Green and Facile Preparation of Chitosan Sponges as Potential Wound Dressings

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ABSTRACT: The aim of this study was to prepare non-leaching chitosan based wound dressings via chemical modification. A green and facile method was applied to fabricate ampicillin grafted chitosan (CSAP) sponges. The morphology, porosity, and swelling behavior of CSAP sponges were analyzed. Specifically, the antibacterial activity of CSAP sponges toward Staphylococcus aureus, Candida albicans, and Escherichia coli was assessed using a series of assays, including bacterial growth curve, nucleic acids leakage, and live/dead staining. As expected, CSAP sponges exhibit excellent antibacterial activity without any leaching. The cytotoxicity assay was carried out on HEK293 cells in vitro, and the result prove the good biocompatibility of the developed sponges. Moreover, the wound healing ability was evaluated using a wound model in vivo, and the result shows that the sponge could speed up wound healing efficiently. Thus, the chemically modified chitosan sponges exhibit great potential as promising wound dressings.

KEYWORDS: Chitosan, Sponge, Nonleaching, Antibacterial, Wound dressing

INTRODUCTION

Skin, as the largest organ, is a protection for the human body from external damage. It has good self-regenerating ability for minor wounds.1 However, it becomes a medical challenge to keep skin wounds sterile and dry in severe injuries. The undesired accumulation of wound exudates such as blood and fluid can promote bacterial growth, cause infections, and lead to delay of the wound healing process.2 Therefore, once the skin is damaged, wound dressings or ointments should be applied. However, frequent changes or reapplication of them on the wound could result in pain and burden for the patient.3 In order to avoid these, wound dressings which are able to provide protection and help recover the damaged skin are welcomed.4

An ideal material for wound dressing should have some advantages, such as high absorbing ability, good oxygen permeability, pain alleviation, easy removal, infection inhibition, wound healing promotion, and reduced scarring. Biopolymer based sponges are considered to be ideal candidates for wound dressings due to their good biocompatibility, high specific surface area, high porosity, and ability to absorb wound exudates well.5,6

Chitosan is a natural polysaccharide, consisting of randomly distributed N-acetyl-D-glucosamine and β-(1,4)-linked D-glucosamine.6,7 Chitosan is derived from the deacetylation of chitin that can be obtained from the exoskeletons of crustaceans and insects and cell walls of fungi.8 Chitosan has various biorelated applications because of its excellent unique features, such as large-scale availability, antitumor properties, antibacterial properties, antioxidant properties, various bioactivities, biodegradability, and biocompatibility.9–11 Since chitosan has unique chem–physical properties, it can be produced as a drug delivery vehicle, artificial skin, and scaffold in the forms of coatings, beads, sponges, fibers, and membranes. Recently, chitosan based sponges have attracted considerable attention in wound dressing applications due to their unique properties and easy preparation.10 It was reported that chitosan can affect macrophage function which could accelerate wound healing with higher angiogenic capability.11 It can also stimulate cell proliferation and promote tissue granulation. Furthermore, chitosan can not inhibit bacterial growth effectively although it exhibits some antibacterial properties. Therefore, many researchers are working on improving the antimicrobial properties of chitosan.13 Chitosan can be functionalized by both physical and chemical methods since chitosan has some reactive functional groups, such as amine and hydroxyl groups leading to its polycationic properties and extensive hydrogel bonding capacity.14 It was reported that silver nanoparticle decorated chitosan cryogels were fabricated through a simple freeze-drying process for point-of-use water disinfection, showing an efficient bactericidal feature.15 Chitosan was...
successfully modified with 3,6-O-N-acetylenediamine which could be applied as a wound dressing.16 A curcumin-loaded chitosan based bio-nano-composite was fabricated in order to reduce dental bacterial biofilm formation.17 Chitosan/ellagic acid films were prepared by solvent casting with high antioxidant and antimicrobial activities, showing great promise in food packaging materials.18 Thymol/chitosan composites with antibacterial and antioxidant activities were developed for oral local delivery.19

