Research Article

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In situ photo-crosslinking hydrogel with rapid healing, antibacterial, and hemostatic activities

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Abstract: Uncontrollable bleeding is still the main cause of post-traumatic deaths due to the blood loss. Moreover, infectious complication of wound is also still a challenging problem for wound healing. Nevertheless, the currently available hemostasis drugs or materials cannot stanch bleeding well due to single function, slow in effectiveness, adhere to wounds easily, poor gas permeability, etc. Therefore, it is of a great significance to utilize a biomedical hemostatic material that can stop bleeding quickly, preventing from bacterial infections, and with good biocompatibility properties. Herein chitosan (CS) was modified with gallic acid (GA) and thrombin (TB) to prepare an antibacterial hemostatic composite dressing. The CS-based composite hydrogel dressing was obtained by acylation modification, ultraviolet curing

crosslinking method and physical mixing. The *in vitro* results showed that our prepared CS-based composite hydrogel has obvious burst release and good degradation property. Moreover, the *in vivo* results showed that it has a strong antibacterial property that is much better than single CS, and it can stop bleeding in 1 min which can promote wound healing. Therefore, the findings of this study is expected to contribute to the future designing of biomedical hemostatic materials with improved properties.

Keywords: chitosan, blend hydrogel, antibacterial, rapid hemostatic, wound dressing

1 Introduction

Advanced local hemostatic materials have been exploited to control bleeding (1), in order to decrease the mortality rate by blood loss and prevent from further complications (2–4). Currently, bandages, fibrin glues, and hemostatic dressings in the form of gels or sponges are the main hemostatic materials. Hemostatic materials achieve the effect of hemostasis by promoting the hemostasis mechanism (5). Nowadays, absorbable medical hemostatic materials include fibrin glue, oxidized cellulose, collagen, chitosan (CS), etc.

Chitosan is a natural broad-spectrum antibacterial agent, and it has obvious inhibitory effects against the growth and reproduction of dozens of bacteria and fungi. Chitosan contains a large number of amino groups and exhibits polycationic properties in acidic conditions. It can interact with various negatively charged microorganisms, change the surface structure of microorganisms and then cause the cells to die through the leakage of intracellular substances (6). In order to improve the antibacterial activity of CS, the positive charge strength of CS molecules can be enhanced by introducing more positively charged groups (7). Mohandas et al. (8) prepared a transparent and flexible CS-based film containing antibacterial drugs by using a suspension of CS floc and antibacterial agents (tetracycline hydrochloride and silver sulfadiazine)

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as raw materials, and glycerol as a plasticizer material; the CS film showed a strong antibacterial effect on *Escherichia coli* and *Staphylococcus aureus*. Chitosan has good hemostatic activity, it can adsorb negatively charged platelets and red blood cells through electrostatic force, and cause aggregation reaction on the wound, block blood vessels to hemostasis. In addition, the amino groups in CS can adsorb fibrinogen and inhibit the activity of fibrinolytic enzyme, which is beneficial to the formation of thrombus; but pure CS hemostatic effect is still limited. At present, the main method to improve the hemostatic properties of CS is to combine it with some coagulation factors and procoagulant substances. Ma et al. (9) invented a thrombin (TB)-CS self-assembled nanoparticle hemostatic preparation, which can be prepared by emulsification crosslinking method. Their report showed that TB-CS nanoparticles not only maintain the stability of TB but also improve the hemostatic properties of CS.

Chitosan can provide a non-protein matrix for the growth of three-dimensional tissues and stimulate the proliferation of fibroblasts and the reconstruction of tissue structure, which are beneficial to promote wound healing. Chitosan can exist in the form of fibers, hydrogels, membranes, and sponges as a biomaterial for wound dressings (10,11). Hydrogel is a unit composite polymer prepared from natural or synthetic polymer chains through crosslinking methods. It can adapt well to wounds of any shape and penetrates deep into the wound (12). Therefore, it can stop bleeding faster and promote wound healing.

In our study, gallic acid (GA) and TB were first used to improve the antibacterial and hemostatic properties of CS by Schiff's base reaction and physical adsorption, respectively. Then, CS was modified by acylation with methacrylic anhydride (MA), and the photosensitive group C=C was introduced. Finally, after the above three products and the photoinitiator are mixed in proportion, the monomers are crosslinked under ultraviolet light to obtain a CS-based composite hydrogel dressing with antibacterial and hemostatic properties. The hydrogel dressing can be beneficial to the early hemostasis and later to enhance antibacterial activity and promote the fast healing of the wound.

