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Antibacterial activity of chitosan coated Ag-loaded nano-SiO_2 composites

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ABSTRACT

A crosslinked chitosan (CCTS) coated Ag-loading nano-SiO_2 composite (CCTS–SLS) was prepared. The structures of CCTS–SLS were characterized by field emission scanning electron microscopy and X-ray photoelectron spectroscopy. The antibacterial properties of CCTS–SLS were measured as the minimal inhibitory concentration and the rate of bacterial growth. The experimental results indicate that the antibacterial activity of CCTS–SLS was affected by the mass ratio of SLS to chitosan, acetic acid concentration and crosslinking time. Moreover, CCTS–SLS exhibited high antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* as a result of the coordinated action of CCTS and SLS.

1. Introduction

With the advance of medical technology, development in antibiotics has been greatly improved to deal with the increasing drug resistance of pathogens. However, monocomponent antibacterial agents have been far from meeting requirements for some special conditions. Therefore, it is necessary to find composite antibacterial agents to solve this problem (Krajewska, 2004; Lei & Bi, 2007; Liu et al., 2006; Petri, Donega, Benassi, & Bocangel, 2007; Wu, Luan, & Zhao, 2006; Xi & Wu, 2006). Silver-loading nano-SiO_2 (SLS) antibacterial material has excellent antibacterial activity but suffers from a change in color as result of the reaction between silver ions and oxygen or sulfur. As inorganic antibacterial agent, the use of SLS is restricted in many fields (Wang, Wen, Wang, & Chen, 2006). Chitosan (CTS) is the second most plentiful natural biopolymer and is relatively cheap (Ma et al., 2008). It has attracted considerable interest due to its biological properties, such as antimicrobial activity, antitumor activity, and immune enhancing effect. However, the antibacterial activity of chitosan is influenced by a number of factors, including the species of bacteria, concentration, pH, solvent and molecular weight (Hernández-Lauzardo et al., 2008).

In this paper, crosslinked chitosan (CCTS) coated Ag-loading nano-SiO_2 composite (CCTS–SLS) was synthesized by an adsorption crosslinking reaction. Compared with CTS, CCTS, SLS, CTS + SLS (the mixture of CTS and SLS) and CCTS + SLS (the mixture of CCTS and SLS), CCTS–SLS composite showed significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Experimental materials

The materials and chemical reagents included: nano-SiO_2 with a particle size of 20 ± 5 nm (Zhoushan Mingri Nano-materials Co. Ltd., Zhejiang, China), $AgNO_3$ (A.R.; Beijing Xingye Chemical Industry Co. Ltd., China), HNO_3 (C.p.; Tianjin Hongfeng Chemical Industry Co. Ltd., Tianjing, China), absolute ethyl alcohol (A.R.; 99.5%; Tianjin Hongfeng Chemical Industry Co. Ltd., Tianjing, China), $NH_3{\cdot}H_2O$ (A.R.; Tianjin Jingtian Chemical Industry Co. Ltd., Tianjin, China), coupling agent (A-1100; Nanjing Shuguang Chemical Industry Co. Ltd., Nanjing, China), chitosan (90% deacetylation; Ningbo Haixin Biological Products Co. Ltd., Zhejiang, China), glacial acetic acid (A.R.; 99%; Tianjin Hongfeng Chemical Industry Co. Ltd., Tianjing, China), HCl (A.R.; Tianjin Jingtian Chemical Industry Co. Ltd., Tianjin, China), glutaric dialdehyde (A.R.; Wuhan Dayang Chemical Industry Co. Ltd., Hubei, China), *E. coli* ATCC 8739, *S. aureus* ATCC 6538 (Shanxi Medical University, Taiyuan, China).

2.2. Preparation of SLS

SLS was prepared by adsorption as follows: 5 g of the nano-SiO_2 powder was added to 50 ml of 0.08 mol/L $AgNO_3$ solution, 1 ml of

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coupling agent A-1100 was added, then the mixture was stirred at 50 °C for 4 h in the dark, with the pH value of the system maintained at 6 by adding nitric acid. The slurry was separated into solid and liquid by vacuum filtration. The separated solid specimen was dispersed into 200 ml of distilled water for washing and then filtered again. The washing and filtration were repeated until no Ag^+ was detected in the filtrate. After that, the specimen was dried at 90 °C to constant weight.

