from 1150 to 1080 cm^{-1} [40] [$\nu_{as(C-O-C)}$ = 1116 cm^{-1} in Figure 2] and a symmetric C—O—C stretching vibration with medium intensity in the range from 890 to 820 cm^{-1} [$\nu_{ss(C-O-C)}$ = 831 cm^{-1} in Figure 2]. The absorption peaks of aryl alkyl ether were observed at 1251 and 1220 cm^{-1}, which belonged to the asymmetric C—O—C stretching vibrations and symmetric C—O—C stretching vibrations, respectively.

The ^{1}H-NMR and ^{13}C-NMR spectra of the modified oligomer and PHGC are shown in Figure 3. The ^{1}H-NMR spectra of PHGC [see Figure 3(1a)] showed sharp peaks at 1.29, 1.45, and 3.14 ppm, which belonged to CH_2 1, 2, and 3, respectively. Compared with ^{1}H-NMR spectrum of PHGC, the ^{1}H-NMR spectrum [see Figure 3(1b)] of the modified oligomer presented new chemical shift peaks at 0.77 ppm (CH_3 4), 2.65 ppm (CH_2 6, 14, and 17), 3.50 ppm (CH_2 10, 11,12, and 15), 3.73 ppm (CH_2 9), 4.05 ppm (CH_2 13 and 16), 6.84 ppm (CH 8), and 7.19 ppm (CH 7). The peaks of the nonyl group (CH_2 5) and CH_3 18 overlapped with the peak of the hexyl group (CH_2 3) at 1.31 ppm and CH_2 1 at 3.17 ppm, respectively. Unsaturated double bonds usually showed chemical shift peak in the range from 4.5 to 5.9 ppm. However, we observed no peaks in this range of the ^{1}H-NMR spectrum of the modified oligomer; this implied that the OPMAC had been completely reacted. The ^{13}C-NMR spectrum of PHGC [Figure 3(2a)] showed sharp peaks at 26.0, 28.5, 40.3, and 157.2 ppm. The peaks at 26.0 and 28.5 ppm were assigned to the methylene groups. The peaks at 40.3 and 157.2 ppm belonged to the C—N and C═N units of guanidine, respectively. As shown in Figure 3(2b), new peaks at 127.4, 113.6, and 69.8 ppm were observed and were assigned to the carbon units of the phenyl groups and C—O—C units. The chemical shift peak of the carbonyl groups was overlapped with that of the C═N units at 150–160 ppm. In terms of the modified oligomer spectra, we concluded that the cationic surfactant (OPMAC) was successfully grafted onto the oligomer guanidine molecular skeleton.

The grafting efficiency of OPMAC was measured via the ^{1}H-NMR spectrum. The following equation was deduced:

$$\text{Grafting efficiency}\,(\%) = \frac{\text{Grafted OPMAC}}{\text{Total OPMAC}} = \frac{\int \text{CH}_2, 8 / \int \text{CH}_2, 2}{N_{\text{OPMAC,CH}_2,8} / N_{\text{PHGC,CH}_2,2}}$$

where $\int$ is the groups peak area of the modified guanidine and N is the number of hydrogens in the molecule.

PHGC, with a M_w around 1800, contained about 12 of the repeat structural units [NC_6H_{12}—NH(C═NH)NH—]. Because the molar feed ratio of —NH(C═NH)NH—/OPMAC was 1:0.1 in the reaction process, $N_{OPMAC,CH2,8}/N_{PHGC,CH2,2}$ was about 2.6:48. According to the ^{1}H-NMR spectrum of the modified guanidine, $\int_{CH2,8}/\int_{CH2,2}$ was about 1:19. Therefore, the grafting efficiency of the modified guanidine was about 97%.

Antibacterial Activity

Determined by the double-dilution method,[41] the MIC of the modified guanidine oligomer for *E. coli* (ATCC25922) was found to be 8 ppm, and the MICs of PHGC and OPMAC were 8 and 64 ppm, respectively. This suggested a synergy of PHGC and OPMAC. The antibacterial rate was measured by an agar plate-counting method.[42,43] The antibacterial rate (X) was calculated by the following equation:

$$X = \frac{A-B}{A} \times 100\%$$

where A and B are the number of colonies detected from the control and treated samples, respectively.