2 Materials and methods

2.1 Materials

Chitosan, tranexamic acid, aminocaproic acid, fibrinogen, and TB were purchased from Bomei Biological Technology

Co., Ltd. (Hefei, China). Gallic acid and glutaraldehyde were purchased from Fuchen Chemical Reagent Factory (Tianjin, China). Yunnan Baiyao was purchased from Yunnan Baiyao Group Co., Ltd. (Yunnan, China). Both HepG2 and human fetal hepatocyte (LO2) cell lines were purchased from the China Center for Type Culture Collection, College of Life Sciences, Wuhan University. New Zealand rabbits and Kunming mice were purchased from Hubei Animal Experimental Center (Wuhan, China). Other chemicals were of analytical grade obtained by commercial means and used without purification.

2.2 Sample preparation

2.2.1 Preparation of CS-GA derivatives

Chitosan loaded with GA (CS-GA) derivatives were obtained by dissolving 3.0 g GA and 1.0 g CS powder in 0.5 mol/mL tris-HCl (pH = 8) and buffer stirring for 12 h. After precipitation in ethanol, the product was washed with distilled water, centrifuged, and lyophilized. (FTIR characteristic peaks: -NH_2 : 1,320/cm, -OH : 3,427/cm, and -C=N : 1,616/cm).

2.2.2 Preparation of CS hemostatic microspheres loaded with TB (CS-TB)

Chitosan microsphere was prepared by emulsion crosslinking(13). Briefly, 2% w/w of CS acetic acid solution, liquid paraffin, and Tween-80 were charged into a flask and stirred at a temperature of 40°C in a water bath, then 5% v/v of glutaraldehyde was added to crosslink for 5 h. Thereafter, CS microspheres were washed with petroleum ether and isopropanol in sequence and lyophilized.

To obtain CS-TB, blank CS microspheres were added to 10 U/mL of TB in 0.9% of NaCl, stirred at a temperature of 37°C for 24 h, centrifuged, and lyophilized.

2.2.3 Preparation of CS hydrogel dressing

The CS composite dressing was modified by using MA to make its adhesiveness and strength better. A certain mass ratio (10.0, 20.0, 30.0, 40.0, and 50.0 wt%) of MA was added to the 20 mL of a 2.0% w/v CS acetic acid solution, and the mixture was stirred for 12 h in a water bath at a temperature of 60°C. After the reaction was complete, the solution was neutralized with 10 wt% of NaHCO_3 solution and then placed in a dialysis bag with a molecular weight

cut of 3,500 Da, which was then placed in 1,000 mL of deionized water, and the deionized water was replaced every 12 h. After 3 days, a pre-polymerized solution of chitosan-methacrylamide (CS-MA) was prepared.

The dialyzed pre-polymerized CS solution was concentrated by evaporation to about 20 mL, and 0.2% w/v of a photoinitiator Irgacure 2959 was added to the solution. The mixture was uniformly stirred by magnetic stirring and poured into a polytetrafluoroethylene plate. The CS hydrogel was prepared by radiating it for 30–90 s with UV curing equipment (14) (UV-10-201, Kunshan, China).

The CS-MA mixture with Irgacure 2959 was added to the antibacterial modified CS-GA and CS-TB and then configured them as mass ratio: CS-MA:CS-GA:CS-TB = 3:1:1. Finally, a composite of CS hydrogel dressing was prepared under ultraviolet light (Scheme 1) (15).

2.3 Sample characterization

The infrared spectrum of CS-GA derivatives was obtained from a Fourier transform infrared spectrometer (Spectrum-2000, PerkinElmer, America).

In order to evaluate the interfacial interaction between blood cells and CS hemostatic microspheres, 100 μ L of rabbit whole blood containing anticoagulant sodium citrate were added to blank CS microspheres and CS hemostatic microspheres and then the samples were incubated for 10 min at a temperature of 37°C. The samples were washed with a phosphate buffer saline (PBS) of pH 7.4 to remove physically adhering blood cells. They were further fixed with 2.5% of glutaraldehyde for 3 h, dehydrated with ethanol, freeze-dried, and observed by scanning electron microscopy (SEM).

Scheme 1: The synthetic route of composite chitosan hydrogel (blue line represents CS, green line represents GA, orange line represents MA, and red ball represents TB; the mass ratio is CS-MA:CS-GA:CS-TB = 3:1:1).

2.4 In vitro release, toxicity, and activity

The cumulative release rate (CR%) is calculated using Eq. 1:

$$\text{CR}\% = \frac{M_{\text{GA}}}{M_0} \times 100\% \quad (1)$$

where M_{GA} is the total mass of GA raw materials fed by grafting reaction and M_0 is the total mass of CS-GA.