2.3. Preparation of CCTS–SLS

One gram of chitosan was added to about 200 ml of commonly used solvent dilute acetic acid solution, because chitosan is soluble in acetic acid solution but insoluble in water. The pH value of the solution was maintained at 2.5 by adding 1 mol/L dilute hydrochloric acid under stirring at 25 °C for 0.5 h. After that, according to SLS-to-CTS mass ratio, SLS was added and stirred at 25 °C for 1.5 h. Dilute ammonia was used to adjust the pH value of the system to 12. The mixture was heated to 65 °C and 0.03 g of 3% glutaric dialdehyde was added dropwise to crosslink chitosan on the surface of SLS particles. After further heating for a certain time, the product was separated by centrifugation and washed several times with absolute ethyl alcohol. Finally, the CCTS–SLS specimen was vacuum dried at 30 °C for 24 h.

2.4. Structure and morphology of the samples

The morphologies of the samples were characterized by field emission scanning electron microscopy (FESEM). The chemical compositions were compared between CS, CCTS, SLS and CCTS–SLS with X-ray photoelectron spectroscopy (XPS) and information on stability obtained using thermogravimetry (TG). The contents of element in samples were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES).

2.5. Antibacterial property of samples

The antibacterial properties of samples were measured by minimal inhibitory concentration (MIC).

Escherichia coli ATCC 8739 and *S. aureus* ATCC 6538 were selected as indicators of experimental bacteria. Luria Bertani (LB) broth was used as a growing medium for both the microorganisms *E. coli* and *S. aureus*. The bacteria were grown aerobically in LB broth at 37 °C for 24 h.

Five grams of sample powder was placed in 45 ml sterilized phosphate buffer saline (PBS) with the pH value of 5. The suspension was diluted to a series of concentrations by adding PBS solution and placed in 45–50 °C water bath.

Ten milliliters of above-mentioned antibacterial solution with different concentrations was added into a plate, then 10 ml of double concentrated MH agar was added under continuous shaking for full mixing.

Cell solution (1–2 μl, $\sim 10^7$ cfu/ml) was taken to inoculate the above-mentioned plate, forming a cell solution quoit with a diameter of about 5∼8 mm. At last, the inoculated plates were cultivated at 37 °C for 24 h.

MIC was determined according to such a standard that the lowest concentration of antibacterial solution needed to prevent visible growth of test microorganism was defined as the MIC against the microorganism.

2.6. Development of bacterial colonies (Hu, Du, & Liu, 2003; Jia, Hou, Wei, Xu, & Liu, 2008; Rivera-Garza, Olguín, García-Sosa, Alcántara, & Rodríguez-Fuentes, 2000; Teng, Zhang, Tang, & WU, 2008)

Escherichia coli ATCC 8739 and *S. aureus* ATCC 6538 were selected as indicators of experimental bacteria. Luria Bertani (LB) broth was used as a growing medium for both the microorganisms *E. coli* and *S. aureus*.

Bacteria were grown aerobically in LB broth at 37 °C for 20 h. The culture solution was centrifuged, and the cells were washed and suspended in distilled water, reaching a final concentration of 10^3 cells/ml.

E. coli or *S. aureus* (10^3 cells/ml) was suspended in 100 mL of distilled water, with the pH value adjusted to 5 by acetic acid. Then 2.5 or 5.0 mg of sample CTS, CCTS, SLS, CTS + SLS, CCTS + SLS, or CCTS–SLS was added to keep in contact with *E. coli* or *S. aureus* under shaking at 37 °C for 24 h. Aliquots of 0.1 ml of above mixture were sampled every hour. These aliquots were diluted in distilled water. Each group of samples was spread on LB agar plates and incubated at 37 °C for 24 h. Bacterial colonies were counted by a cell counter.

3. Results and discussion

3.1. Morphologies of samples

The morphologic changes of samples can be seen clearly from the FESEM images shown in Fig. 1. Sample CTS took the shape of irregular long strips interconnecting with each other, which is typical for macromolecular structure because of the straight chain polysaccharide by β-(1,4) glycosidic linkage. Sample CCTS was a kind of porous and membranous materials, typical for crosslinked polymer. Sample SLS was in the shape of superfine powder. Sample CCTS–SLS had aggregated particle structures. The formation of CCTS–SLS was showed in Fig. 2.

