# Unique biological performance of the silver nanoparticles prepared by using carboxymethyl chitosan and sunlight

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In this paper, the study of the biological performance of the silver nanoparticles prepared by a method which had been reported by us was presented. The silver nanoparticles prepared by this method contain a minute amount of silver chloride. In this work, it was found that *Escherichia coli* bacteria was more sensitive to the silver nanoparticles containing more silver chloride and *Staphylococcus aureus* bacteria was more sensitive to the silver nanoparticles with the less silver chloride content. The antibacterial sponge which can be used as a dressing was successfully prepared by the original reaction of this novel method.

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## 1. Intoduction

Metallic nanoparticles have attracted great attention because of their size-dependent physical and chemical properties [1]. The conventional method for preparing metallic nanoparticles, which is based on the chemical reduction, is simple and effective, but removal of the agents used is both cost- and time-consuming; furthermore, the residual agents show biological toxicity [2]. Thus, there is an increased interest in using dispersing or reducing agents based on biomaterial systems [3]. Over the last decade, polysaccharide based biopolymers such as, alginate [4], starch [5, 6], and chitosan [7, 8] have been used as "green" alternatives [9] to conventional agents due to their low cost, nontoxicity and environment-friendly processing. In most cases, the chemical dispersants for controlling growth of metallic nanoparticles was replaced by these biopolymers and toxic reductants were still used. Some researchers had reported that some polysaccharides or their derivatives can be used both as dispersants and reductants [10, 11], but the reducing reaction must be initiated by γ-rays [11], UV-rays [12], or heat [13].

A "greener" and handier method for preparing silver nanopartiles had been created by our group [14]. Following this method, silver nanoparticles can be prepared by using silver chloride and commercially available carboxymethyl chitosan (CMCS) under solar irradiation. This method was called as "solar reduction in CMCS" method (SRIC method) by us. CMCS is one of important derivatives of chitosan and obtained by carboxylation of the hydroxyl and amine groups of chitosan using monochloroacetic acid. Thus commercially available CMCS contains a small amount of chlorine. And, the suspension of silver chloride would be produced in the mixture of commercially available CMCS and silver nitrate. The photolysis of silver chloride is just the key of this method. In this method, AgCl was reduced to silver nanoparticles and chloride radicals in CMCS matrix after being irradiated by sunlight. The silver nanoparticles prepared by this method contain a minute amount of silver chloride, and the content depends on the $Ag^+$: $Cl^-$ molar ratio and irradiation time of the reaction mixture.

The antibiotic activity of the silver nanoparticles with different contents of silver chloride was examined in this work. Finally, a potential application of the products of this method was shown: it was attempted that the sponge loading silver nanoparticles which can be used as antibacterial dressing was prepared just by using CMCS and the original reaction solution of SRIC method.

## 2. Materials and methods

### 2.1. Materials

All compounds were used as received. Pharmaceutical grade carboxymethyl chitosan (95.1 % substituted ratio) was obtained from Honghai Biotechnology Co., Ltd. (Qingdao, China). Silver nitrate (analytical reagent), sodium chloride (analytical reagent) and agar were obtained from Kelong Chemical Reagent Factory (Chengdu, China). Yeast extract and tryptone were obtained from Oxoid Ltd. (Basingstoke, England). Ultrapure water was obtained from a Millipore Milli-Q Plus filtration system. All microbial strains were purchased from ATCC (Manassas, VA).

### 2.2. Preparation of silver nanoparticles

An aqueous solution of commercially available CMCS (0.1 % (w/v)) was prepared and stirred overnight. As determined by ion chromatography (ICS-3000, Dionex, USA), the concentration of chloride ions in the stock solution of commercially available CMCS was 14 mg/L (approximate 0.4 mM). Different amounts of a 0.6 M aqueous solution of $AgNO_3$ (200 μL, and 67 μL) were added dropwise under magnetic stirring into 100 mL of the CMCS stock solution. The reaction solutions were then irradiated by sunlight for 7 hours. The mixtures were stirred with a magnetic stir bar during the irradiation.

### 2.3. Characterizations of silver nanoparticles

Before characterization, the nanoparticles were purified from the reaction solution by centrifugation. The precipitates were rinsed three times and re-suspended in ultrapure water.