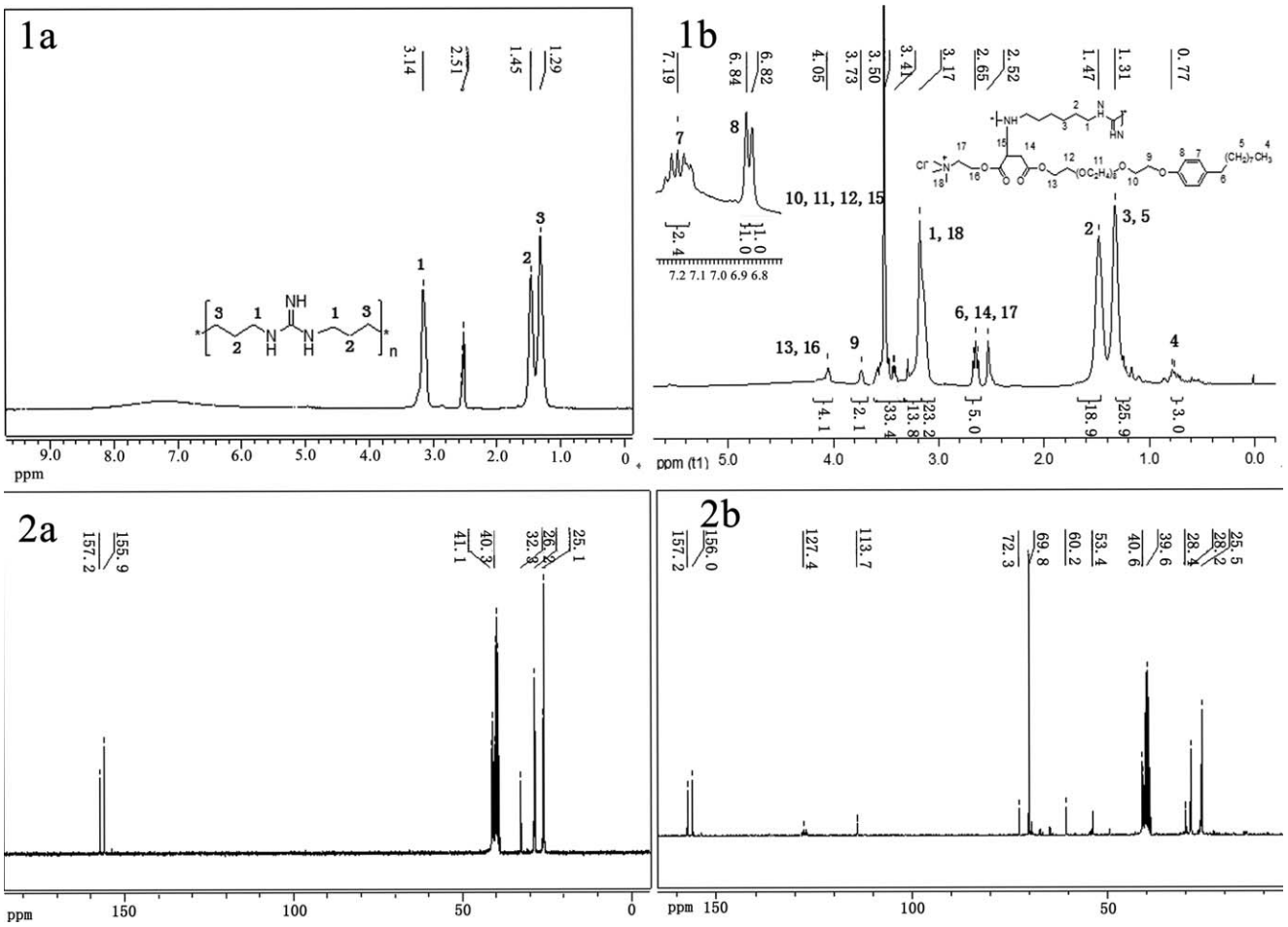


Figure 3. ^{1}H-NMR and ^{13}C-NMR spectra of the PHGC and modified oligomer in hexadeuterated dimethyl sulfoxide: (a) PHGC and (b) modified oligomer.