Fig. 3 shows the XPS spectra of CTS, CCTS, SLS and CCTS–SLS. In Fig. 3(a and b), the appearance of elements in CTS was the same as in CCTS. Compared with CCTS and SLS, CCTS–SLS composite included the nitrogen, carbon and oxygen elements of sample CCTS and the oxygen, silicon and silver elements of sample SLS. Silver contents of SLS and CCTS–SLS were calculated as 0.69 wt% and 0.48 wt%, respectively, by ICP-AES measurement. However, the content of SLS in composite CCTS–SLS was 71 wt%, as can be measured from TG analysis (Fig. 4). Therefore, the content of silver in SLS of CCTS–SLS was calculated to be 0.68 wt%, very close to that of SLS, suggesting that CCTS was coated well on the surface of SLS particles by crosslinking reaction and had little effect on the content of silver in SLS of CCTS–SLS.

3.2. Antibacterial activities of CCTS–SLS

Many factors during the preparation of CCTS–SLS had an important influence on the MIC values of CCTS–SLS, such as mass ratio (SLS: CTS), acetic acid concentration, and crosslinking time. The effects of these factors on the antibacterial activity of CCTS–SLS against *E. coli* and *S. aureus* were shown in Fig. 5 (The typical values for the parameters are: SLS to CTS ratio 1.0, acetic acid concentration 1%, crosslinking time 1.5 h).

Some structural characteristics were obtained by crosslinking CCTS on SLS (Fig. 2). At first, the crosslinking reaction reduced the inter- and intra-molecular hydrogen bonds of CTS because of the formation of a Schiff base. Second, the condensation reaction between the –OH on the surface of SLS and the –OH of C_6 in CCTS resulted in a new network structure and further reduced the inter- and intra-molecular hydrogen bonds. Third, the –OH of C_3 in CCTS was exposed outside molecular chains with increased degree of freedom. Furthermore, the unreacted $-NH_2$ was protonized to form $-NH_3^+$ in acid solution. Therefore, the hydrophilicity and dispersibility of composite was greatly improved, which was helpful in promoting the synergistic effect between CCTS and SLS.

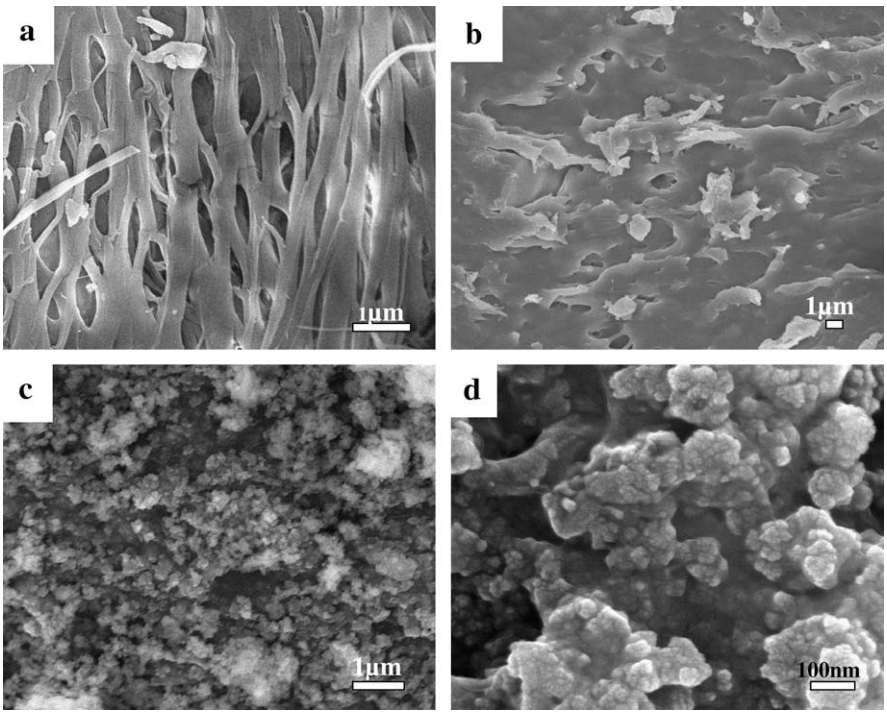


Fig. 1. FESEM images of samples (a) CTS, (b) CCTS, (c) SLS and (d) CCTS–SLS.