The reactions were monitored using a UV-3600 UV-vis spectrophotometer (Shimadzu, Japan). Transmission electron microscopy (TEM) was conducted on a LIBRA 200 TEM (ZEISS, Germany) at an accelerating voltage of 200 kV. The TEM samples were prepared by slowly evaporating a drop of the nanoparticle solutions on a copper grid covered by a carbon-supported film at room temperature. X-ray diffraction (XRD) experiments were performed on a D/MAX-2500PC X-ray diffractometer (Rigaku, Japan) using Cu Kα radiation. The tube voltage and current were 40 kV and 150 mA, respectively. The purified nanoparticle suspensions were coated on glass plates and dried. The obtained films were used for the XRD measurements.

### 2.4. Bactericidal test

The antibacterial activity of the silver nanoparticles prepared by this method was tested against Gram-negative *E. coli* and Gram-positive *S. aureus* bacteria. These bacteria were cultured in a Luria-Bertani (LB) liquid nutrient medium with pH = 7. The culture medium was incubated at 37 °C until the $OD_{600}$ value of the solution reached 0.5.

1 μL inoculum was incubated with 100 μL original or diluted silver nanoparticle solution (silver content = 0.4 mM) at 37 °C for different time durations. Then, the mixture was spread over half the surface of the agar and 100 μL physiological saline solution containing 1 μL inoculum was spread over the other half of the agar surface as a control. The plates were incubated at 37 ◦C for 24 h.

### 2.5. Preparation of the sponge loading silver nanoparticles

10 mL CMCS solution (1 %, w/v) and 10 mL unpurified reaction solution ($Ag^+$: $Cl^-$ = 3:1) were mixed. The mixture was then poured into a 24-well plates (1 mL each well), frozen overnight at -50 °C and freeze dried overnight in an Edwards freeze dryer.

### 2.6. Characterization of the sponge loading silver nanoparticles

The morphology of the sponge was observed by scanning electron microscopy (SEM) with an S-3400N (Hitachi, Japan) at the acceleration voltage of 15 kV.

### 2.7. Antibacterial activity of the sponge loading silver nanoparticles

Bactericidal effect of the sponge loading silver nanoparticles was studied by an agar diffusion method. A sponge was placed on the surface of nutrient agar which had been previously seeded with 100 μL physiological saline solution containing 1 μL inoculum of *S. aureus* ($OD_{600}$ = 0.5). The plate was incubated at 37 °C for 24 h.

## 3. Results and discussion

### 3.1. Characterizations of silver nanoparticles

The characteristic peaks of silver nanoparticle could be found in the UV-vis spectra (Fig. 1, a, the bottom) and XRD spectra (Fig. 1, a, the right inset) of the products of this method. It could also be found that the UV-vis extinction peak of the mixture shifted from 410 nm to 416 nm (Fig. 1, a, the bottom). It may be due to the differences in the shape of the silver nanoparticles (Fig. 1, b and c). Alike the description in our previous work, the silver nanoparticles prepared by SRIC method contain a minute amount of silver chloride, and the content depends on the $Ag^+$: $Cl^-$ molar ratio of the reaction mixture (Fig. 1, a, the right inset) when the irradiation time was the same.

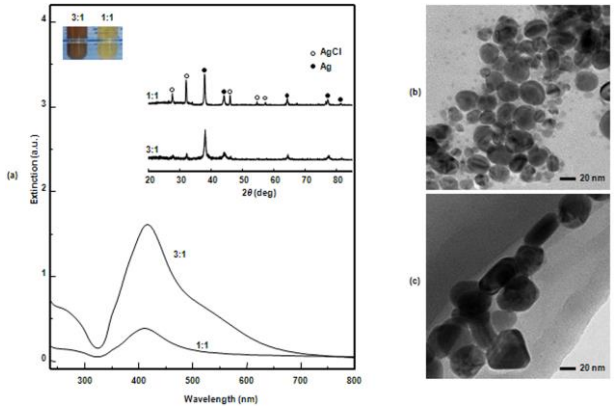


*Fig. 1. (a): UV-vis spectra of the reaction mixture containing different molar ratios of silver and chloride ions after irradiating with sunlight for 7 hours. The left inset shows the photograph of the solutions. The right inset is XRD spectra of the silver nanoparticles purified from the reaction mixtures. (b): TEM image of the particles purified from the reaction mixture in which molar ratio of silver and chloride ions was 3:1. (c): TEM image of the particles purified from the reaction mixture in which molar ratio of silver and chloride ions was 1:1.*

### 3.2. Bactericidal test

In this test, the bacterial strain which was treated with the silver nanoparticles prepared by SRIC method and the control containing untreated inoculum were cultured on the same agar plate.

