A Novel Asymmetric Wettable AgNPs/Chitosan Wound Dressing: In Vitro and In Vivo Evaluation
Donghui Liang, Zhong Lu, Hao Yang, Jingting Gao, and Rong Chen

ACS Appl. Mater. Interfaces, Just Accepted Manuscript • DOI: 10.1021/acsami.5b11160 • Publication Date (Web): 22 Jan 2016
Downloaded from http://pubs.acs.org on January 25, 2016

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
For submission to *ACS Appl. Mater. Interface*

**A Novel Asymmetric Wettable AgNPs/Chitosan Wound Dressing: In Vitro and In Vivo Evaluation**

Donghui Liang, Zhong Lu*, Hao Yang, Jingting Gao, Rong Chen*

*School of Chemistry and Environmental Engineering, Key Laboratory for Green Chemical Process of Ministry of Education, Wuhan Institute of Technology, Xiongchu Avenue, Wuhan 430073, PR China*

*Corresponding author: Prof. R. Chen, E-mail address: rchenhku@hotmail.com

Prof. Z. Lu, E-mail address: zhongluwit@163.com
Abstract

A novel silver nanoparticles (AgNPs)/chitosan composite dressing with asymmetric wettability surfaces was successfully prepared via a simple two-step method for biomedical applications as wound healing materials. Firstly, AgNPs were assembled into the chitosan sponge which was prepared by lyophilization process. Then one side of the sponge was modified by a thin layer of stearic acid. The incorporation of AgNPs into chitosan dressing could enhance the antibacterial activity against drug-sensitive and drug-resistant pathogenic bacteria. The asymmetric surface modification endows the dressing with both highly hydrophobic property and inherent hydrophilic nature of chitosan. The hydrophobic surface of the dressing shows waterproof and anti-adhesion for contaminant properties, whereas the hydrophilic surface preserves its water-absorbing capability and efficiently inhibits the growth of bacteria. Furthermore, the AgNPs/chitosan composite dressing displays improved moisture retention and blood clotting ability compared to the unmodified dressings. Cytocompatibility test evaluated in vitro and in a wound infection model illustrates the non-toxic nature of the composite dressing. More importantly, the in vivo wound healing model evaluation in mice reveals that the asymmetric AgNPs/chitosan dressing promotes the wound healing and accelerates the reepithelialization and collagen deposition. The silver accumulation in mice body treated by the composite dressing is far lower than that of the clinically used Acasin™ nanosilver dressing treated mice. This work indicates the huge potential of the novel AgNPs/chitosan wound dressing with asymmetrical wettability for clinical use.

Keywords: asymmetric wettability; chitosan dressing; silver nanoparticle; antibacterial
activity; anti-adhesion; *in vivo.*
Introduction

Skin, the largest organ of human body, plays an important role in homeostasis and prevention of invasion by microorganisms.\textsuperscript{1-2} Therefore, an ideal non-toxic, non-allergenic and non-adherent skin wound dressing is of significant importance in our daily life, which could absorb excess exudates and maintain a moist environment on wound surface. Meanwhile, it should possess good antimicrobial and biocompatible properties to promote wound healing, as well as self-cleaning ability to prevent contamination.\textsuperscript{3-7} In recent years, biomaterial-based wound dressings have been widely used, such as natural abundant chitosan, due to its non-toxic, biocompatible, biodegradable, analgesic, moisture retentive and readily available properties.\textsuperscript{8-13} Different forms of chitosan as wound dressings, such as hydrogels, membranes, scaffolds and sponges were reported and some chitosan-based wound dressing products such as Syvek-Patch\textsuperscript{®}, Chitopack C\textsuperscript{®}, Tegasorb\textsuperscript{®}, HemCon Bandage\textsuperscript{TM} and KytoCel\textsuperscript{®} are commercially available.\textsuperscript{5,14-17}

Unfortunately, up to now, there is almost no clinically used wound dressing satisfying with all the requirements of the ideal skin wound dressing. Recently, much attention has been paid to the coating with asymmetric properties. For examples, the reported asymmetrically wettable cotton fabrics exhibited stable ultra-water-repellent property on the one side and good water-absorbing capability on the other side.\textsuperscript{18-19} However, their antibacterial property was not mentioned. The superhydrophobic surface of asymmetric free-standing poly (ethylene-imine)-Ag/poly (acrylic acid) film was reported to possess the abilities of self-cleaning and anti-bacterial adhesion, while the hydrophilic surface could deliver bactericidal silver ions.\textsuperscript{20} But the poor water-absorption ability and the complex
preparation process are not satisfied.