The combination of CCTS and SLS by crosslinking methodology resulted in coordinated antibacterial function of CCTS–SLS. First, SLS releases silver ions slowly, which damages the proliferation of microbial cells. Second, swelling of CCTS on the surface of SLS prevents SLS particles from agglomerating and precipitating, which favors the diffusion of silver ion, increases the contact probability of bacteria, and moreover, deters the Ag^+ ions from changing into black silver oxide to give less antibacterial activity (Dai et al., 2008). Third, the competitive reaction between Ag^+ ions and H^+ ions with the unreacted $-NH_2$ groups of CCTS and the steric hindrance of $-NH_2$ groups in crosslinked CCTS inhibit the complex reaction of the amino groups in CCTS and silver ions, decrease the loss of silver ions, and thus maintain the antibacterial activity of SLS in CCTS–SLS. Finally, the protonated $-NH_3^+$ of CCTS in acid solution can adsorb the electronegative substances in the bacteria cell to induce the leakage of nutritious substances, and form a polymeric membrane around bacteria cell, which prevents the transport of essential nutrients into the cell and thus results in the death of cell (Choi et al., 2001; Hu, Jou, & Yang, 2003; Shi, Neoh, Kang, & Wang, 2006).

As the mass ratio of SLS to CTS changed from 0.5 to 3.0, the minimum MIC values of CCTS–SLS against *E. coli* and *S. aureus* were 750 μg/ml and 1000 μg/ml, respectively, which occurred at the point where the mass ratio of SLS to CTS was 2.0. This result indicates that the antibacterial activity of CCTS–SLS did not change monotonically with the mass ratio. The reason was that the increase or decrease of mass ratio influenced on the coordinated action of CTS and SLS. The lower mass ratio suggested higher content of CCTS in CCTS–SLS, resulting in a decrease of Ag release in CCTS–SLS. The higher mass ratio suggested higher SLS in CCTS–SLS. Therefore, the reduced content of CCTS on the surface of SLS particles resulted in the poor dispersion of CCTS–SLS in solution and less antibacterial activity.

When the acetic acid concentration reached 1.5%, the minimum MIC values of CCTS–SLS against *E. coli* and *S. aureus* were 1000 μg/ml and 1200 μg/ml, respectively. Chitosan did not dissolve in water but in acetic acid solution (Xing et al., 2008). Therefore, a lower acetic acid concentration led to insufficient dissolution of CTS, which was unfavorable to the crosslinking of CTS. On the other hand, higher acetic acid concentration caused the breaking of the glycosidic bond of CTS into molecular segments, resulting in the degradation of the main molecular chain in CTS (Zhao & Jiang, 2007).

The crosslinking time of 2 h gave the MIC values of 1000 μg/ml and 1250 μg/ml against *E. coli* and *S. aureus*, respectively. With shorter crosslinking time, CTS did not crosslink thoroughly on the surface of SLS, which decreased the antibacterial activity of CCTS. With longer crosslinking time, CTS extensively crosslinked with SLS was unfavorable for the release of silver ion from SLS. At the same time, the molecular chains of CTS were hydrolyzed for a long time under the basic condition of the crosslinking reaction, thus decreasing the antibacterial activity of SLS in the coordinated action.

The optimized values for the parameters are: SLS to CTS ratio 2.0, acetic acid concentration 1.5%, and crosslinking time 2 h. The satisfying MIC values of CCTS–SLS against *E. coli* and *S. aureus* were 250 μg/ml and 300 μg/ml, respectively. This suggests that the CCTS–SLS composite showed strong antibacterial activity.

3.3. Time course of antibacterial activity of CCTS–SLS

Fig. 6 shows the number of viable cells of *E. coli* or *S. aureus* suspended in solution after keeping in contact with 2.5 mg of samples for different times (all of the blank and the control tests had no effect on the bacterial growth). It can be clearly observed that CCTS showed little antibacterial activity because CCTS was insoluble in acid solution or water; and CCTS + SLS also showed little antibacterial activity because the insoluble flocculating agent of CCTS could adsorb Ag^+ ions from SLS in solution, resulting in the less antibacterial activity of SLS. After *E. coli* suspended in solution was in contact with CTS, SLS and CTS + SLS for 9 h, 6 h and 7 h, respectively, and with CCTS–SLS for 3 h, the number of *E. coli* cells went down to zero. This implies that CCTS–SLS exhibited improved antibacterial timeliness and excellent antibacterial function against *E. coli*.