To verify that the chemical blending could enhance the antibacterial activity, we observed the antibacterial effects of OPMAC, PHGC, and modified guanidine at different concentrations against *E. coli* (Figure 4). As shown in Figure 4, when we treated *E. coli* with antibacterials for 1 h, the *E. coli* inhibition rate increased with increasing concentration; for example, with modified guanidine, the *E. coli* growth inhibition rates were 34.2, 45.3, 65.4, 99.9, and 100% when the concentrations were 10^{-1}, 10^{0}, 10^{1}, 10^{2}, and 10^{3} μg/mL, respectively. At higher concentrations (100 and 1000 μg/mL), the bacteria could be effectively killed by PHGC and modified guanidine. At 10 μg/mL, the antibacterial rate of the modified guanidine was 65.4%; however, those of OPMAC and PHGC were 30.3 and 50.0%, respectively. The results suggest that the antibacterial effect of the antibacterials against *E. coli* became stronger in the order OPMAC < PHGC < Modified guanidine. Therefore, there were synergistic effects of the modified guanidine with chemical blending with enhanced antimicrobial activity.

The antibacterial rate in the presence of the antibacterials at 100 ppm with different contact times are listed in Table I. As shown in Table I, all of the samples were able to reach a high sterilization efficiency in a short time (5 min), and the prolonged contact time (60 min) unlikely killed more bacteria. The antibacterial rate of antibacterials against *E. coli* became stronger in the order OPMAC < Physical blending mixture (OPMAC and PHGC) < PHGC < Modified guanidine. The value of the antibacterial rate of the physical blending mixture (99.86%) was

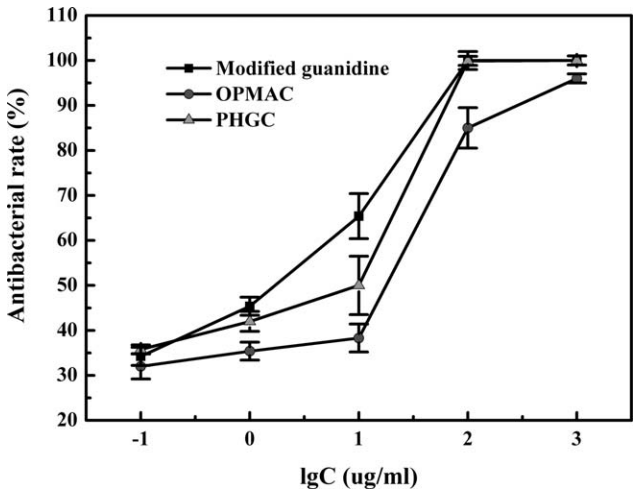


Figure 4. Antibacterial activities of the modified guanidine, OPMAC, and PHGC with various concentrations against *E. coli*.

Table I. Antibacterial Rate in the Presence of PHGC, OPMAC, Physical Mixture, and Modified Guanidine Oligomer with 100 ppm for Different Contact Times

Contact time (min)	Antibacterial rate of OPMAC (%)	Antibacterial rate of PHGC (%)	Antibacterial rate of the physical blending mixture (%)	Antibacterial rate of the modified oligomer (%)
5	84.00	99.19	98.68	99.20
15	84.60	99.62	99.51	99.72
30	85.20	99.79	99.77	99.91
60	85.00	99.90	99.86	99.99

between the values of 85.00% (OPMAC) and 99.90% (PHGC); this suggested that the combined effect between OPMAC and PHGC was indifferent. In other words, the physical doping of OPMAC and PHGC could not improve their antibacterial activity. The antibacterial rate of the modified guanidine within 60 min was up to almost 100%; however, those of OPMAC and PHGC were 85.00 and 99.90%, respectively. That further implied that the chemical combined effect between OPMAC and PHGC was synergism. Namely, the PHGC combined cationic surfactant (OPMAC) by chemical bonds could effectively improve the antibacterial properties of an antiseptic. Meanwhile, the antibacterial performance was slightly improved through synergy because of the introduction of the phenyl groups and quaternary ammonium groups in the modified oligomer.