In this work, we designed and prepared a sponge-like silver nanoparticles (AgNPs)/chitosan composite dressing with asymmetric wettability through a simple two-step method. Firstly, AgNPs were assembled into the chitosan sponge which was prepared by lyophilization process. Then one side of the sponge was modified by a thin layer of stearic acid. The incorporated AgNPs could improve the antibacterial activity against drug-sensitive and drug-resistant pathogenic bacteria. The surface modification could endow the AgNPs/chitosan dressing with both hydrophobic and hydrophilic surfaces. The highly hydrophobic surface possessed the properties of self-cleaning by minimizing the water and contaminant adhesion. To maintain the comfortable, water-absorbing and hemostatic properties of the wound dressing, the downward surface which contacts with the wound kept its original hydrophilic property. To develop the potential application of AgNPs/chitosan composite dressing with asymmetric wettability, the antibacterial adhesion and infiltration abilities of the hydrophobic upper surface and the bactericidal activity as well as the wound healing activity of the hydrophilic surface were investigated in vitro and in vivo. To the best of our knowledge, chitosan-based dressing with asymmetrical wettability has not been reported.

Materials and Methods

Materials

Chitosan (degree of deacetylation \( \geq 95\% \)) and stearic acid \((\text{CH}_3\text{(CH}_2)_{16}\text{COOH})\) were purchased from Aladdin (China). Silver nitrate \((\text{AgNO}_3)\) was purchased from GmbH & Co.
KG (Germany). Sodium borohydride (NaBH₄) was purchased from Zhanyun Chemical Co. Ltd. (China). Polyvinylpyrrolidone (PVP, Mw 55,000), amoxicillin (C₁₆H₁₀N₃O₅S·3H₂O, potency ≥ 900 µg per mg) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, C₁₈H₁₆BrN₅S) were purchased from Sigma-Aldrich. Acetic acid (CH₃COOH), absolute ethanol (C₂H₅OH), absolute methanol (CH₃OH), dimethyl sulfoxide (DMSO), absolute ether (C₂H₅OC₂H₅), formalin (40% methanol solution, HCHO), sodium chloride (NaCl), nitric acid (HNO₃), 30% hydrogen peroxide solution (H₂O₂) and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co. (China). All the chemicals were used without further purification.

The bacteria strains of Staphylococcus aureus (S. aureus, ATCC 9118), Escherichia coli (E. coli, CCTCC AB 93154), Pseudomonas aeruginosa (P. aeruginosa, CCTCC AB 93066) and cell strains of human embryonic kidney cell (HEK293), human normal hepatocyte (L02) were obtained from China Center for Type Culture Collection (CCTCC). Methicillin-resistant S. aureus (MRSA), drug-resistant E. coli (DREC) and drug-resistant P. aeruginosa (DRPA) were obtained from Wuhan Third Hospital. Luria-Bertani (LB) medium was used in growing and maintaining all the bacteria. Dulbecco’s minimum essential medium (DMEM) was used for the cell culture.

**Preparation of AgNPs/chitosan composite dressings**

In the preparation of chitosan dressing, 0.5 g chitosan was dissolved in 25 mL 1% (v/v) acetic acid solution with continuous stirring under room temperature, and poured into Teflon plate and kept at -20°C overnight. After that, the frozen sample was lyophilized for 24 h, then immersed in 5% NaOH (w/w) solution for 2 h. Finally, the sample was rinsed with
deionized water to neutral to obtain chitosan dressing and named as CS.

In a typical preparation of AgNPs/chitosan dressing, the as-prepared CS was soaked into 0.1, 0.2 and 0.5 mmol/L AgNPs solutions prepared at 4°C in dark for 24 h according to our previous work. The obtained AgNPs/chitosan dressing was labelled as CS-Ag0.1, CS-Ag0.2 and CS-Ag0.5 in the sequence of the concentration of AgNPs solution mentioned above.

Asymmetric wetting modification to AgNPs/chitosan composite dressings

The prepared AgNPs/chitosan dressings were soaked in deionized water for at least 24 h, then frozen at -20°C for 12 h. After that, 2 mL stearic acid solution (20 mmol/L in alcohol) was poured uniformly onto one surface of each frozen dressing separately and kept the dressings freezing at -20°C for 30 min. Subsequently, the frozen samples were lyophilized to obtain asymmetrically wettable AgNPs/chitosan dressings and labelled as CS-Ag0.1-S, CS-Ag0.2-S and CS-Ag0.5-S, respectively.

Characterization

The microstructure of the prepared dressing was characterized by scanning electron microscopy (SEM, Hitachi S-4800, operating at 5 kV). The AgNPs/chitosan dressing was dissolved by 1% acetic acid, then the AgNPs in the chitosan solution was characterized by transmission electron microscope (TEM, Philips Tecnai G220, accelerating voltage of 200 kV) and energy dispersive X-ray spectrum (EDX). The silver content was determined by flame atomic absorption spectroscopy (FAAS, Shanghai Spectrum SP-3530).