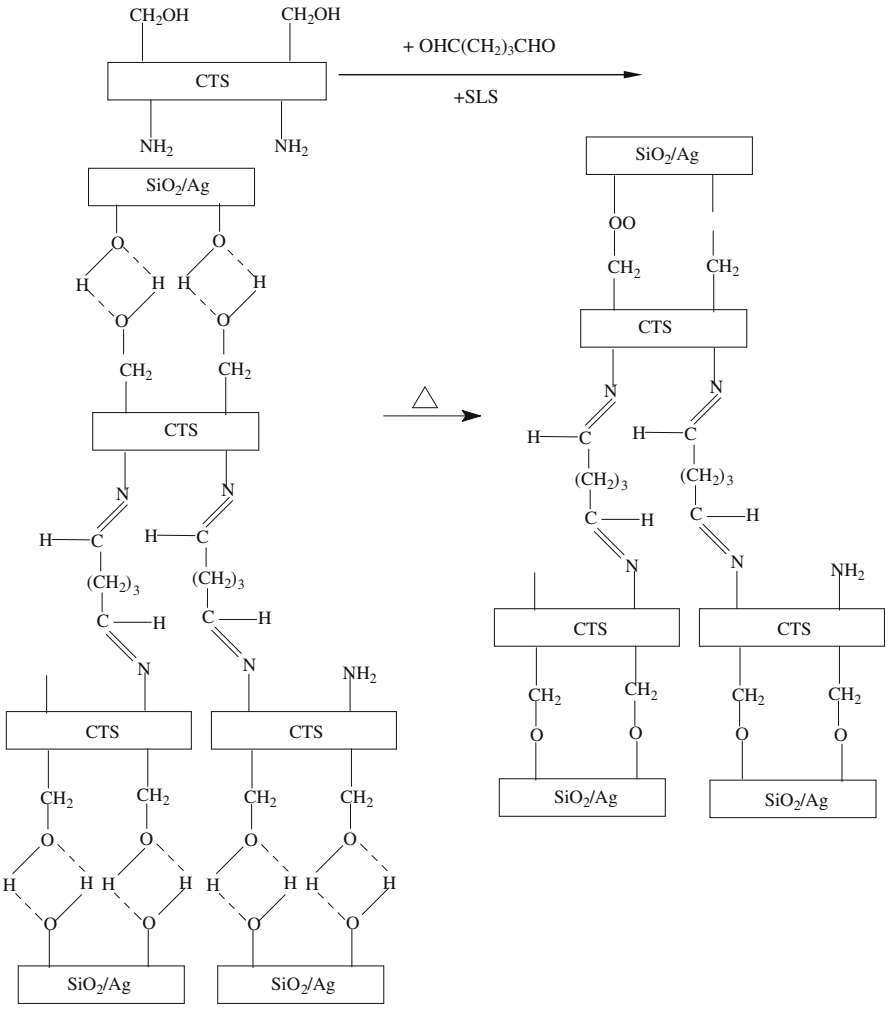


Fig. 2. Formation of CCTS–SLS.

For *S. aureus*, 7 h of contact time was needed to obtain the same result for CCTS–SLS, shorter than that of CTS (9 h), SLS (10 h) and CTS + SLS (8 h), also suggesting the powerful antibacterial activity of CCTS–SLS. The above-mentioned results do show that the antibacterial effect of CCTS–SLS against *E. coli* was stronger than against *S. aureus*. This can be explained by the structural difference between two microorganisms: *S. aureus* has a thicker cellular wall than *E. coli*, thus needs longer contact time to achieve the same effect as *E. coli*.