It was shown in Fig. 2 that the growth of *E. coli* was completely inhibited after being incubated with the silver nanoparticles containing less silver chloride for 4 hours (Fig. 2, a) or incubated with the silver nanoparticles containing more silver chloride for 15 minute (Fig. 2, b). In other words, *E. coli* was more sensitive to the silver nanoparticles containing more silver chloride. But in the test of *S. aureus* (Fig. 2, c and d), the bacteria was more sensitive to the silver nanoparticles containing less silver chloride. So, in practical application, the different silver nanoparticles with different content of silver chloride should be chosen according the class of bacteria.

### 3.3 Characterization and antibacterial activity of the sponge loading silver nanoparticles

The sponge is soft (Fig. 3, a) and multiporous (Fig. 3, b). Silver nanoparticles can also be observed (Fig. 3, c). The antibacterial activity test of the sponge loading silver nanoparticles was performed against *S. aureus* bacteria on nutrient agar plates. The clear zone surrounding the sponge was observed (Fig. 4, d), meaning that the bacterial strains were sensitive to the sponge.

Besides of sponge, CMCS can be used in diverse ways: as a gel [15], a film [16], or a sphere [17]. Thus, the new method for preparing silver nanoparticles gives us more options for the product form.

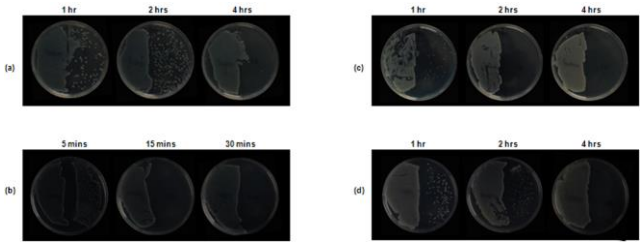


*Fig. 2. (a) and (c): Antibacterial effect of the unpurified reaction mixture in which molar ratio of silver and chloride ions is 3:1 on E. coli (a) and S. aureus bacteria (c) at different time points. (b) and (d): Antibacterial effect of the unpurified reaction mixture in which molar ratio of silver and chloride ions is 1:1 on E. coli (b) and S. aureus bacteria (d) at different time points. The procedure: 1 μL inoculum was incubated with the original or diluted silver nanoparticle solution (silver content = 0.4 mM) at 37 °C for different time durations. The mixture was spread over the right half surface of the agar and a physiological saline solution containing 1 μL inoculum was spread over the left half of the agar surface as a control. The plates were incubated at 37 °C for 24 h.*

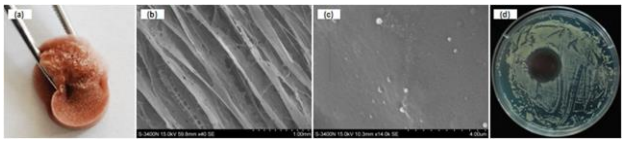

*Fig. 3. (a): Photograph of the sponge which was prepared by using the reaction mixture of SRIC method and CMCS handled by tweezers. (b) and (c): SEM image of the sponge at different magnifications (the scale for the photograph of SEM can be found at the bottom of the corresponding picture). (d): Zone of inhibition produced by the sponge with S. aureus bacteria.*

## 4. Conclusions

In summary, we presented the biological performance of the silver nanoparticles prepared by a method which had been reported by us. Following this method, the silver nanoparticles can be prepared by using silver nitrate and commercially available carboxymethyl chitosan in presence of sunlight. The silver nanoparticles prepared by this method contain a minute amount of silver chloride, and the content depends on the $Ag^+$: $Cl^-$ molar ratio and irradiation time of the reaction mixture.

The antibacterial activity of the silver nanoparticles prepared by this method was tested against Gram-negative, *E. coli*, and Gram-positive, *S. aureus*, bacteria. *E. coli* was more sensitive to the silver nanoparticles containing more silver chloride. But in the test of *S. aureus*, an opposite result was observed.

Finally, the antibacterial sponge loading silver nanoparticles was prepared successfully by using CMCS and the original reaction solution of this new method for preparing silver nanoparticles.

The properties of the product of this novel method match well the criterion for the antibacterial dressing material which is needed wildely, so it is worth to pay more attention to this method and its product.

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