Asymmetric wettability measurement

The wettability of the CS-Ag0.2-S was measured via sessile drop contact angle
measurements with a KRÜSSDSA100 contact angle system at room temperature. At five different positions, a water droplet of 4 µL was dispensed onto the surface of dressing and the contact angle was measured.

**Porosity measurement**

The porosity of the prepared dressing was determined by using the reported method. The dressing was immersed in absolute ethanol until it was saturated. The weight of the dressing was measured before and after the immersion. The porosity \( P \) was calculated by the equation (1).

\[
P = \frac{m_2 - m_1}{\rho V} \times 100\% \quad (1)
\]

In this equation, \( m_1 \) and \( m_2 \) are the weight of the dressings before and after immersion in alcohol respectively. \( V \) is the volume of the dressing before immersion, which was calculated by the formula of length \( \times \) width \( \times \) height of the dressing. \( \rho \) is the density of alcohol. All samples were triplicate in the experiment.

**Degree of swelling and moisture retention capacity**

The test dressing was immersed into deionized water. After swelling for 2 h, the dressing was taken out and gently blotted. Then it was immediately weighted. The degree of swelling \( (DS) \) was calculated by the equation (2).\(^6\)

\[
DS = \frac{m_w - m_0}{m_0} \times 100\% \quad (2)
\]

In this equation, \( m_0 \) and \( m_w \) are the weight of dressings before and after immersion. All samples were triplicate in the experiment. In order to measure the moisture retention capacity of the dressing, the wet dressing was placed in a glass dryer at room temperature, and the \( DS \) was determined every hour. The moisture retention time was recorded as the
value of the $DS$ reduced to 100%.

**Mechanical property evaluation**

The tensile strength and elongation at break of the dressing (50 mm in length × 15 mm in width × 2 mm in height) were measured by a universal testing machine (SANS, CMT8202) at room temperature with the crosshead speed of 20 mm per minute. The average value of five measurements was given for each sample.

**In vitro antibacterial test**

The antimicrobial activity of AgNPs/chitosan dressing was tested by an inhibition zone method. The strains of drug-sensitive, *E. coli*, *P. aeruginosa* and drug-resistant MRSA, DREC, DRPA were used to evaluate the antibacterial activity of the dressing. After 40 µL bacteria suspension (1×10$^8$ CFU/mL) was spread on LB agar plate, the sterile dressing (with a diameter of 1 cm, and sterilized by autoclaving) was placed onto the surface of the agar. After 48 h incubation at 37°C, the diameter of inhibition zone was measured.

**In vitro cytotoxicity studies**

*In vitro* cytotoxicity of the dressings was estimated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. HEK293 and L02 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C and in 5% CO$_2$. A piece of sterile dressing (with a diameter of 1 cm) was placed in a well of a 24-well plate, then seeded with the cell suspension at a concentration of 1×10$^5$ cells. After 1~4 days of incubation, 0.5 mL of 5 mg/mL MTT was added into the surface of dressing. Followed by 4 h incubation, 1 mL of dimethyl sulfoxide (DMSO) was added to the well and further incubated for 10 min. The cell viability was
determined by measuring the absorbance at 490 nm on a microplate reader (Multiskan MK3).

The blank control without dressing was also carried out under identical conditions. All the
experiments were performed in triplicates.

**Antibacterial activity test using a mimetic wound infection model**

A human cell-based wound model was used to mimic a skin scenario. The wound model
was established by adding HEK293 cells to a 24-well plate (1×10^5 cells/well) and incubating
in 5% CO₂ at 37°C for 24 h to form the cell monolayer. Then cell-free media was removed
and fresh growth media was added. Subsequently, 10 µL of microbial suspension (S. aureus
or P. aeruginosa, 1×10^5 CFU/mL) was added to the surface of cell monolayer, which served
as wound infection. Then, the dressing was placed on top of the bacteria exposed to cell layer,
and further incubated for 12 h. After that, 10 µL of suspension from each well was dropped
on LB agar and incubated at 37°C for 24 h to determine the viability of the bacteria. In the
meantime, the dressing was removed from the well and 1 mL of fresh growth media with 10%
(v/v) penicillin-streptomycin mixture was added into the well to kill the living bacteria, then
followed by further incubation at 37°C for 12 h. The viability of cells in the well was
determined by MTT method. The dressing treated with amoxicillin served as a positive
control, and the dressing without any treatment served as a blank control. All the
experiments were performed in triplicates.

**Bacteria infiltration and adhesion evaluation**

For bacteria infiltration evaluation, the sterile quadrate pieces of CS and CS-S (2 cm × 2
cm) were placed on an LB agar plate with the hydrophobic surface of CS-S up. Then, 200 µL
of *E. coli* (1×10⁶ CFU/mL) suspension was dropped on each surface of CS and CS-S separately and incubated at 37°C for 24 h. The bacteria growth in the plate after the removal of the dressing was observed.