Fig. 7 shows the number of viable cells of *E. coli* or *S. aureus* suspended in solution after keeping in contact with 5.0 mg of samples for different times (all of the blank and the control tests had no ef-

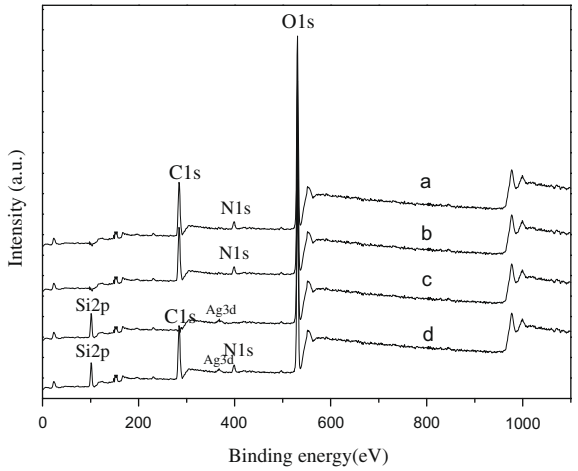


Fig. 3. XPS spectra of (a) CS, (b) CCTS, (c) SLS and (d) CCTS–SLS.

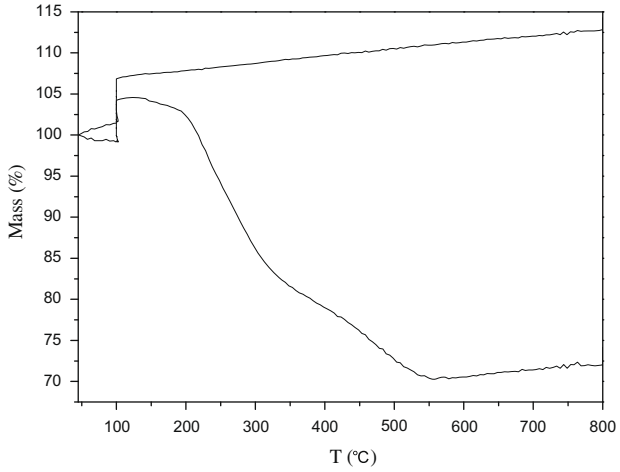
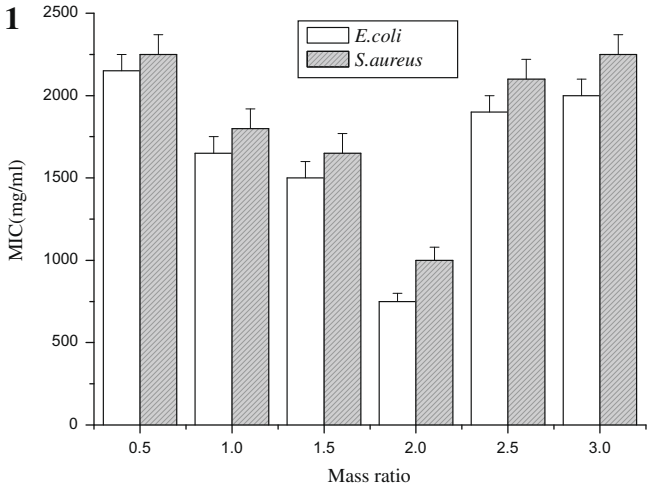
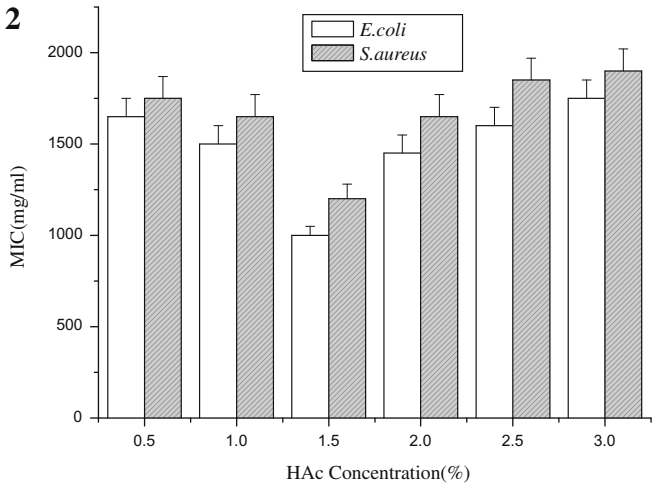