Dynamic Monitoring of the Antimicrobial Behavior of Guanidine Oligomer

As we all know, big organic intracellular components (proteins and nucleic acids) have a strong absorbance at 260 nm,[44] so the amount of cytoplasm that leaks out from the membrane of bacteria cells is an appropriate indication of the membrane integrity. The UV absorbance of the *E. coli* suspension at 260 nm in the presence of modified guanidine oligomers with various concentrations was measured at the same time intervals, and the antimicrobial activity, represented by the OD_{260nm} ratio, was dynamically monitored. The results are shown in Figure 5(a).

As shown in Figure 5(a), the OD_{260nm} ratio only reached 1.05 at a low concentration (25 ppm); this revealed that the membrane permeability was less disrupted, and most of the bacteria were alive. However, at concentrations of 400 ppm, the OD_{260nm} ratio was higher than 1.52; this suggested that much more of the big molecular organic intracellular components had leaked from the bacterial membrane. It also implied that the OD_{260nm} ratio had reached the maximum value at the low concentrations (25 and 50 ppm) at 20 min, whereas at the high concentrations (>100 ppm) of the modified oligomer, the OD_{260nm} ratio rapidly reached a plateau within 5 min. This result was consistent with the conclusion for the antibacterial rate. The relationship between the concentration of the modified guanidine oligomer and the final OD_{260nm} ratio was investigated when the bacterial suspension was treated with the modified guanidine oligomers for 30 min. The results are shown in Figure 5(b). The OD_{260nm} ratio was proportional to the concentration when the modified guanidine oligomer concentration was below 100 ppm, and the OD_{260nm} ratios reached a plateau when the guanidine oligomer concentration was above 200 ppm. This implied that the bacterial membrane was damaged severely and most of the *E. coli* were dead when the guanidine oligomer concentration was sufficiently high; this observation was identical to the findings reported by other researchers.[1,28]

Antimicrobial Mechanism of the Guanidine Oligomer as Revealed by SEM

SEM, which could clearly provide information about the bacterial surface, was used to reveal the morphological changes in

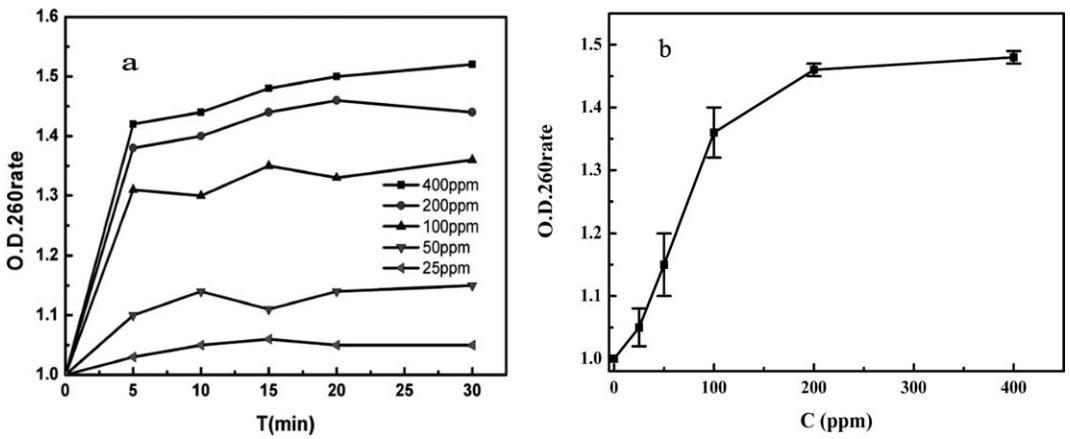
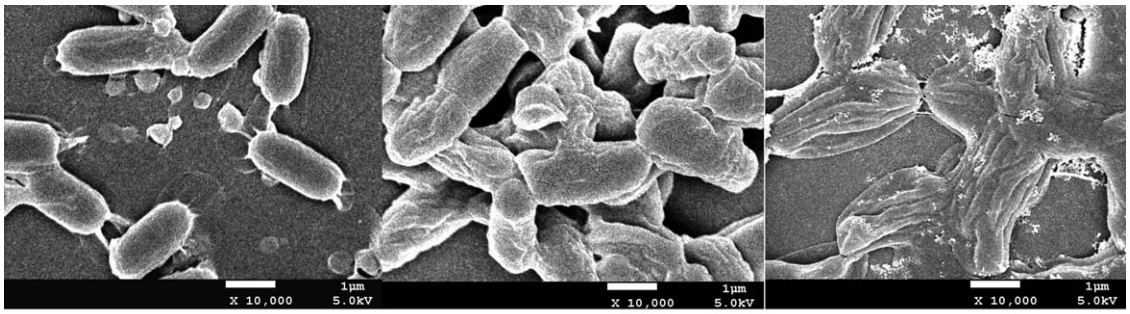


