Preparation and characterization of silver nanoparticles on silk fibroin/ carboxymethy lchitosan composite sponge as anti-bacterial wound dressing

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Abstract. The infection in burn wounds covered by biologic dressings leads to wound deepening and chronic wounds. The introduction of silver nanoparticles (AgNPs) into biologic dressings is a beneficial method to prevent wound infection and simultaneously promote wound healing. In this study, an AgNP-loaded silk fibroin (SF)/carboxymethylchitosan (CMC) composite sponge was fabricated. AgNPs with a mean diameter of 4.9 nm was synthesized in SF solution in situ. While CMC was incorporated and chemically crosslinked, SF was insolubilized by ethanol annealing. SEM imaging determined that the AgNP-loaded SF/CMC sponge was more porous than the pure SF sponge. Anti-bacterial results, measured by disk-diffusion and bacterial suspension assay, showed that the AgNP-loaded SF/CMC sponge demonstrated effective anti-bacterial activity against *S. aureus* and *P. aeruginosa*, and that its anti-*P. aeruginosa* activity was higher than that of AQUACEL® Ag. The introduction of CMC improved the water absorption capacity, retention ability, and water vapor transmission rate of the sponge, which are all important properties of wound dressings.

Keywords: Silver nanoparticles, silk fibroin, carboxymethylchitosan, wound infection, wound healing

1. Introduction

Biologic dressings are used to create a moist environment for burn wounds and to promote wound repair and regeneration [1]. However, such an environment is also an ideal place for the colonization of pathological bacteria, such as *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Therefore, it is beneficial to develop a novel biologic dressing to promote both wound healing and anti-infection.

Antibiotics or antiseptics have long been encapsulated into and delivered by biologic dressings to prevent wound infection. Functioning as temporary epidermal substitutes, these dressings act as delivery systems for sustained drug release [2, 3]. However, due to the growing number of antibiotic-resistant microorganisms, it is contentious to apply topical antibiotics to wound surfaces [4]. As an

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anti-bacterial substance, silver has a low probability of resistance and demonstrates broad-spectrum anti-microbial activity [5]. Commercial biologic dressings containing silver ions are widely available, such as AQUACEL® Ag [6]. Ag nanoparticles (AgNPs) have been encapsulated into various polymeric matrices [7, 8]. It has been suggested that AgNPs are more effective than other forms of silver in bacterial suppression [9]. Thus, incorporating AgNPs into biologic dressings is a promising strategy in infection prevention and the simultaneous improvement of wound healing.

This study sought to develop a hybrid silk fibroin/carboxymethylchitosan sponge loaded with AgNPs, which is able to prevent wound infection and simultaneously promote wound healing. AgNPs were synthesized in situ in regenerated silk fibroin solution, and then supplemented with carboxymethylchitosan. The AgNP-loaded sponge was prepared by lyophilizing, physical crosslinking of silk fibroin, and chemical crosslinking of carboxymethylchitosan. The anti-bacterial activity of the sponge was measured by disk diffusion and a bacterial suspension assay. Derived from *Bombyx mori*, silk fibroin demonstrates excellent biocompatibility and robust mechanical strength, but its application as a wound dressing is limited due to its high degree of crystallinity. In this study, in order to improve the internal structure of the silk fibroin-based sponge, carboxymethylchitosan was introduced to decrease the degree of crystallinity of silk fibroin. Therefore, it was hypothesized that the loading of AgNPs could result in a silk fibroin/carboxymethylchitosan hybrid sponge, with sustained anti-bacterial potential and improved physichemical properties, which is promising as an excellent anti-bacterial wound dressing.

2. Materials and methods

2.1. Preparation of silk fibroin (SF) solution

Impurity-removed *Bombyx mori* silk cocoons were boiled and degummed in 0.02 M Na_2CO_3 solution at 100°C for 45 minutes, washed five times with distilled water, and then dried overnight at 50°C to obtain SF. The resulting SF was dissolved in a ternary solvent, $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8 molar ratio) at 70°C for five hours. The SF solution was then dialyzed against distilled water for three days. The fibroin concentration was determined by analytical balance after undergoing water evaporation overnight.