Ampicillin is a β-lactam antibiotic which belongs to the penicillin family. It is quite effective to treat a number of bacterial infections mediated by Gram-negative and Gram-positive strains.20 The extensive use of antibiotics usually leads to the development of tolerance against antibacterial action. Moreover, multidrug resistant superbugs are becoming alarmingly common in recent years which is due to the excessive and improper use of antibiotics.21−23 Thus, some policies have been established in order to limit the use of antibiotics.24 It is crucial to develop novel effective antibacterial agents. Therefore, our study was to prepare ampicillin grafted chitosan (CSAP) sponges without any leaching in order to limit the usage of ampicillin. In this study, CSAP sponges were developed by a facile and green method. The CSAP sponges were analyzed by FTIR, SEM, and XPS, respectively. The swelling behavior and porosity were tested. The antimicrobial activity of the CSAP sponges was investigated against *S. aureus* ATCC 6538, *C. albicans* CMCC(F) 98001, and *E. coli* ATCC 25922, respectively. The biocompatibility and cell morphology on HEK293 cells were investigated through MTT assay. Furthermore, the wound promotion efficacy of CSAP sponges was examined in vivo.

### RESULTS AND DISCUSSION

**Morphologies.** The pictures of chitosan (CA) and CA₅ sponges are displayed in the Supporting Information (Figure S1). There is no obvious difference in appearance between CA and CA₅ sponges; both of them display as blue sponges. The porosity and surface morphology of CSAP sponges are important factors for wound dressing application, since they directly affect the absorption of exudates and blood capacities.25 Figure 1 shows the cross-section morphologies of the prepared CA (A) and CA₅ sponges (B). As shown in Figure 1A, the CA sponge presents a well-interconnected porous 3D network structure with a quite smooth pore wall. The cross-section morphology and the structural integrity of the sponge were not influenced at all after being grafted with ampicillin (Figure 1B); the CA₅ sponge still keeps the interconnected 3D network structure. Besides, no obvious difference in the porosity between CA and CA₅ sponges was observed.

Moreover, the porosities of the fabricated sponges were calculated and the result is shown in Figure S2. It can be seen that the calculated porosity of CA sponge was 76.1%, and the porosities of CSAP sponges were in the range of 74.9%−75.5%. Although slightly reduced porosities were observed for the
CSAP sponges, they still maintain very high porosity. These highly porous structures can absorb exudates and blood from wounds, which enables the fabricated sponges to have great potential in wound dressings. Figure 1C–F displays the element mappings of Si, N, and S and EDS spectrum of CA sponge. The uniform distribution of Si, N, and S elements confirmed that ampicillin was successfully grafted onto CA sponge via APTES. XPS spectrum of CA$_5$ sponge was shown in Figure S3. The successful fabrication of CSAP was further proven from the existence of Si, N, and S elements.

**FTIR Analysis.** The successful chemical modification of the CA sponge was confirmed by FTIR, as displayed in Figure 2A.

![Figure 2](image-url)  
**Figure 2.** (A) FTIR spectra of CA (a), CA$_1$ (b), CA$_2$ (c), CA$_3$ (d), CA$_4$ (e), and CA$_5$ sponges (f) and (B) swelling behaviors of CA and CSAP sponges at different time intervals.

FTIR spectra of free ampicillin, and chitosan-APTES were shown in Figure S4. For the FTIR spectrum of CA (Figure 2a), a broad peak existing between 3500 and 3100 cm$^{-1}$ corresponds to the stretching vibrations of N–H and O–H.$^{26}$ Two peaks at 1650 and 1560 cm$^{-1}$ are attributed to the C=O in the amide group and N–H bending vibration of the amine group, respectively.$^{27}$ The bands between 1100 and 1000 cm$^{-1}$ are assigned to C=–N and C=–O stretching vibrations.$^{28}$ In the case of chitosan-APTES, two peaks at 1069 and 1020 cm$^{-1}$ appear, which are assigned to the siloxane groups (Si–O–Si) from APTES.$^{29}$ FTIR spectra of ampicillin were shown in Figure S4b and c. Figure S4c shows the characteristic peaks of ampicillin between 2000 and 1300 cm$^{-1}$. Five main characteristic bands were seen at 1770 cm$^{-1}$ (C=O stretching of β-lactam), 1693 cm$^{-1}$ (primary amide C=O stretching), 1603 cm$^{-1}$ (C=–C stretching of benzene ring), 1506 cm$^{-1}$ (both C–N stretching and N–H in-plane bending in secondary amide group), and 1455 cm$^{-1}$ (aromatic ring –C=C–C stretching), respectively.$^{20,27,30}$ For CSAP, some characteristic peaks of ampicillin (1693 and 1455 cm$^{-1}$, et al.) are shown in the FTIR spectra (Figure 2A), the others are merged into the large peaks of chitosan. The existence of characteristic peaks of ampicillin in the CSAP spectra reveals that ampicillin was grafted successfully onto CA sponges.