Figure 5. (a) Dynamic UV absorption of *E. coli* in the presence of the modified guanidine oligomers at various concentrations. (b) Relationship between modified guanidine oligomer concentration and the final O. D. ratio with a contact time of 30 min.



a. E.coli

b. E.coli after treated with 8 ppm of modified oligomer for 30minc

c. E.coli after treated with 80 ppm of modified oligomer for 30min

Figure 6. Morphology of *E. coli* treated with the modified guanidine oligomer at different concentrations.

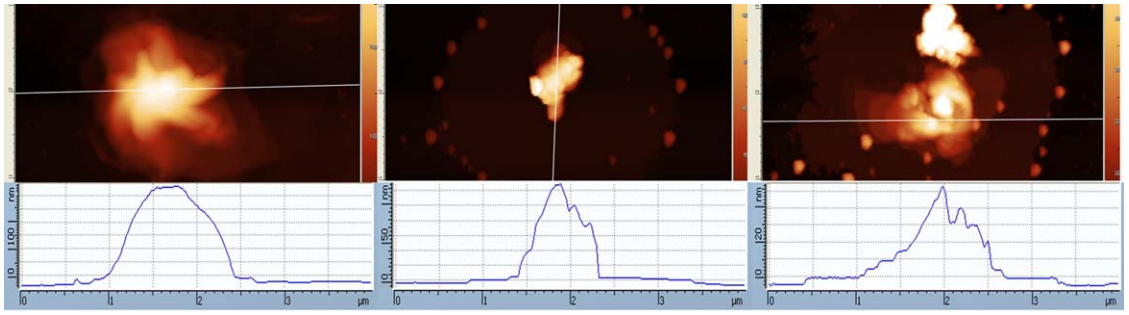
the microbial cells after treatment with the modified guanidine-based oligomer. *E. coli* showed a different shapes corresponding to the treatment with different concentrations of the modified oligomer. Figure 6 shows the morphological images of the fresh *E. coli* and *E. coli* after the treatment. As shown in Figure 6(a), the inherent *E. coli* had an ellipsoid shape, its surface membrane was structured and integrated, and there were no indentations or grooves on the cell surface. When the bacteria were treated with the modified oligomer at low concentration (8 ppm), there were slight grooves on the surface of *E. coli* [Figure 6(b)], but the *E. coli* cells still retained their intact shape, and the cell profile was legible. This indicated that maybe the low-molecular-weight species, such as potassium ions and phosphate, had leaked, but the cell membrane had not collapsed, and the *E. coli* cells were still alive. Hence, the UV absorption of the *E. coli* suspension treated with a low concentration of modified oligomer increased slightly. At this concentration, the modified guanidine in the *E. coli* suspension interacted directly with the cytoplasmic membranes by electrostatic interactions. The distance between the phospholipid head groups in a closely packed monolayer is roughly equivalent to the length of a hexamethylene grouping.[1] Therefore, PHGC in the modified guanidine molecule could bind firmly to the anionic sites found on the membrane surface, and this led to the maximum disturbance in the fluidity of the cell membrane. QACs in the modified guanidine molecule were able to provide a long-chain lipophilic group, entering the molecular structure of the membrane and perturbing the membrane-located physiologies. Both of these made the antibacterial activity of the modified guanidine efficient. As shown in Figure 6(c), however, the membrane of *E. coli* completely collapsed after treatment with a higher concentration (80 ppm) for 30 min; this indicated that most of the intracellular components leaked via the deformed membrane. The results were also consistent with those observed in the UV test.