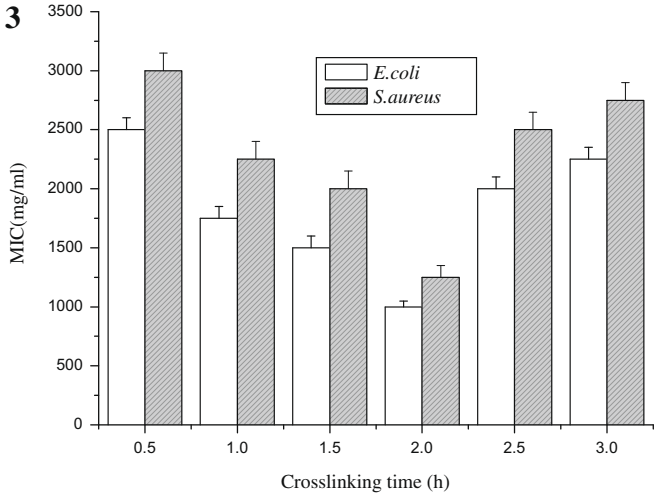
Fig. 4. The TG curves of SLS and CCTS.



Condition: Acetic acid concentration 1%, crosslinking time 1.5h and others see the description of section 2.3.



Condition: SLS to CTS mass ratio 1.0, crosslinking time 1.5h and others see the description of section 2.3.



Condition: SLS to CTS mass ratio 1.0,acetic acid concentration 1% and others see the description of section 2.3.

Fig. 5. Effects of different factors on the antibacterial activity of CCTS–SLS against *E. coli* and *S. aureus* (1) SLS-to-CTS mass ratio, (2) acetic acid concentration, and (3) crosslinking time.

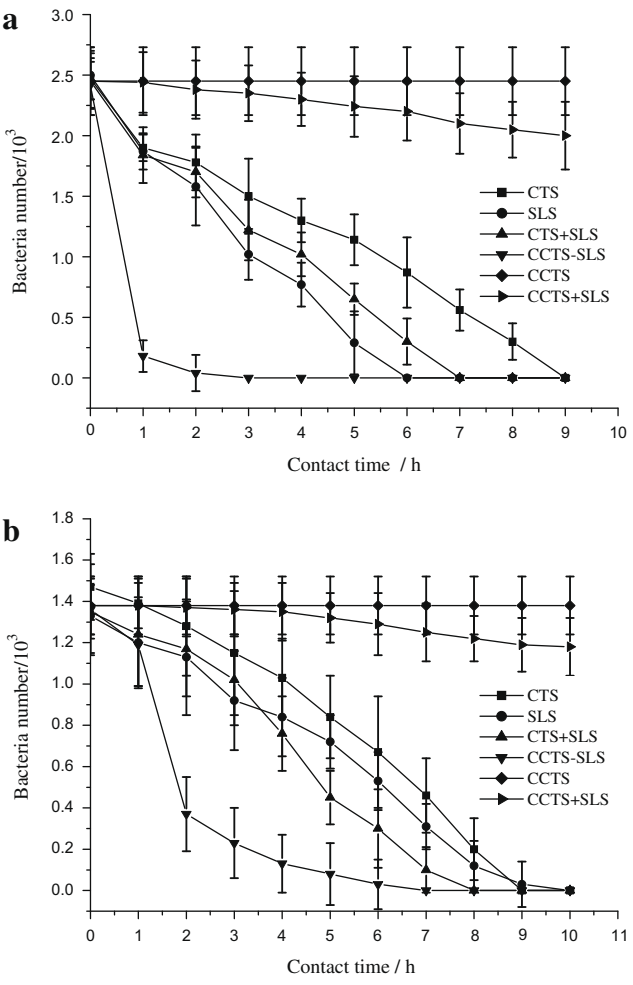


Fig. 6. Time course of antibacterial activity of 2.5 mg samples against (a) *E. coli* and (b) *S. aureus*.

fect on the bacterial growth). It can be clearly observed that CCTS and CCTS + SLS showed little antibacterial activity even at this higher quality of sample. After *E. coli* suspended in solution was in contact with CTS, SLS and CTS + SLS for 7 h, 4 h and 5 h, respectively, and with CCTS–SLS for 2 h, the number of *S. aureus* cells went down to zero. This implies that the antibacterial activity of SLS against *E. coli* was greatly improved by CCTS crosslinking. For *S. aureus*, 4 h of contact time was needed to obtain the same result for CCTS–SLS, shorter than that of CTS (7 h), SLS (6 h) and CTS + SLS (6 h), also suggesting the powerful antibacterial activity of CCTS–SLS.