The antibacterial properties of CS-GA derivatives were studied by the inhibition zone method (16).

About 7×0.25 g of CS hemostatic microspheres were weighed to be tested (W_1) in each group and 50 mL of human body simulating fluid (SBF) was added. The sample was incubated at a temperature of 37°C for a set time. The sample was filtered, washed, dried, and the final mass W_2 of the sample was recorded (17). The sample weight loss rate $\omega\%$ was calculated according to Eq. 2:

$$\omega\% = \frac{W_1 - W_2}{W_1} \times 100\% \quad (2)$$

The water absorption capacity is used to measure the swelling capacity of the hydrogel, thereby reflecting the hemostatic ability of the hydrogel. Suitable dry hydrogel (m_d) was weighed and placed in room temperature with PBS buffer (pH = 7.4), it was then taken out after 120 min and its wet weight (m_w) was weighed. The swelling ratio (SR%) of the hydrogel was calculated according to Eq. 3:

$$\text{SR}\% = \frac{m_w - m_d}{m_d} \times 100\% \quad (3)$$

The piezoresistive test is used to characterize the compression resistance property of the hydrogel. CS-MA pre-polymerized solutions with different MA mass ratios were selected and CS hydrogels of the same specifications (a cylinder with a radius of 10 mm and a height of 15 mm) with a crosslinked curing mold were prepared. The piezoresistive test was performed using the universal mechanical tester (CMT-6104, Shanghai, China), and the compression speed was 10 mm/min until the gel broke. The stress strength (SS) of the gel is calculated according to Eq. 4:

$$\text{SS} = \frac{F}{S} \quad (4)$$

where F is the compressive force on the cross-section of the hydrogels, S is the crosssectional area of the sample.

HEPG2 and LO2 cell lines were separately cultured in DMEM medium supplemented with 10% (v/v) FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C, 5% of CO₂, and 95% of relative humidity.

To determine the biocompatibility of composite CS hydrogel, LO2 cell line was used for *in vitro* experiment. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Briefly, LO2 cells were seeded in 96-well plates at the density of 5×10^3 cells/well. After 12 h of incubation, the corresponding wells were added with 100 µL of different concentrations of CS composite hydrogel in DMEM media. After 24 and 48 h, 20 µL of MTT (5 mg/mL) solution were added into each well. After incubating for 4 h at 37°C, we aspirated all fluids from the plate and then added 200 µL DMSO into each hole and placed in a shaker for 15 min in dark. Thereafter, the absorbance of each well was measured at 490 nm with a Microplate reader (Infinite F200 Pro, TECAN, Switzerland). The cell survival rate can be calculated according to the absorbance. Similar experimental setup was used for HEPG2 cells. All experiments were carried out in quintuplet.

2.5 Wound healing from animal studies

0.05 g of sample and 0.1 mL of 0.01 mol/L PBS (pH = 7.4) were mixed to a suspension at a temperature of 37°C for 2 min. Next 1 mL fresh whole blood of New Zealand rabbits was added to the above mixed liquids. The clotting time was observed and recorded. There is no hemostatic drug application for control groups.

Thirty Kunming mice were randomly divided into 3 groups (10 mice in each group with equal number of males and females and weight of 30 ± 2 g) and each mouse was anesthetized with 0.150 mL of sodium pentobarbital (1%, 50 mg/kg). Group A was treated with Yunnan Baiyao, group B was treated with the CS-TB, and group C was treated with the composite CS hydrogel. The Kunming mice' tails were cut off 3 cm from its tip. After the blood flowed out, the dried Yunnan Baiyao, CS-TB, and composite CS gel were applied to the wounded section of the mice' tails, respectively, and the complete hemostasis time was recorded.

Another similar three groups of Kunming mice were fixed on the dissection table (ZH-BXT, China), the right femoral villi were cut, anesthetized with sodium pentobarbital, and disinfected with medical alcohol. A one-shaped wound with a length of about 1 cm was cut on the surface of the skin with a knife. The Yunnan Baiyao, sugar-TB microspheres, and composite CS hydrogel were sprinkled on the wound. The scar length of the wound was measured seven days later, and the wound healing was observed and recorded.

All animal experiments were carried out complying with the "Regulations on the Administration of Experimental Animals" (second edition, revised in 2013) submitted by the National Science and Technology Commission.

Figure 1: The cumulative release profile of GA from CS-GA in a PBS medium at pH 7.4 for 72 h (37°C, immersion in SBF, mean values $\pm$ SD [$n = 3$]).