The bacterial adhesion was measured according to the reported literature. Briefly, 100 μL of 1×10⁶ CFU/mL microbial suspension was dropped on both sides of CS-S or CS-Ag0.2-S quadrate pieces (2 cm × 2 cm), then the dressing was soaked in LB broth. After incubation at 37°C for 24 h, the dressing was fixed with 2.5% glutaraldehyde for 15 min. Then the dressing was thoroughly washed with PBS and deionized water, and dehydrated by absolute methanol. Both surfaces of the dressings were examined via SEM.

**Whole-blood clotting study**

The blood-clotting ability of CS-S and CS-Ag0.2-S was determined by procoagulant ratio (PR) according to the literature. Blood drawn from human ulnar vein was stored in anticoagulated BD Vacutainer (Nouhua 9NC, containing sodium citrate). Each dressing (20 mg) was added with blood and placed in centrifuge tubes. Then 10 μL of CaCl₂ solution (0.2 mol/L) was added to initiate whole-blood clotting. Blood without dressing was used as blank. The PR of the dressing was calculated, based on the equation (3).

\[
PR = \frac{T_a - T}{T_0} \times 100\% \quad (3)
\]

In the equation, *T* and *T₀* are the blood-clotting time in the presence and absence of dressing, respectively. For comparison, the blood-clotting ability of clinically available gauze and pledget were also analyzed.

**In vivo animal experiment**

*In vivo* animal experiment was approved by the animal experimental center of Wuhan
University. Twenty BALB/c mice, about 20 g of weight and 6–8 weeks of age of each, were evenly divided into four groups (each group contained five mice). On the day of wounding, the mice were anaesthetized by aether, and a partial thickness wound with a length of 7 mm was created on the back of each mouse. The prepared wounds of three groups were tightly covered with the CS-Ag0.2-S, CS-S and commercial Acasin™ nanosilver gauze (AGT Pharm Co. Ltd, Shenzhen), respectively. Wounds without covering dressing were kept as negative control. The dressing materials were changed at every two days. During the changing of dressings, the area of the wound was measured and photographs were taken. The healing ratio ($HR$) is defined by equation (4), where $S_0$ is the wound area at day of wound created and $S$ is the wound area at day of the changing of dressing.

$$HR = \frac{S_0 - S}{S_0} \times 100\%$$

(4)

For histological examination, skin tissue samples were excised at the day 14 after injury and then fixed with 10% formalin. After staining with hematoxlin-eosin (H&E) and Picro-Sirius red (PSR) independently, the samples were observed by an optical microscope (DMI3000B, Leica).

The accumulated trace silver content in blood, liver, spleen and kidney of the mice on postoperative day 14 was determined by FAAS. After carefully weighting, each organ was soaked in 5 mL of nitric acid solution (with 1:4 dilution) at 80°C till completely dissolved, then 0.1 mL of 30% $H_2O_2$ was added into. The mixtures were kept at room temperature overnight. After centrifuging at 3,000 rpm for 10 min, the supernatant liquid was collected to determine the content of trace silver.

Meanwhile, the exudate from the wound on day 2 and day 14 after surgery was collected
using sterile swabs and cultured in LB broth at 37°C for 4 h. Subsequently, 20 µL of the suspension was spread on LB agar plate and cultured at 37°C overnight, then the colonies were counted.

Results and discussion

Characterization of AgNPs/chitosan dressing

Figure 1A demonstrates the photographs of the formation of AgNPs/chitosan dressing. After being immersed into colloid AgNPs, composite AgNPs/chitosan dressing with yellow color was prepared. Figure 1B shows the SEM images of chitosan dressing and AgNPs/chitosan dressing, respectively. It is observed that both the surfaces of CS before (b1) and after (b2) AgNPs decoration displayed interconnected micro-porous structure with pore size in the range of 20~100 µm. Well-dispersed AgNPs are distributed on the surface of AgNPs/chitosan dressing, as depicted in Figure 1B (b3). TEM image of the AgNPs in the dressing illustrate the particle size is about 20 nm (Figure 1C). Figure 1D shows the EDX spectrum of the nanoparticles on the surface of AgNPs/chitosan dressing, indicative of the presence of metallic silver content in the composite dressing. The silver content in AgNPs/chitosan dressings is also quantitatively determined by FAAS. As summarized in Table 1, the silver content in the dressing shows a linear increase in the range of 0.36~2.19 mg/g with the increase of AgNPs concentration used in preparation of AgNPs/chitosan dressing.
Figure 1. (A) Photographs of CS, AgNPs solution and AgNPs/chitosan dressing. (B) SEM images of CS (b1) and CS-Ag0.2 (b2 and b3, white arrows in b3 indicate the AgNPs). (C) TEM image and (D) EDX spectrum of AgNPs of CS-Ag0.2 dressing.