These results for the time course of the antibacterial activity are quite different from those obtained with 2.5 mg of samples, indicating that as the antibacterial materials increased from 2.5 to 5.0 mg, better antibacterial effect was obtained against *E. coli* and *S. aureus*. For two dosages of CCTS–SLS, the contact time required to kill all the *E. coli* or *S. aureus* bacteria decreased from 3 to 2 h and from 7 to 4 h, respectively, indicating that CCTS–SLS had more powerful antibacterial activity at the higher level of 5.0 mg.

4. Conclusions

Crosslinked chitosan coated Ag-loading nano-SiO_2 composite were prepared by crosslinking CTS on the surface of SLS particles.

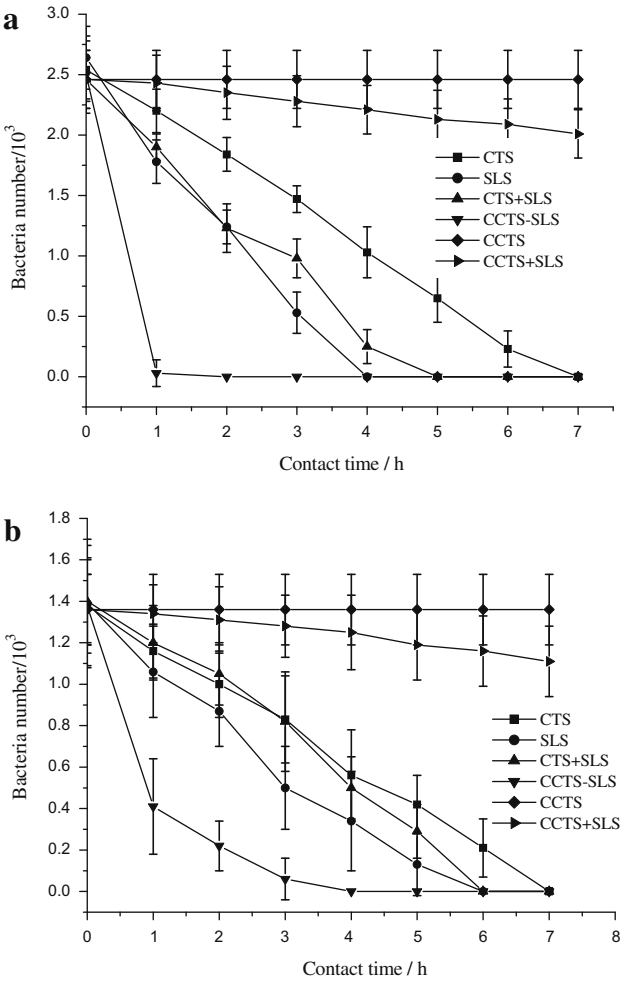


Fig. 7. Time course of antibacterial activity of 5.0 mg samples against (a) *E. coli* and (b) *S. aureus*.

The mass ratio of SLS to CTS, acetic acid concentration and crosslinking time during crosslinking reaction affected the antibacterial activity of the CCTS–SLS. The optimized values for the parameters are: SLS to CTS ratio 2.0, acetic acid concentration 1.5%, and crosslinking time 2 h. The minimum inhibitory values of CCTS–SLS against *E. coli* and *S. aureus* were 250 μg/ml and 300 μg/ml, respectively.

CCTS–SLS showed powerful antibacterial activity against *E. coli* and *S. aureus*, much higher than separately used CTS or SLS. The antibacterial effects of CCTS–SLS against *E. coli* and *S. aureus* were different because of the difference in the structures of the two microorganisms. The antibacterial properties of CCTS–SLS were greatly enhanced with prolonged contact time and increased dosage of the antibacterial materials. When the dosage of the antibacterial composite increased from 2.5 mg to 5.0 mg, the contact time needed to kill all the viable cells of *E. coli* suspended in the solution of 100 ml (10^3cells/ml) decreased from 3 to 2 h, and for *S. aureus*, it decreased from 7 to 4 h.

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