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An injectable serotonin-chondroitin sulfate hydrogel for bio-inspired hemostatic adhesives with high wound healing capability

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The biocompatibility, hemostatic performance and wound healing capability are key limitations for currently available hemostatic agents. To overcome these problems, a hydrogel inspired by a platelet coagulation mediator is developed in this work as a new class of hemostatic adhesive with improved performance and wound healing capability. The hydrogel is prepared using highly biocompatible serotonin and chondroitin sulfate (CS), both of which are natural components of body. The structure, physical and biological and hemostatic properties of the hydrogel are characterized in detail. It is demonstrated that serotonin acts as a crosslinker to form adhesive hydrogels and a blood clotting mediator for rapid hemostasis. Chondroitin sulfate regulates cell behaviors and fates to facilitate wound healing. The serotonin-conjugated chondroitin sulfate hydrogel exhibits improved hemostatic capability in vivo and rapid wound healing after hemostasis. In addition, the wound healing capability of the hydrogel are further improved with aloe vera powder, confirming the versatility of the hydrogel system. Therefore, the chondroitin sulfate-serotonin hydrogels exhibit the potential for effective hemostasis and wound healing.

Introduction

In war and clinical trauma, massive blood loss caused by incompressible injury may lead to tissue disease and even death. Many hemostatic agents have been developed to manage uncontrolled bleeding. The commonly used hemostatic materials on the market include tourniquets, dressings, coagulation powders, etc. Among them, hydrogels received wide attention because of their good viscoelasticity and functionality. Particularly, in situ injectable hydrogels can closely adhere the material to irregular wounds through minimally invasive injection techniques, and to perform well in humid and highly dynamic environments. However, the biocompatibility, hemostatic performance and wound healing capability are key limitations for currently available injectable hemostatic hydrogels. The delay of wound healing due to the irregular loss of the wound also leads to an increase in mortality. For example, the cyanoacrylate-based tissue adhesive has the risk of inhibiting collagen remodeling and causing inflammation in the wound. Thus, it is extremely desired to develop injectable and biocompatible hydrogels with high capability for hemostasis and wound healing.

Recently, various injectable hemostatic hydrogels are developed based on synthetic polymer (polycyanocrylate, polyethylene glycol, polyurethane and polyester) and naturally derived polysaccharide (chitosan, cellulose, hyaluronic acid, algicin acid and chondroitin, etc.). Compared with synthetic polymer, natural polysaccharides possess excellent biocompatibility. Chondroitin sulfate (CS) is a sulfated glycosaminoglycan that is abundant in the extracellular matrix of human tissues. It has been reported that chondroitin sulfate can regulate cell functions, such as cell migration and receptor binding. And the CS-based hydrogels possess high wound healing ability and biological activity at the cellular level. From the point of view of effective hemostasis, serotonin is an ideal candidate because serotonin is a natural component in the human body and can simulate the natural coagulation pathway through platelet activation. During coagulation, serotonin is released from the activated platelets and induces the secretion of platelet granules from activated platelets inversely. The platelet granules contain various hemostatic factors including fibrinogen, with Willebrand factor, platelet factor 4 and platelet factor V, which facilitate fast hemostasis. In addition, serotonin is reported to decrease apoptosis and increase the cell survival significantly in human fibroblasts and neonatal keratinocytes, and the endogenous serotonin pathway contributes to regulating the skin wound healing process. Therefore, serotonin-chondroitin sulfate injectable hydrogels are expected to possess high biocompatibility, hemostatic performance and wound healing capability. To our best knowledge, serotonin-chondroitin sulfate injectable hydrogels have not been fabricated.

In this study, a new injectable in-situ hydrogel based on serotonin and chondroitin sulfate is developed. To guarantee the proper cross-linking, degradability and non-toxicity, the enzyme-catalyzed cross-linking methods is used. Horseradish peroxidase (HRP) is an efficient and specific biocatalyst in horseradish that induce cross-linking to form a hydrogel in combination with H2O2. This enzymatic cross-linking can be carried out under mild conditions, and endows the hydrogel with injectability, adjustable gel kinetics and controlled mechanical properties. The functional evaluation of chondroitin sulfate-serotonin (CSS) hydrogel system is carried out by establishing a mouse liver hemorrhage model and a mouse back injury model. In order to confirm the versatility of serotonin-
modified chondroitin sulfate and promote wound healing remarkably, aloe vera powder (AVP) is added to the hydrogel system. Aloe powder is a kind of curdlan obtained from freeze-dried aloe gel, which can stabilize the collagen on the wound surface and resist inflammation. The functional evaluation of CSS hydrogel system is carried out by establishing a mouse liver hemorrhage model and a mouse back injury model. CSS hydrogels doped with aloe powder are demonstrated to possess excellent hemostatic and wound healing capacity, which provides a new strategy for design and develop hemostatic materials.