Antimicrobial Mechanism of the Guanidine Oligomer as Revealed by AFM

AFM was also used to reveal the morphological changes in the *E. coli* cells after treatment with the modified guanidine-based oligomer. Figure 7 shows the morphological and profile images of *E. coli* that was treated with various concentrations of oligomer.

Figure 7(a) presents the morphological and profile images of the *E. coli* cells; it shows that there were no leaked residues around and that the *E. coli* cells kept their ellipsoid shape with a middle high above 250 nm and a length of 1.5 μm or more.

The influence of different concentrations of the modified guanidine-based oligomer on the bacterial morphology was also investigated. The profile image in Figure 7(b) showed that *E. coli*'s surface had a slight depression phenomenon when it was treated for 30 min with 8 ppm modified guanidine oligomer (MIC), and the height of the bacterial cells decreased from 250 to 150 nm. This indicated that *E. coli*'s contents may have



a. E.coli (control)

b. E.coli after treated with 8 ppm of modified oligomer for 30min

c. E.coli after treated with 80 ppm of modified oligomer for 30min

Figure 7. AFM morphology of *E. coli* treated with the modified guanidine oligomer at different concentrations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

leaked from the bacterial membrane, and this led to the shrinkage of the cells. This was confirmed by the results from AFM, in which a little leakage was found around the *E. coli* cells. Meanwhile, the morphology of *E. coli* also could explain the corresponding OD_{260nm} ratio results, in which the OD_{260nm} ratio of the treated samples increased slightly at a low concentration of modified oligomer. Hence, the low-molecular-weight species of protoplast, such as potassium ions and phosphate, leaked at first, then a little bit of big molecular organic intracellular components leaked, but the cell membrane did not collapse. As shown in Figure 7(c), the membrane of *E. coli* was damaged severely and was decomposed into debris when the cells were treated with modified oligomer at 80 ppm (>MIC) for 30 min. Because of the collapse of the *E. coli* membrane, most of the intracellular components leaked; this led to the death of *E. coli*. The height in the AFM graph decreased to 40 nm; this implied that the intracellular components leaked, and the *E. coli* cells were dead. The collapse of the *E. coli* membrane left massive amounts of cytoplasm in the suspension so that the OD_{260nm} ratio at a high guanidine oligomer concentration became much higher. The AFM image results were consistent with those observed from the UV tests and SEM images.

CONCLUSIONS

In this study, we designed a modified guanidine-based oligomer with a novel structure. According to the results of FTIR, ^{1}H-NMR, and ^{13}H-NMR spectroscopy, the modified oligomer was synthesized successfully. Michael addition was an effective method for the grafting of the double-bond-containing compound onto the oligomeric guanidine skeleton. The antimicrobial activity and antimicrobial mechanisms were investigated. The results show that the introduction of the phenyl groups and quaternary ammonium groups improved the antimicrobial ability of obtained modified guanidine oligomers. Compared with those modified by physical blending, guanidine oligomers modified through chemical reaction much more effectively improved their antibacterial properties. In this study, the UV_{260nm} absorption was used to reveal the dynamic antimicrobial process of the modified guanidine oligomer. The results indicate that the low concentrations of modified oligomers did not severely damage the membrane permeability of the *E. coli* cells. However, high concentrations of it surely killed the *E. coli* cells in a very short time because of its solubility for the *E. coli* membranes; this resulted in the leakage of the cell contents. This conclusion was also proven by SEM and AFM imagery.

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