Table 1. Silver content in the AgNPs/chitosan composite dressings.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AgNPs solution(mmol/L)</th>
<th>Silver content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-Ag0.1</td>
<td>0.1</td>
<td>0.36±0.08</td>
</tr>
<tr>
<td>CS-Ag0.2</td>
<td>0.2</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>CS-Ag0.5</td>
<td>0.5</td>
<td>2.19±0.24</td>
</tr>
</tbody>
</table>

Asymmetric wettability

Figure 2A shows the asymmetric wetting behavior of the AgNPs/chitosan dressing after stearic acid modification (CS-Ag0.2-S). The modified upward surface of CS-Ag0.2-S coated
with white stearic acid layer shows hydrophobic property against water droplets (b1 in Figure 2B). However, the unmodified downward surface of CS-Ag0.2-S still presents yellowish-brown color and readily adsorbs the water drops (b2 in Figure 2B). The asymmetric wettability of CS-Ag0.2-S was further assessed by water contact angle measurement. The water contact angle of the stearic acid modified surface is 135°, which exhibits highly hydrophobic property (c1 in Figure 2C). The unmodified surface shows super hydrophilic behavior and absorbs the water droplet immediately (c2 in Figure 2C). Figure 2D shows the SEM images of the surfaces of stearic acid modified chitosan and AgNPs/chitosan dressing (CS-S and CS-Ag0.2-S). Both the modified surfaces are smooth and have a little amount of pores, which is ascribed to the presence of stearic acid layer (d1 and d3 in Figure 2D). The unmodified surfaces of CS-S and CS-Ag0.2-S still present sponge-like and well interconnected micro-porous structure (d2 and d4 in Figure 2D), which illustrates that the addition of AgNPs almost has no influence on the porosity of dressings.
Figure 2. (A) Photographs of asymmetric wetting modification. (B) Hydrophobic (modified, b1) and hydrophilic (unmodified, b2) surface of CS-Ag0.2-S respectively. (C) Water contact angles of hydrophobic surface (c1) and hydrophilic surface (c2) of CS-Ag0.2-S respectively. (D) SEM images of hydrophobic and hydrophilic surface of CS-S (d1, d2) and CS-Ag0.2-S (d3, d4).

Physical and mechanical properties

Figure 3A shows the porosity of the different dressings. All the dressings show high porosity in the range of 63~68%, indicating that the asymmetric wetting modification and addition of AgNPs almost has no effect on the porosity of the chitosan dressings. The high
porosity of the dressings could benefit to absorb exudate from the wound surface, and prevent the wound infection. Furthermore, the presence of large volume of porosity is also beneficial for the transfer of nutrients and oxygen to the cells attached on the dressings. The degree of swelling of the dressings is illustrated in Figure 3B. The degree of swelling of the chitosan and AgNPs/chitosan dressings after being fully immersed in water are in the range of 1100~1400%, while the degree of swelling of the asymmetric wetting CS-S and CS-Ag-S dressings are in the range of 900~1100%, which is lower than that of the unmodified dressings. However, the moisture retention capacity of the asymmetric wettable dressing is obviously longer than that of unmodified dressing. For example, the moisture retention time of the AgNPs/chitosan dressings is about 10~12 h, whereas it is more than 14 h for the modified asymmetric wettable dressings.

Although the unmodified dressings show higher degree of swelling at early exposure time in dry air, the degree of swelling of unmodified dressing decreases faster than that of the asymmetric wettable dressings with the exposure time increases. When the exposure time was extended to 5 h, the asymmetric wettable dressings still possessed high degree of swelling, compared with the unmodified dressings. The longer moisture retention time of asymmetric wettable dressing is ascribed to its highly hydrophobic surface, which could effectively retard the evaporation of water from the inner of dressing. According to the moist wound healing theory, moist wound treatment could promote wound healing, remove the dressing painlessly without destroying fresh formed tissue and reduce scar formation.35

The tensile strength and elongation at break of the dressings were measured. As depicted in Figure 3C and 3D, chitosan dressing possesses a tensile strength of 0.48 MPa, which is
higher than that of the reported chitosan dressing. The incorporation of AgNPs into chitosan dressing results in the decrease of the tensile strength at the range of 18% to 68%, compared with chitosan dressing. However, it is still sufficient for wound care application. The asymmetric wettability modification of dressing almost have no influence on the tensile strength. Elongation value at break indicates the flexibility of the dressing. Compared with the chitosan dressing, the values of elongation at break of AgNPs/chitosan dressing increase by 18~50%, which indicates the enhanced flexibility of AgNPs/chitosan dressing. Meanwhile, the asymmetric wettability modification has a slightly influence on elongation at break values of the dressing. The flexible nature of AgNPs/chitosan dressing and asymmetric wettable AgNPs/chitosan dressing would be supportive for the application of the dressing over wound surface.