Results and discussion

The preparation process of CSS polymer is shown in Figure 1A. The serotonin-modified chondroitin sulfate polymer is obtained by activating the carboxyl group on CS with the catalysis of EDC and NHS and subsequent amination reaction of the amino group on serotonin. The chemical structures of CS, serotonin and CSS are characterized by $^1$H NMR (Figure 1B). In contrast to CS and serotonin, a specific peak of CSS between 6.8 and 7.5 ppm (indole groups in aromatic protons) confirms the successful combination of serotonin and the main chain of the CS. Compare the $^1$H NMR spectrum of CSS to that of CS, two new signal peaks appear at 2.4 ppm and 2.9 ppm , which is attributed to methylene and methyl peaks of CS-NHS. In addition, the enhanced peaks between 3.2 and 3.4 ppm are due to the methylene on serotonin. The peaks between 3.5 and 4.6 ppm in the CSS spectrum represent methyne, methylene, methyl, and hydroxyl groups on the main chain of chondroitin sulfate. Meanwhile, in the UV-visible absorption spectrum, a new absorption peak appears in the synthesized CSS polymer at a wavelength of 280 nm, demonstrating the successful introduction of indole groups (Figure 1C). The results show the grafting rate of serotonin increases significantly with the enhancement of serotonin feed. The grafting reaction is further analyzed with FTIR spectra (Supporting Information, Figure S1A). Compared with the CS spectrum, the new peaks at ~1027 cm$^{-1}$ and ~1624 cm$^{-1}$ in the CSS spectrum confirm the successful engraftment of indole groups on CS.

The substitution degree of CSS with different feed molar ratios is determined by $^1$H NMR spectroscopy (Supporting Information, Figure S1B). By comparing the signal integral area of the aromatic protons peak in serotonin with that of the indicated proton in the CS main chain methyl (~2.0 ppm), the DS values of indole groups are 8.0, 13.0 and 16.0%. The three products with DS of 8.0, 13.0 and 16.0% are defined as CSS 1, CSS 2 and CSS 3 (Table 1). The higher the degree of substitution, the higher the content of serotonin bound on the CS main chain. Serotonin not only plays the role of being oxidized and cross-linked in the enzyme-catalyzed cross-linked hydrogel, but also acts with the nucleophile in the biomolecules to adhere to the tissue surface. Therefore, the product with the highest degree of substitution (16.0%) is selected for subsequent experiments.

CSS hydrogels are prepared by in-situ crosslinking through HRP-mediated chemical reactions in the presence of H$_2$O$_2$ (Figure 2A). The whole catalytic cycle is initiated by the interaction between H$_2$O$_2$ and the resting ferric state of HRP [Fe(III)], and then two single-electron reduction steps are performed to obtain two equivalents indole radicals. The generated indole radical forms intermolecular covalent linkages through the carbon-carbon bonds between the ortho-carbons of the aromatic ring or through the carbon-oxygen bonds between the ortho-carbons and phenolic oxygen, thus preparing CSS hydrogels. A simple and efficient method is used to quickly form CSS hydrogel by mixing CSS/H$_2$O$_2$ and CSS/HRP solution. Through the oxidation of CSS polymer solution with HRP/H$_2$O$_2$ enzyme within 1 min, the color of the pre-gel solution immediately changes from colorless to light yellow, and finally to brown, indicating the sol-gel transition process due to the bonding between 5-hydroxyindole oxidation products in serotonin (Figure 2B). In a complete catalytic cycle, fixing the concentration of HRP or H$_2$O$_2$ will generate a phenol free radical. The polymer network consists of phenolic compounds passing through the normal carbon on the aromatic ring and the phenolic oxygen. The cross-linking between carbon-carbon bonds or carbon-oxygen bonds between positive carbon and phenolic oxygen forms the polymer network. Due to the substrate specificity and efficiency, mild reaction conditions and good cell compatibility, this cross-linking strategy is safe and suitable for biomedical applications.