2.2. Preparation and characterization of AgNPs in SF solution

Fresh $AgNO_3$ solution (0.05 wt%) was mixed with SF solution (1.5 wt%) at a volume ratio of 1:4. The solution was then irradiated under ultraviolet light at a wavelength of 365 nm for six hours. An ultraviolet visible (UV-vis) spectrometer (UV-1800, Shimadz) was used to measure the UV-vis absorption spectrum of the AgNPs. Transmission electron microscopy (TEM, JEM-2100) was used to observe the morphology of the AgNPs, from which the mean diameter of the particles was measured and determined by ImageJ.

2.3. Preparation of AgNP-loaded SF/CMC sponge

An equal volume of carboxymethylchitosan (CMC) solution (1.2 wt%) was supplemented into and mixed with the SF solution containing the AgNPs. After bubbles were removed by centrifugation, an equal volume (570 µL or 3 mL) of the resulting solution was pipetted into each well of 6-well or 24-

well cell culture plates (Costar, Corning Inc.). The solutions were prefrozen at -20°C for 30 minutes, frozen at -80°C overnight, and then lyophilized. The lyophilized sponge was then crosslinked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-Hydroxysuccinimide, (EDC/NHS, 50 mM and 12.5 mM) solution containing 90% (v/v) ethanol for 12 hours. After crosslinking, the sponge was washed three times with distilled water to remove unreacted substances, and then lyophilized again to obtain the AgNP-loaded SF/CMC sponge. AgNP-null SF and SF/CMC sponges were prepared at the same concentrations of SF and CMC as that of the AgNP-loaded SF/CMC sponge.

2.4. Scanning electron microscopy (SEM)

Morphology of the sponges was observed by SEM (S-4800, Hitachi Instruments). The internal structures of the sponges, exposed by cross-sectional dissection, were coated with gold using an ion sputterer (E-1010; Hitachi) at 50 mTorr and 5 mA for 30 seconds, and viewed by SEM at a voltage of 1.0~2.0 kV.

2.5. Anti-bacterial activity of the sponges

The anti-bacterial activity of the sponges against both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria was investigated using the Kirby-Bauer disc diffusion method and bacterial suspension assay.

During the Kirby-Bauer disk-diffusion method, 50 μL of bacterial suspension at log-phase was uniformly inoculated and cultured on a Mueller-Hinton (M·H) Agar plate (Oxoid, Thermo Fisher). After a bacterial biofilm formed on the plate, the fully water-absorbed sponge, AQUACEL® Ag (positive control), and SF/CMC sponge (negative control) were placed on top of the film, and zones of inhibition were observed after 24 hours.

During the bacterial suspension assay, 50 μL of bacterial suspension was inoculated overnight in 10 mL of M·H medium under constant shaking at 200 rpm at 37°C. The bacterial solution was then diluted in M·H medium to an O.D. of 0.07 at 600 nm. 400 μL of the solution was then added into a test tube filled with 5 mL of M·H medium and containing AgNP-loaded SF/CMC sponge, AQUACEL® Ag, SF/CMC sponge, or no test material (blank control). The test tube was then wrapped in tin foil, and placed at 37°C under constant shaking at 50 rpm for 24 hours. Then, 100 μL of the bacterial solution was removed from the test tube for O.D. measurement.

2.6. Water absorption capacity and retention test

The water absorption capacity (WAC) of the samples was measured using a modified British Pharmacopoeia (BP) method. First, a circular sample, 2 cm in diameter, was weighed (W) and then immersed in 50 mL of BP solution A (142 mM NaCl, 2.5 mM $CaCl_2$) in a 100-mL beaker. After incubation at 37°C for one hour, the sample was removed by forceps and weighed again (W'). WAC (%) was calculated as (W'-W)/W×100%.

The water retention ability (WR) of the samples was tested using a modified method from that described by Liu, et al [10]. Briefly, the fully water-absorbed sample was placed in a desiccator containing silica gel at 37°C for 24 hours. The weights of fully water-absorbed samples (W'), samples removed from the desiccator (W), and dry samples (W_0) were recorded. The WR (%) per hour was calculated as (W'-W)/(W_0×24)×100%.

2.7. Measurement of water vapor transmission rate

The water vapor tranmission rate (WVTR) was measured in accordance with the standard ASTM D1653. Briefly, a Perm Cup (Gardner Company, America) was filled to 4 mm from the top with distilled water and then sealed by the circular freeze-dried sample of 9.5 cm^2. The assembly was weighed, kept in an environment of 25±0.5°C and 40±2% relative humidity for 24 hours, and then weighed again. WVTR (g/m^2/h) was calculated by the following equation: WVTR $= \frac{(W'-W)}{A \times 24}$, where W' and W represent the assembly weight before and after 24 hours of water evaporation, respectively, and A represents the transmission area of the sealing sample.