**Swelling Study.** The abilities to hold and absorb water are essential features for wound dressings that can absorb exudates, metabolites, and body fluids.$^{31}$ The swelling performances of CA and CSAP sponges were displayed in Figure 2B. The swelling ratio of CA sponge was 3240% after 24 h. There are many hydrophilic groups including amino and hydroxyl groups, resulting in very high swelling ability.$^{32}$ Similar swelling ability of CSAP sponges was observed after 24 h, which is in the range of 3210–3250%. However, quite different swelling behaviors were observed for the CSAP sponges in the first 3 h that the swelling ratios of CSAP sponges were much lower than that of CA sponge. This phenomenon could be due to the existence of APTES in the CSAP sponges that reduces the hydrophilic groups.

**Antibacterial Growth Behaviors.** The antibacterial growth kinetics of *S. aureus*, *C. albicans*, and *E. coli* with CSAP sponges were studied (Figure 3A–C). The growth behavior was monitored by measuring the optical density at 600 nm (OD$_{600}$) using a UV–vis spectrophotometer. There are similar antibacterial performances for CA and CSAP sponges against the tested three strains. It can be seen that bacterial growth without any sponges (curve a) shows a typical bacterial growth curve with three phases in 8 h. It is clearly observed that both CA and CSAP sponges delayed the progress of bacteria to exponential growth. There is stronger bacterial inhibition growth activity for CA grafted with a higher amount of ampicillin due to the smaller OD$_{600}$ value. Furthermore, the OD$_{600}$ value remains almost zero after 8 h of incubation with the CA$_5$ sponge. These results demonstrate that the incorporation of ampicillin into CA sponges can significantly enhance the antibacterial activity of CA. Moreover, a disk diffusion method was conducted to demonstrate any leaching effect existence for the fabricated sponges against *S. aureus*, *C. albicans*, and *E. coli*. Pictures of the inhibition zones are shown in Figure S5. No zones of inhibition can be detected after 24 h incubation, which proves that there is no ampicillin leaching from the prepared sponges. Thus, the fabricated CSAP sponges exhibited great antimicrobial performance with no leaching.

**Nucleic Acid Leakage.** It is known to all that the UV light absorption capacity of nucleic acids is at the wavelength of 260 nm. The existence of nucleic acids in the bacterial suspension indicates the membranes of bacterial cells are destroyed. Therefore, the leakage of nucleic acids in the bacterial suspension can be used to reveal the amount of bacterial membranes damage. Thus, the bacterial suspension was measured at the wavelength of 260 nm (OD$_{260}$) in order to detect whether the inhibition effect on the bacterial growth was due to the cell membrane damage. The result is shown in Figure 3D–F. There was a similar trend for the tested three strains. There was nearly no change in the OD$_{260}$ values of the control group, which means little membrane damage happened. However, there were higher OD$_{260}$ values for the bacterial...
suspension treated with a CA sponge, indicating some leakage of intracellular contents occurred. It is stated that CA has some antibacterial performance due to polycationic CA binding to the bacterial cell wall with negatively charged causing the leakage of nucleic acids and proteins. Significant differences of the OD\textsubscript{260} values after treatment with CSAP sponges were observed. The OD\textsubscript{260} values increased with increasing the initial additive amount of ampicillin, suggesting that more and more nucleic acids leak into the bacterial suspension through the damaged bacterial membrane. The OD\textsubscript{260} values of S. aureus, C. albicans, and E. coli suspensions for the control were 0.014, 0.034, and 0.015, respectively, and that for treatment of CA\textsubscript{5} sponges increased to 0.42, 0.557, and 0.658, respectively. These significant changes could be due to the existence of damages to the bacterial membrane that higher OD\textsubscript{260} values suggested the fabricated sponges have higher cell membrane damage capacity. The result revealed that the fabricated CSAP sponges have a great damaging effect on the cell membranes of S. aureus, C. albicans, and E. coli. This is consistent with the bacterial growth curve results.