Figure 3. Physical and mechanical properties of the dressings. (A) Porosity; (B) Degree of swelling and moisture retention capacity; (C) Tensile strength; (D) Elongation at break.
Cell viability and antibacterial activity

As shown in Figure 4A, all the dressings could obviously enhance the growth of human embryonic kidney cells (HEK293) and human normal hepatocyte cells (L02). Although the cell viability treated with AgNPs/chitosan dressing decreases with the increase of silver content in dressing, it is still much higher than that of the control cell. Cell viability results indicate that both chitosan and AgNPs/chitosan dressings show noncytotoxicity toward the HEK293 cells and L02 cells. The enhancement of cell growth on the dressings is probably due to the excellent biocompatibility of chitosan and the highly porous structure of the dressings. Figure 4B shows the inhibition zones of different chitosan dressings against drug-sensitive bacteria (*S. aureus, P. aeruginosa* and *E. coli*) and drug-resistant bacteria (MRSA, DRPA and DREC). It is found that no inhibition zone is observed for CS and CS-Ag0.1 dressings toward all strains. CS-Ag0.2 and CS-Ag0.5 exhibit obvious toxicity toward drug-sensitive *S. aureus, E. coli* and drug-resistant MRSA, DREC. No distinct inhibition zone is observed for AgNPs/chitosan dressing against *P. aeruginosa* and DRPA.

To explore the practical application of AgNPs/chitosan dressing, the antibacterial activity is evaluated by a simulated wound infection model. As illustrated in Figure 4C, a monolayer culture of HEK293 cells incubated with *S. aureus* or *P. aeruginosa* suspension and a dressing were used to mimic the infection treatment scenario, where the wound surface exposed to bacteria and the dressing. Figure 4D shows the viability of bacteria and cells in the wound infection model after the treatment with different chitosan dressings. After 12 h incubation, the HEK293 cell viability cultured with *S. aureus* or *P. aeruginosa* is in the range of 15~25% in control and CS treated groups, while the bacteria viability is at 90~100%. The low cell
viability and high bacteria viability in control and CS treated groups illustrate the limited anti-infective activity of the chitosan dressing and strong wound cell damage caused by bacteria. However, the AgNPs/chitosan dressing exhibits excellent antibacterial activity against both *S. aureus* and *P. aeruginosa*, with the cell viability being around 100% and the bacteria viability being below 15%. It indicates that the AgNPs/chitosan dressing possesses the ability of inhibiting bacterial infection and promotes the proliferation of the wound cells. It is also noted that CS-Ag0.2 presents highest cell viability (120–140%) and wonderful bacteria inhibition activity among three kinds of AgNPs/chitosan dressings, which is in accordance with the results of *in vitro* antibacterial activity and cell viability. As a positive control, amoxicillin shows high antibacterial activity against Gram-positive *S. aureus*, and the bacteria viability is lower than 10%. In the meantime, the HEK293 cell viability cultured with *S. aureus* is higher than 100% due to the excellent antibacterial effect of amoxicillin. However, amoxicillin shows poor antibacterial activity against Gram-negative *P. aeruginosa*, resulting in higher bacteria viability and lower HEK293 cells viability.
Figure 4. (A) Viability of cells incubated with chitosan or AgNPs/chitosan dressing; (B) Antibacterial activity of chitosan and AgNPs/chitosan dressing; (C) A wound infection model to assess the antibacterial activity of the dressings. (D) Viabilities of bacteria and cells in the wound infection model after the treatment with the different dressings.

Bacteria infiltration and adhesion properties

Figure 5A displays the bacteria infiltration result of the chitosan and the stearic acid modified chitosan dressings. When bacteria suspension was dropped on the hydrophobic
surface of the CS-S dressing, the suspension remained a spheric droplet on the upward surface of CS-S, whereas the hydrophilic surface of CS absorbed the suspension rapidly into the dressing (a1 in Figure 5A). After 24 h incubation, the bacterial suspension still kept sphere-like droplet on the upward hydrophobic surface of CS-S (a2 in Figure 5A) and there was no bacteria growing on the agar plate when the CS-S dressing was removed (a3 in Figure 5A). However, the bacteria infiltrated from upward surface of the CS dressing into the agar plate and randomly grew in the covered area. It illustrates that the chitosan dressing after asymmetric wettability modification possesses excellent infiltration resistance towards the bacteria.