3. Results and discussion

3.1. Synthesis of AgNPs

Silk fibroin can reduce Ag$^+$ to Ag by the strong electron donating property of its tyrosine (Tyr) amino acid residue. Meanwhile, UV light irradiation is an effective method to assist the synthesis of AgNPs or Ag clusters on silk fibers [11]. In this study, AgNPs were synthesized from Ag$^+$ in SF solution under 6-hours of UV light irradiation. The formation of AgNPs was confirmed by color transition of the solution from colorless to brown, as shown in Figure 1(a). This color transition is correlated with surface plasmon resonance (SPR) of AgNPs. UV-vis absorption spectrum of the solution displays this characteristic SPR band at ~440 nm, as shown in Figure 1(a). As shown in Figure 1(b), the synthesized AgNPs were spherical in shape, and had a mean diameter of 4.9 ± 1.9 nm, calculated by the average measurement of 100 nanoparticles.

3.2. Morphology of AgNP-loaded SF/CMC sponge

Pore structure and porosity are key parameters of a wound dressing which affect the water absorption capacity, water vapor transmission, and modulus of the dressing. Although one of the primary advantages of SF for biomaterial application is its assembly into an insoluble matrix in the
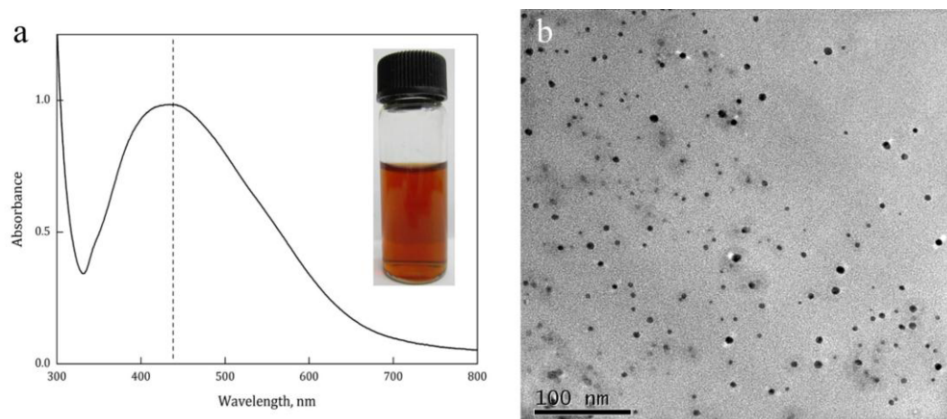

Fig. 1. Synthesis and characterization of AgNPs in SF solution. (a) Image and UV-visible absorption spectrum of silver nanopartcles in SF solution; (b) TEM-observed morphology of AgNPs.
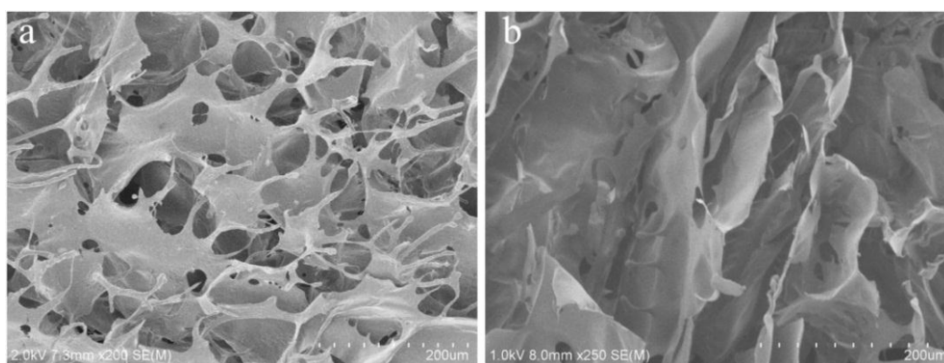
Fig. 2. SEM micrographs of (a) AgNP-loaded SF/CMC sponge; and (b) pure SF sponge.
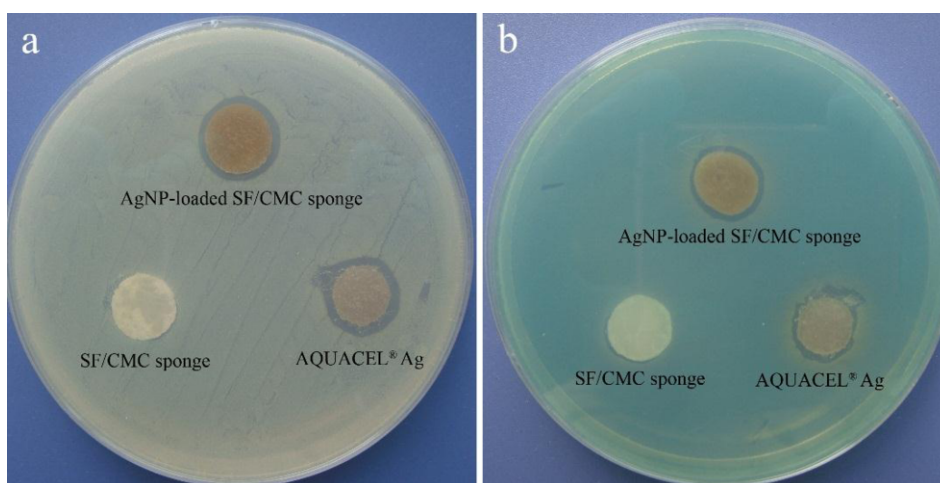