**Live/Dead Assay.** The live/dead assay was carried out using a fluorescence microscope based on the LIVE/DEAD BacLight Bacterial Viability Kit (L13152) containing propidium iodide (PI) and SYTO 9. The strains treated with CSAP sponges were stained with PI and SYTO 9 for 15 min under dark conditions. Fluorescent green illustrates live bacteria with intact cell membranes, while fluorescent red shows dead bacteria with damaged membranes. Representative fluorescence microscopy images are displayed in Figure 4, together with the control comprising untreated live bacteria. As expected, untreated S. aureus cells (the control) are almost alive (green), only a few S. aureus cells are dead (red) (Figure 4A). Most S. aureus cells treated with CA\textsubscript{5} sponges were dead with only a little live bacteria being seen from Figure 4D. This result means that CA\textsubscript{5} sponges can induce S. aureus cell death. Similar results can also be found for both C. albicans and E. coli. Therefore, this test provides direct evidence that CSAP sponges exhibited great antimicrobial activity against S. aureus, C. albicans, and E. coli, which is consistent with antibacterial growth curves.
Cytotoxicity Assay in Vitro. Biomaterials with good biocompatibility are considered to be the primary requirement for wound dressing application. MTT assay was conducted in order to test the effect of CSAP sponges on the growth of HEK293 cells. The MTT result in term of cell viability is shown in Figure 5. It can be found that cell growth was not inhibited by CSAP sponges at all after incubation for 24 h with cell viabilities of more than 95%. This result demonstrates that the fabricated CSAP sponges had good biocompatibility since no cytotoxic effect was detected at all.

Cells morphologies treated with CA and CA₅ sponges for 24 h were stained and studied using confocal microscopy. The fluorescent microscopy pictures of HEK293 cells are shown in Figure 5. No difference in the cell shape and morphology among the cells treated with the control and the fabricated CA and CA₅ sponges was observed. Therefore, the results show that the CSAP sponges are nontoxic to cell morphology, making them promising candidates for wound dressing applications.

Wound Healing Capacity in vivo. The wound healing capacity of the sponges were determined on the backs of mice. Figure 6 displays optical pictures of wound healing processes after treatment with CA and CA₅ sponges for 2, 4, 6, 8, and 10 days, respectively. Wounds with no treatment or dressing were used as control. The wound covered with CA sponge healed better than the control, although the wound healing ability of the CA sponge is slower than that of CA₅ sponge. The wound contraction for the CA₅ sponge had an advantage over both CA and the control group that the CA₅ sponge exhibited the best wound healing capacity after 10 days. The percentages of wound area closure at different healing times were calculated.

As shown in Figure 6, the wound size tends to contract with the increase of healing time. The CA₅ sponge exhibited a good wound healing capacity with a 25% wound closure ratio after 2 days of treatment, while the wound healing closure is 15% and that of the control (bare wound) is only 10%. On day 6, the wound treated with CA₅ sponge appears significant closure to 85%, whereas the wounds treated with CA and the control show 60% and 45% wound closure, respectively. After 10 day of treatment with the CA₅ sponge, the wound was healed completely without any scars and the wound contraction is 100%, which shows a highly improved wound healing ability. This indicates that the CA₅ sponge could be beneficial and effective for accelerating wound healing. Chitosan based biomaterials are reported to promote wound healing, because the depolymerization of chitosan gradually occurs to release N-acetylglucosamine, which is beneficial for the deposition of ordered collagen, initiating fibroblast proliferation and stimulating the production of hyaluronic acid at the wound site. This great wound healing capacity of CA₅ sponge may be attributed to the excellent antibacterial performance, biocompatibility, and good swelling ability based on the CA sponge.

Histopathological study was carried out to further compare the wound healing efficiencies of the control, CA, and CA₅ sponges. Figure 7 presents the histological result of wound tissues of the control and treated with CA and CA₅ sponges. There are still some inflammatory cells in the wound site without any treatment after 10 days. Meanwhile, the inflammatory cells were completely diminished at day 10 treated with CA and CA₅ sponges. Interestingly, the group treated with CA₅ sponge clearly regenerated more blood vessels...
than the CA sponge did. These results indicate that the CA₅ sponge can effectively enhance the wound healing process. These results demonstrate that the CA₅ sponge has excellent wound healing ability.