In order to analyze the gel kinetics of CSS hydrogels, the gelation time of CSS polymer solutions with different concentrations of HRP and H$_2$O$_2$ is measured. Unless otherwise specified, the final optimized CSS polymer concentration for preparing CSS hydrogel is fixed at 5wt%. The gel time of the designed injectable hydrogel should meet the clinical needs within an appropriate range. When the concentration of H$_2$O$_2$ is lower than 2mM, the hydrogel cannot be formed. When the concentration of H$_2$O$_2$ is higher than 8mM, the gel time greatly increased, which does not meet our standard for preparing hydrogels. Therefore, the concentration of H$_2$O$_2$ is selected between 2mM and 8mM for the test. The concentration of HRP is selected in the range of 6 U/ml to 24 U/ml. As shown in Figure 2C, all CSS hydrogels are formed within 1 min. When the concentration of H$_2$O$_2$ is fixed, the gelation time decreases significantly with the increase of HRP concentration because the high content of enzyme (HRP) can trigger more indole free radicals for effectively crosslinking the polymer. When the concentration of HRP is fixed, the gel time decreases with the increasing H$_2$O$_2$ level from 2.0 mM to 4.0 mM, but when the concentration of H$_2$O$_2$ continues to increase, the gel time increases instead. The similar phenomenon has been observed with the previous research, which

| No. | Samples | Theoretical feeding molar ratio | DS (%) |
|-----|---------|--------------------------------|--------|
| 1   | CSS 1   | 1:1:1                          | 8      |
| 2   | CSS 2   | 1:3:3                          | 13     |
| 3   | CSS 3   | 1:5:5                          | 16     |

Table 1 Synthesis of CSS with different degrees of substitution (DS)
attributed to reduction of the HRP activity with excessive H$_2$O$_2$. The initial increase in H$_2$O$_2$ concentration can promote the cross-linking process, but the gel time will continue to increase under the influence of excessive H$_2$O$_2$ level. Compared with the wound healing CS-based hydrogels with good biocompatibility and hemostatic capacity, the advantage of CSS hydrogel in this work is that the gelling time is controllable and can be adapted in about 30s, which is necessary to the treatment of emergency bleeding.

The gel kinetics of CSS hydrogels are further analyzed with rheological tests (Supporting Information, Figure S2). The frequency sweep test determines that the linear elastic region of hydrogel is in the range of 0.1%-10% strain. At this region, the storage modulus of the hydrogel is always higher than the loss modulus, confirming the stability of obtained hydrogels (Supporting Information, Figure S2A). The time for intersection of storage modulus and loss modulus is usually considered as the gel point, representing the transition of viscous fluid from solution to gel. After that, the storage modulus increase rapidly with time and is always higher than the loss modulus, suggesting that the elastic behavior in the hydrogel system is dominant. As time goes by, the two moduli of the hydrogel finally reach a plateau, which indicates that the gelation is complete and a stable hydrogel is formed (Supporting Information, Figure S2B). The rheological results are generally consistent with the gelation time tested by the rotor stirring method.

The storage modulus of crosslinked hydrogels under varied oxidation conditions are measured with the rheometer. As shown in Figure 2D, the average storage modulus of CSS hydrogels increases proportionally with the increase of H$_2$O$_2$ and HRP concentrations, and the H$_2$O$_2$ concentration has a significant effect on the elastic behavior of the hydrogel. As the concentration of H$_2$O$_2$ and HRP increases, more indole radicals are oxidized for crosslinking reactions, resulting a higher mechanical strength.

The microstructure of CSS hydrogel is analyzed by scanning electron microscope (SEM). As shown in Figure 3A, the hydrogel has a porous structure with the irregular shape, which is conducive to absorbing excess exudates on the wound surface and increasing the concentration of red blood cells and platelets at the wound to accelerate blood clotting. In addition, the porous structure speeds up wound healing through facilitating cell migration and proliferation, nutrients supply and waste removal. Due to the increased crosslinking density, the internal pores of hydrogel shrink with the increase of HRP concentration. Swelling degree reflects the interaction between the solution and the hydrogel, the structure of hydrogel and the degree of internal crosslinking. The swelling capacity of CSS hydrogel is calculated by the mass change of the initial dry gel and the wet gel after being placed in PBS buffer (PH 7.4) for a certain time. When the CSS polymer concentration is constant, the swelling degree of CSS hydrogel increases continuously with the elongation of the incubation time, and the swelling equilibrium is reached in about 12 hours. When the CSS polymer concentration increases from 2 wt% to 10 wt%, the equilibrium time is about 12 h, but as the CSS polymer concentration increases, the equilibrium swelling degree of the hydrogel decreases from 68% to 48% (Figure 3B). The high swelling hydrogel can effectively adsorb exudate from the serum to concentrate coagulation factors and cells, thereby accelerating coagulation at the wound site.