Fig. 3. Anti-bacterial properties against (a) *S. aureus* and (b) *P. aeruginosa* using the Kirby-Bauer disk diffusion method (24 hours after treatment).

absence of chemical crosslinkers, this structural transformation is intense, and thus difficult to modulate in order to fabricate SF-based scaffolds with controlled silk II (β-sheet) formation [12]. In this study, SF was combined with CMC to reduce the silk II content of SF. The AgNP-loaded SF/CMC sponge had a porous structure, as shown in Figure 2(a), while the homogeneous SF sponge had an irregular sheet-like structure which lacked porosity, as shown in Figure 2(b). Moreover, no visible phase separation was observed in AgNP-loaded SF/CMC sponges, as shown in Figure 2(a). SF was combined with other polymers, such as collagen, gelatin, and chitosan, in order to fabricate versatile biomaterials with regard to biocompatibility and tunable physical properties [13-15]. The incorporation of another polymer can improve the porosity of the integral materials, partially accomplished by interrupting the secondary structural transformation of SF.

3.3. Anti-bacterial activity of AgNP-loaded SF/CMC sponge

The anti-bacterial activity of the composite material was tested by disk diffusion and suspension. As shown in Figures 3(a) and 3(b), results of the disk diffusion analysis demonstrated the formation of clear rings around AgNP-loaded SF/CMC sponges and AQUACEL® Ag after 24 hours, whereas no