2.6 Statistical analysis

All data are presented as the mean values $\pm$ standard deviation (SD). Statistical analysis of all data was performed using 1-way ANOVA (StatView) whereas p values < 0.05 were considered to be statistically significant ($n = 3$).

3 Results and discussion

The cumulative release profile of GA from CS-GA in a PBS medium of pH 7.4 for 72 h is shown in Figure 1. The release rate of GA reached 13.3% within 0.5 h after being immersed into the release medium, showing a significant

burst phenomenon. In the first 4 h after entering the medium, CS-GA released rapidly, and the cumulative release rate reached 45.0%. After that, the release rate of CS-GA decreased significantly, and the cumulative release rate was 54.6% at 8 h, showing a well sustained release capacity. At the end of the experiment, the cumulative release rate of CS-GA was 67.0% at 72 h, and about 33% of GA had not yet been released.

As it can be seen from Figure 2, there was an obvious inhibition zone around the small round filter paper soaking CS and CS-GA. The inhibition zone width of CS was about 1 mm and the inhibition zone width of CS-GA was about 3 mm. It indicates that CS itself has a certain inhibitory effect against *E. coli*, while the CS-GA derivative has better antibacterial effect than CS. The possible antibacterial mechanism of CS is that it contains a large number of amino groups. Under acidic conditions, it exhibits polycationic properties and interacts with the microorganisms which have many negatively charges to change the surface structure of them. The leakage of intracellular substances can kill the microorganisms. However, the CS of the simple component has a weak antibacterial effect. Gallic acid is a natural polyphenolic antioxidant with a certain antibacterial effect. The antibacterial property of GA was enhanced after grafting to CS molecules (18). Because the phenolic hydroxyl groups of GA can form benzoquinone and then react with the amino groups in CS to form Schiff base imines ($\text{CH}=\text{N}$) bridging bond, the bond has strong anti-bacterial activity (19). Moreover, both CS and GA have good biocompatibility. Therefore, the CS molecule can be chemically modified to improve its antibacterial property, expanding the application range of CS.

Figure 3 shows that the blood of Yunnan Baiyao group (A) and blank CS microsphere group (B) were not

Figure 2: Antimicrobial effect of CS (a) and CS-GA (b) against *E. coli*.

Figure 3: Clotting time *in vitro*: (A) Yunnan Baiyao group, (B) blank chitosan microsphere group, and (C) the chitosan hemostatic microsphere group.

coagulated, but the hemostatic microsphere group (C) was coagulated within 30 s. The hemostatic effect of TB is better than that of Yunnan Baiyao. The hemostasis effect of one component CS is not obvious. The loaded TB of CS hemostatic microspheres can rapidly convert soluble fibrinogen in plasma into insoluble fibrin clots to make blood coagulation efficient and stable (20). The introduction of high-efficiency hemostatic TB can optimize the hemostatic performance of CS and expand its application range. And CS can be used as a protein immobilization carrier to improve the stability and high activity of TB.

It can be seen from Figure 4 that the quality of the microspheres gradually decreased with the extension of time. In the first two weeks, the mass of the microspheres

Figure 4: Degradation rate of hemostatic chitosan microspheres *in vitro* (37°C, immersion in SBF, mean values $\pm$ SD [$n = 3$]).

was not reduced to a large extent, and the weight loss rate was about 15.0%. The microspheres degradation rate was slower. But after 2 weeks, the degradation rate increased significantly. By the fourth week, 45.5% of the CS hemostatic microspheres were degraded. At the same time, the degradation rate of the microsphere control group without adding lysozyme was about 4.6% within 4 weeks, indicating that the degradation of CS hemostatic microspheres had little relationship with HAC-NaAc buffer (pH = 6.0). The results show that the CS microspheres have good degradation properties in the SBF.

Comparing the blood cell aggregation shown in Figure 5a and b, the blood cell aggregation on the surface of the modified CS-TB was better than that on the surface

Figure 5: SEM image of hemostatic materials in contact with blood. (a) Chitosan microspheres (2,700 $\times$, scale bar = 10 μ m, and WD 7.9 mm). (b) Hemostatic chitosan microspheres (1,800 $\times$, scale bar = 10 μ m, and WD 8.0 mm).

Figure 6: Content of MA effect on gel strength (cylinder with $d = 20$ mm and $h = 15$ mm; compression speed = 10 mm/min).

of blank CS microspheres whose blood cells were less and more dispersed. Therefore, the CS hemostatic microsphere can not only adsorb blood cells physically, but also promote blood cell aggregation (21).