■ CONCLUSION
In summary, novel chitosan based sponges were successfully fabricated via a green and facile method. There is no significant difference in porosity and swelling behaviors between CSAP and CA sponges. It was observed that the fabricated CSAP sponges showed excellent antimicrobial activity toward S. aureus, C. albicans, and E. coli with nontoxic activity to HEK293 cells. Most importantly, CSAP sponges exhibited accelerated wound healing capacity in vivo. Therefore, the great antibacterial and wound healing performances of the developed CSAP sponges, as well as their good swelling behaviors, make them competitive candidates to be applied as potent wound dressings.

■ EXPERIMENTAL SECTION
Preparation of CSAP Sponges. (3-Aminopropyl)triethoxysilane (APTES) ethanol solution was added into chitosan acetic acid solution to achieve concentrations of 0.8 and 2 wt %, respectively. Then it was stirred at 150 rpm at 25 °C for 24 h and dialysis for 3 days. A 10 mg/mL ampicillin-MES solution was prepared and different volumes were added into the above mixture, followed by adding EDC and NHS, with final concentrations of S and 12.5 mM/L, respectively. After reacting for 4 h, 0.08% genipin solution was added and stirred for 0.5 h. The mixture was centrifugated at 4000 rpm for 3 min and kept at 45 °C for 36 h. The final weight ratio of ampicillin to chitosan is 1, 2, 3, 4, and 5 wt %, respectively (named as CA₁, CA₂, CA₃, CA₄, and CA₅, respectively). The chitosan sponge was prepared by the same method except no ampicillin was added (named CA).

Characterization. The morphologies of cross-section of chitosan and CSAP sponges were examined by JSM-7600F SEM. The morphology and CSAP sponges were analyzed by measuring bacterial growth kinetics toward instrument (Thermo Fisher, USA). The microographies were obtained by confocal microscopy (Leica DM2500, Germany).

Antibacterial Activity. The antibacterial activity of CSAP sponges was analyzed by measuring bacterial growth kinetics toward S. aureus, C. albicans, and E. coli according to previous methods. Bacteria suspension with concentration of 1 × 10⁶ CFU/mL treated with sterilized CSAP sponges was performed and the growth behaviors were monitored at fixed interval at 150 rpm at 37 °C by measuring OD₆₀₀ value. All experiments were run in triplicate.

Leaching of CSAP sponges was evaluated using disk diffusion method. About 1 × 10⁴ CFU/plate of test strains were loaded on the TSA. The sterilized chitosan and CSAP sponges with 1 cm diameter were placed on top of the lawns. They were incubated at 37 °C for 24 h. The inhibitory effect was evaluated.

Live/Dead Bacterial Cell. Bacterial live/dead fluorescent staining tests were carried out in order to confirm bacterial death behaviors. Suspensions (1 × 10⁸ to 1 × 10⁹ CFU/mL S. aureus, C. albicans, and E. coli) were incubated with CA₅ sponges at 150 rpm at 37 °C for 2 h. Then the mixture was stained with SYTO 9 and PI (LIVE/DEAD BacLight Bacterial Viability Kit, L13152) and imaged with fluorescence microscopy (Olympus, IX53).

Cytotoxicity Tests. An MTT assay was carried out on HEK293 cells according to previous methods. Chitosan and CSAP sponges were cut into a square of 5 mm × 5 mm. The cells treated with chitosan and CSAP sponges were fixed and stained with DAPI and FITC-phalloidin. The cell morphologies were obtained by confocal microscopy (Leica DM2500, Germany).

In vivo Wound Healing Evaluation. The wound healing ability was evaluated on mice according to our previous study. The wounds were treated using chitosan and CA₅ sponges, and the bare wounds were applied as the control. The sponges were replaced every 2 days, and the wound area was imaged and measured. The sizes of all images were the same.

The area of wound closure (Aₕ₋ₐ) is calculated as follows:

\[ Aₕ₋ₐ(\%) = \left( \frac{A₀ - A}{A₀} \right) \times 100\% \]  

Where A₀ is the created wound area and A is the area of wound at the dressing changing day.

After day 10, the skin wound tissue samples were excised and fixed with 10% formalin. Then the samples were stained with hematoxylin and eosin (HE) and observed using an optical microscope (Eclipse CI, Nikon).

■ ASSOCIATED CONTENT
5 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b01468.

Images of HE-stained histopathological sections after 10 days of treatment: (black arrows) inflammatory cells; (white arrows) microvessels.

Figure 7. Images of HE-stained histopathological sections after 10 days of treatment: (black arrows) inflammatory cells; (white arrows) microvessels.

Notes
The authors declare no competing financial interest.

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