zone of inhibition was observed around AgNP-null SF/CMC sponges. To further measure the anti-bacterial activity of the AgNP-loaded SF/CMC sponge, suspension assays were conducted by determining the O.D. of the bacterial solution in the presence and absence of the sponge. For the two bacterial strains, solutions of the sample treated with the AgNP-loaded SF/CMC sponge was more transparent than that treated with an SF/CMC sponge and the blank control, as shown in Figures 4(a) and 4(b). The O.D. of the sample treated by the AgNP-loaded SF/CMC sponge was significantly lower than that of the samples treated by the AgNP-null SF/CMC sponge and the blank control, as shown in Figures 4(c) and 4(d). The O.D. of the sample treated by the AgNP-loaded SF/CMC sponge was lower than that treated by AQUACEL® Ag, as shown in Figures 4(c) and 4(d). Both the disk diffusion method and bacterial suspension assay results showed a similar trend regarding the anti-bacterial activity of the AgNP-loaded SF/CMC sponge. It is worth noting that the AgNP-loaded SF/CMC sponge was more effective against *P. aeruginosa*, a bacterial strain particularly known for its drug resistance.
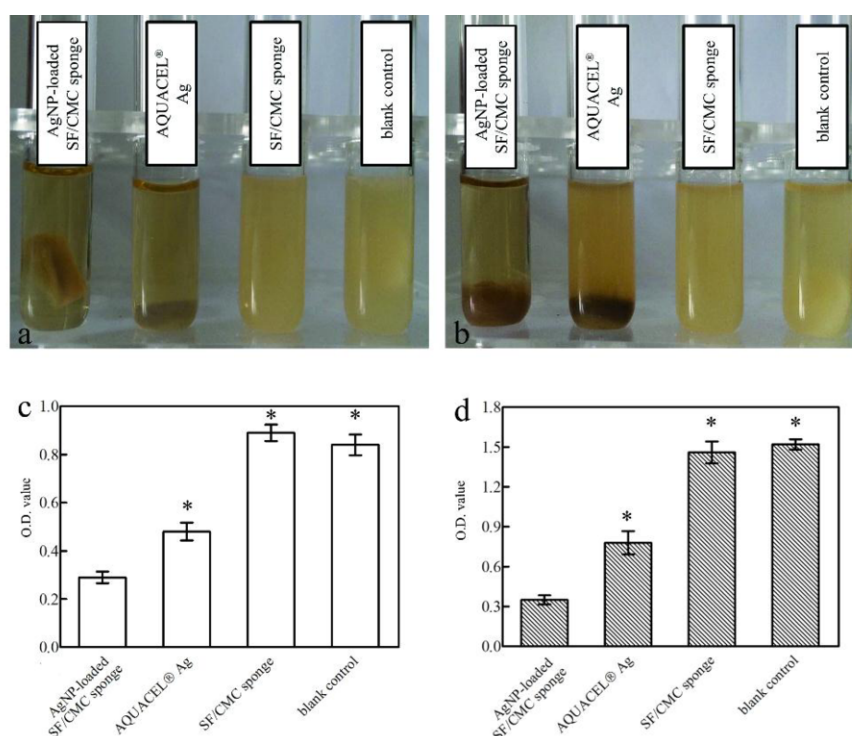

Fig. 4. Anti-bacterial activity against *S. aureus* and *P. aeruginosa* determined by bacterial suspension assay. (a) Sample images of *S. aureus* solutions; (b) sample images of *P. aeruginosa* solutions; (c) O.D. values of *S. aureus* solutions; (d) O.D. values of *P. aeruginosa* solutions (24 hours after treatment). * represents statistical significance, in comparison to AgNP-loaded SF/CMC sponge.

Table 1

WAC, WR, and WVTR of sponges

	WAC (%)	WR (%)	WVTR (g/m^2/h)
SF sponge	623±36	43.3±3.8	163.64±22.98
SF/CMC sponge	1487±157	83.8±5.2	109.23±19.36
AgNP-loaded SF/CMC sponge	1496±149	82.6±5.5	115.43±18.53

3.4. WAC and WR

WAC and WR are key material properties that determine the performance of biologic dressings in maintaining a moist environment for wound healing. The WACs of the SF/CMC sponge and the AgNP-loaded SF/CMC sponge were comparable and more than twice that of the SF sponge (see Table 1). Similarly, water retention ratios of the SF/CMC sponge and the AgNP-loaded SF/CMC sponge were also comparable and approximately twice that of the SF sponge (see Table 1). These results indicate that the presence of CMC improves the water absorption and retention ability of SF/CMC composite sponges, and is not weakened by AgNP synthesis and loading.

3.5. WVTR

An appropriate moist environment is important for the facilitation of wound healing. Insufficient removal of wound exudate leads to wound edema and increased risk of infection, and excessive water loss leads to wound hydration. In this study, the average WVTRs for AgNP-loaded SF/CMC and SF/CMC sponges were 115.43 and 109.23 $g/m^2/h$, respectively, both lower than that of the SF sponge (see Table 1). The AgNP-loaded SF/CMC sponges in this study had a WVTR of 111.92 $g/m^2/h$, which is similar to that of gelatin, alginate, and borax-based wound dressings, and is appropriate for maintaining a fluid balance on the wound bed [16].

4. Conclusion

An AgNP-loaded SF/CMC sponge was developed for use as an anti-bacterial wound dressing. The sponge demonstrated anti-bacterial activity against *S. aureus* and *P. aeruginosa*, with anti-*P. aeruginosa* activity higher than that of AQUACEL® Ag, a commercial Ag^+ dressing. In addition, the sponge displayed improved water absorption capacity and retention abilities, and an optimal water vapor transmission rate. This AgNP-loaded SF/CMC sponge is a promising wound dressing candidate for the prevention of wound infection and the simultaneous promotion of wound healing.

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