It was reported that MA can be used to modify gelatin to obtain stable and highly biological gelatin fibers (22). Therefore, we tried to use MA for CS modification and found that when 20% of MA was added, the gel strength of CS could reach the highest value of 16.73 MPa, which showed that CS hydrogel had mechanical strength and adhesive force to a certain extent (Figure 6). Moreover, the swelling ratio of the hydrogel was 81% after absorbing PBS buffer for 120 min. Therefore, the hydrogel is also expected to be used as a biological tissue adhesive (23) for wound bonding and wound healing.

The survival rates of the composite CS hydrogel on HepG2 and LO2 cells at 24 and 48 h are shown in Figure 7. We found that as the concentration of the composite CS hydrogel increased, the survival rate of HepG2 and LO2 cells gradually decreased. But in the concentration range of 0.01–1% (v/v), the survival rates of the two cell types were higher than 90% at 24 and 48 h, therefore, its cytotoxicity could be considered as low. In particular, when the concentration of the composite CS hydrogel is 0.01%, the survival rates of the two cell types were higher than 95% at 24 and 48 h. We could basically consider that the composite CS hydrogel was not toxic to the cells.

Yunnan Baiyao is a well-known component of traditional Chinese medicine, including bitter ginger, borneol, stasis grass, and other herbs from Southern China (24) and it has been used as a hemostatic drug to counteract external or internal bleeding (25). Besides hemostatic

Figure 7: The cell survival rate of composite chitosan hydrogel on HepG2 and LO2 cells for 24 and 48 h.

performance, it was proved to have anti-inflammatory activity (26). Therefore, Yunnan Baiyao can be an ideal control group. The hemostasis test of mouse tail bleeding model and the hemostasis time is shown in Figure 8. The average hemostasis time of Yunnan Baiyao was about 66.67 s, and the average hemostasis time of CS hemostatic microspheres was about 33.33 s. The average hemostasis time of the gel was 58.33 s. The shorter the hemostasis time, the better the hemostasis performance. Therefore, the hemostatic effect of CS hemostatic microspheres is significantly better than that of Yunnan Baiyao group, and the hemostatic performance of CS composite gel is slightly better than Yunnan Baiyao group.

In Figure 9, the hemostatic materials used in the experiments can all adhere to the wound well, and the hair around the wound of the mice grew again, the length of the wound shortened obviously, and the healing condition was better. The average wound length of the mice in the hemostatic microsphere treated group was not much different from that of the Yunnan Baiyao treated group, while the average wound length of the mice in the composite gel group was slightly shorter than that of the hemostatic microsphere and Yunnan Baiyao group. The results confirm that the overall healing effect of the mice in the composite gel group is the best.

4 Conclusion

In summary, we have prepared CS composite hemostatic hydrogel dressing with antibacterial and hemostatic

Figure 8: Hemostasis model in mouse tails. (a) Representative macroscopic appearance of mouse tail samples. Group A: Yunnan Baiyao, Group B: CS-TB microsphere, Group C: CS composite hydrogel; (b) quantitative measurement of hemostatic time. *, **, and Δ indicate a significant difference, $p < 0.05$.

effects. Gallic acid was grafted onto CS by chemical modification to modify the antibacterial property of CS. Both CS and GA-CS derivatives have antibacterial effects; however, the modified CS derivative has better antibacterial properties. To modify the hemostatic properties of CS, we used the CS microspheres prepared by the emulsion crosslinking method and made it adsorb the TB drug physically. The hemostatic property of CS was evaluated by *in vitro* clotting time and the mouse tail docking experiment. The results show that TB/CS hemostatic microspheres can be biodegraded and have certain adsorption properties and significant hemostasis effect, and its hemostasis time is significantly shorter than that

of Yunnan Baiyao. The CS-based composite hydrogel dressing was obtained by acylation modification, ultra-violet curing crosslinking method, and physical mixing. The hemostatic properties of the hydrogel dressing and the ability to promote wound healing were investigated by mouse tail docking experiment and mouse wound healing experiment. The experimental results show that the hemostatic effect of CS-based composite hydrogel dressing is slightly better than that of Yunnan Baiyao, and it has a certain promoting effect on wound healing and better biocompatibility. The CS-based composite hydrogel dressing is expected to be an excellent biomedical hemostatic material.

Figure 9: Observation of mouse skin wound. (a) Representative macroscopic appearance of wound closure before treatment, 0 days, and 7 days after treatment of skin wound of Group A: Yunnan Baiyao, Group B: CS-TB microspheres, and Group C: CS composite hydrogel; (b) quantitative measurement of wound length. *, **, and Δ indicate a significant difference, $p < 0.05$.

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