The bacterial adhesion properties of the stearic acid modified chitosan and AgNPs/chitosan dressing were characterized by SEM images. As shown in Figure 5B, when bacteria were incubated with CS-S, almost no bacterium was found on the stearic acid modified hydrophobic surface, whereas large quantities of bacteria adhered on the unmodified hydrophilic surface. It illustrates that the hydrophobic surface of CS-S possesses excellent anti-adhesion property for microorganism. Noticeably, almost no bacterium is found on the both hydrophobic and hydrophilic surfaces of CS-Ag0.2-S, indicating that the presence of silver nanoparticles in AgNPs/chitosan dressing could efficiently inhibit the bacterial growth on the hydrophilic surface of the dressing.

The good bacteria infiltration resistance and anti-adhesion properties of the asymmetric wettable AgNPs/chitosan dressing imply that when it is covered on skin wound, the upward hydrophobic surface could prevent wound infection from bacteria in air. Meanwhile, the excellent antibacterial activity of AgNPs endows the hydrophilic downward surface with
good capacity of killing the bacteria on the wound surface, resulting in the improved wound healing ability.

**Figure 5.** Photographs of bacteria infiltration (A) and SEM images of bacteria adhesion (B).

**Evaluation of in vitro blood clotting**

Blood clotting test was conducted to assess the hemostatic potential of the AgNPs/chitosan
dressing. As summarized in Table 2, the clotting time of CS-S is obviously shorter than that of gauze and pledget, which are commonly used in clinic wound care. The AgNPs/chitosan composite dressing (CS-Ag0.2-S) could further shorten the clotting time and lead to the increase of advance rate of clotting from 30% to 44%. Generally, the clotting behavior of wound dressing is related to the chemical composition, morphological feature and microstructure of the materials. As a cationic polymer, chitosan is a natural hemostat, and its hemostatic ability is attributed to the adsorption of negatively charged red blood cell, blood fibrinogen and plasma proteins. Hence, the highly porous chitosan dressing could enhance the adsorption ability. The blood-clotting capability of chitosan dressing was further enhanced after the incorporation of AgNPs because $\text{Ag}^+$ can denature the anticoagulant proteins and affect the intrinsic pathway of blood coagulation.

Table 2. Blood clotting evaluation of dressings.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Gauze</th>
<th>Pledget</th>
<th>CS-S</th>
<th>CS-Ag0.2-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time(s)</td>
<td>720±10</td>
<td>617±12</td>
<td>567±5</td>
<td>503±4</td>
<td>403±9</td>
</tr>
<tr>
<td>Advance rate of clotting</td>
<td>-</td>
<td>14%</td>
<td>21%</td>
<td>30%</td>
<td>44%</td>
</tr>
</tbody>
</table>

**In vivo evaluation of wound healing and antibacterial effect**

**In vivo** study was conducted in BALB/c mice to investigate the wound healing ability of the prepared asymmetric wettable AgNPs/chitosan dressing (Figure 6A). Figure 6B shows the photographs of an **in vivo** wound healing process after treatment by CS-S, CS-Ag0.2-S and Acasin™ dressing. Acasin™ dressing is a kind of nanosilver gauze and wildly used in the major hospitals in China. All the dressings achieve good healing after 12 days, compared with the untreated wound. More importantly, CS-Ag0.2-S shows excellent healing effect only after 4 days. Moreover, the regenerated skin after CS-Ag0.2-S and CS-S treatment is
smooth and similar to normal skin without scar formation, indicating their good healing ability for the skin tissue. The extent of wound closure at different healing time is also quantitatively evaluated, as depicted in Figure 6C. After 2 days, the wound treated with CS-Ag0.2-S achieves closure to 32%, compared with CS-S and Acasin™ dressing treated wound as well as untreated wound, which only shows 20%, 24% and 15% wound closure respectively. After 6 days, the wound closure after CS-Ag0.2-S treatment significantly enhance to 90%, which is about 20% higher than that of being treated with CS-S and Acasin™ dressing. The complete wound closure was observed on day 8 for CS-Ag0.2-S, while it was realized on day 10 for CS and Acasin™ dressing. The in vivo wound healing results elucidate that the asymmetric wettable AgNPs/chitosan dressing could accelerate wound healing, especially at the initial stage. Besides, the antibacterial activity of the dressing on mice wound is shown in Figure 6D. The bacteria grow well no matter on dressing-treated or untreated wound at day 2. However, there is obvious decrease of the number of bacterial colonies on the Acasin™ and CS-Ag0.2-S dressing treated wound, compared to the untreated and CS-treated wound at day 14, which is probably attributed to the excellent antibacterial capacity of AgNPs. It also well explains the phenomena that there is no obvious fester on the wound treated with Acasin™ and CS-Ag0.2-S, but it exists in untreated and CS-S treated wound. The silver content in the organs and blood of mice after 14 days of treatment is determined, as summarized in Table 3. There is no silver detected in blank and CS-S treated groups. For Acasin™ nanosilver dressing treated group, silver is widely distributed in the blood, liver, spleen and kidney, and the highest content of silver (1.818 µg/g) is presented in the spleen of mice. However, there is no detectable silver...
existing in the liver, spleen and kidney of CS-Ag0.2-S treated mice. The silver content is merely 0.01 µg/mL in blood, which is about a quarter of that in blood of Acasin™-treated mice. The low silver accumulation in CS-Ag0.2-S treated mice illustrates its good biosafety in a practical application.

**Figure 6.** (A) Treatment to the mice after operation; (B) Photographs of untreated and dressings-treated wound; (C) Evaluation of the wound area closure; (D) Bacteria isolated from the mice wounds.
Table 3. Silver content in the organs and blood of mice.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dressing (mg/g)</th>
<th>Blood (µg/mL)</th>
<th>Liver (µg/g)</th>
<th>Spleen (µg/g)</th>
<th>Kidney (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acasin™</td>
<td>12.18±0.83</td>
<td>0.037±0.014</td>
<td>0.032±0.002</td>
<td>1.818±0.032</td>
<td>0.147±0.040</td>
</tr>
<tr>
<td>CS-S</td>
<td>0.82±0.11</td>
<td>0.010±0.001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Histological observations

Figure 7 shows the histological observations of wound tissue on day 14 after the operation. H&E staining of the untreated and Acasin™-treated wound show obvious fibroblasts and endothelial cells, which do not develop to fibrocytes and blood vessels (a2 in Figure 7A). The reconstructive venules and arterioles are clearly visible in CS-S and CS-Ag0.2-S treated wound (a3 in Figure 7A). It indicates that the asymmetric wettable chitosan dressing could improve the wound tissue repair and angiogenesis capabilities. As we known, when skin wound occurs, the neoformative fibroblast with little collagen will replace the necrotic tissue. If the containing collagen increases, fibroblasts would develop into fibrocytes in microanatomy and accordingly the wound executes healing process in macrocosmic. Hence, it is important to determine the collagen deposition while wound healing. The extent of collagen deposition was estimated by histomorphometry on sections stained with PSR (Figure 7B, where the collagen was stained into red). After 14 days of healing, the collagen deposition at the CS-Ag0.2-S and Acasin™ treated wound is much denser than that of the untreated and CS-S treated wound, and the CS-Ag0.2-S treated wound displays the most density of collagen. The white colored empty spaces in Figure 7B (b2) indicate the lack of collagen. Compared with CS-S and CS-Ag0.2-S treated groups, the untreated and...
Acsin™-treated wound tissue show sparse and untidy collagen fibres and obviously lack of collagen. The results reveal that CS-Ag0.2-S treated wound site showed excellent tissue repair, outstanding angiogenesis capabilities and enhanced deposition of collagen fibers in ordered arrangement.

**Figure 7.** Micrographs of wound tissues stained with H&E (A) and PSR (B). Hereinto, a1 and b1 are the low-magnification images (100x), a2, a3 and b2 are the high-magnification images (400x). In a2, the black and cyan arrows indicate the bands of the endothelial cells and the fibroblast respectively. In a3, red and blue arrows indicate the reconstructed arteriole
and venule, respectively. In b1, black line segments indicate the thickness of collagen deposition.

Conclusions

In summary, a novel AgNPs/chitosan composite dressing with asymmetric wettability surfaces was successfully prepared by the simple two-step method. The asymmetrically wettable AgNPs/chitosan dressing shows high porosity, enhanced moisture retention time and blood-clotting capability, which is helpful for accelerating wound healing. Via stearic acid modification, the upward surface of the dressing is hydrophobic while the opposite surface is hydrophilic. The hydrophobic surface displays bacterial infiltration resistance and anti-adhesion properties, and the hydrophilic surface could extensively absorb the exudates of wound and efficiently inhibit the growth of bacteria. More importantly, in vitro and in vivo antibacterial studies illustrate the excellent antibacterial activities against drug-sensitive and drug-resistant pathogenic bacteria. In vivo wound healing evaluation in mice shows that the asymmetrically wettable AgNPs/chitosan dressing possesses faster wound healing compared with chitosan and clinically used Acasin™ nanosilver dressing, and it also exhibits excellent re-epithelialization and dense collagen deposition properties in histological observation. The lowest silver accumulation was also observed in the asymmetrically wettable AgNPs/chitosan dressing. Moreover, in vitro cytocompatibility study reveals that the dressing could enhance cell growth. The results demonstrate that the prepared asymmetrically wettable AgNPs/chitosan dressing has potential application in burn, chronic and diabetic wound infections.
Acknowledgments

This work was supported by the Key Cooperation Program of Wuhan Science and Technology Bureau (201260523183), the National Natural Science Foundation of China (21371139, 21201135) and the High-tech Industry Technology Innovation Team Training Program of Wuhan Science and Technology Bureau (2014070504020243).

References


TOC