Figure 2. (A) Schematic illustration of the CSS hydrogel formation and potential enzyme crosslinking mechanisms. (B) Color change of CSS hydrogels formed by enzymatic oxidation of CSS solution. (C) Gelation time of CSS hydrogels crosslinked using different concentrations of HRP and H$_2$O$_2$ (n=3). (D) Average elastic modulus (G’) of CSS hydrogels at different concentrations of HRP and H$_2$O$_2$ (n=5).

Figure 3. (A) Microstructure of the crosslinked CSS hydrogels formed by using different concentrations of HRP at a fixed concentration of H$_2$O$_2$ (4 mM): (i) 6 U/ml HRP (ii) 12 U/ml HRP (iii) 18 U/ml HRP (iv) 24 U/ml HRP (B) Measurement of swelling property of CSS hydrogels formed with different concentrations of pre-gel solution upon incubation in PBS (PH 7.4) at 37°C (n=3). (C) Enzymatic degradation profile of CSS hydrogel formed.
with 18 U/ml HRP and 4 mM \( \text{H}_2\text{O}_2 \) by chondrosulphatase treatment (n=3). (D) Adhesive force of CSS hydrogels formed using different concentrations of HRP at a fixed concentration of \( \text{H}_2\text{O}_2 \) (4 mM). (E) Photographs of the hydrogels adhered to skin tissues were observed under torsion. (F) Schematic illustration of the chemistry for tissue adhesiveness of CSS hydrogel.

The biodegradability of hydrogel is related to its composition, physicochemical properties and physiological conditions. The hydrogel formed in 5 wt% CSS polymer solution is used for enzymatic degradation by 0.01 U/ml chondrosulphatase. With the decomposition of the CS backbone by chondrosulphatase, the content of the hydrogel continues to decrease, confirming the biodegradability of CSS hydrogels. The CSS hydrogel is completely degraded by chondrosulphatase at about 36 hours (Figure 3C), indicating that the CSS hydrogel can be removed naturally in the body after hemostasis is achieved.

The adhesion force of CSS hydrogel is measured with a rheometer through an adhesion separation experiment. When the concentration of \( \text{H}_2\text{O}_2 \) (4 mM) is fixed, the adhesion force decreases with the increase of HRP concentration (Figure 3D). The faster oxidation rate due to the increasing ratio of enzyme (HRP) to substrate (\( \text{H}_2\text{O}_2 \)) accelerates the internal cross-linking reactions between the conjugated serotonin molecules and CS.\(^{14}\) This may lead to the decreased interactions between oxidized serotonin and other substrates, resulting in the reduced adhesion. The hydrogel is formed in situ on the surface of the pig skin, and the hydrogel adheres stable on the skin even the pig skin is bent, stretched and inverted (Figure 3E). The stable adhesion of CSS hydrogel to tissues because serotonin derivatives (including serotonin free radicals and tryptamine diketone produced during serotonin oxidation) can bind to protein molecules containing amines, thiols and phenols (Figure 3F).\(^{45}\) Therefore, the physical and chemical properties of hydrogels, such as gelation time, modulus, swelling degree, degradation rate and adhesion strength, can be adjusted by tuning the concentration of the hydrogel pre-polymer solution and enzyme solution. Based on these results, CSS hydrogels cross-linked with 4 mM \( \text{H}_2\text{O}_2 \) and 18 U/ml HRP is confirmed as the suitable adhesive for subsequent experiments and further application.

![Figure 3](image3.png)

Figure 3. (A) SEM images of L929 cells adhered to the surface of CSS hydrogel. (B) Blood clotting index (BCI). Inset is the photograph of an uncoagulated blood cell ruptured in water. (C) Total blood loss from the damaged livers at 120 s treated with hydrogels, commercial gum (Control, positive control), and without treatment (NT, negative control) (n = 5, *p < 0.05, **p < 0.01). (D) Schematic illustration of the mouse liver hemorrhage model. (E) Gross view of the bleeding mouse liver treated with hydrogels, commercial gum (Control), and untreated (NT) every 30 s for 2 min.

The adsorption capacity and porous structure of the hydrogels provide active sites for the adhesion and aggregation of blood cells, facilitating the thrombus formation. To confirm the induced adhesion of platelets and red blood cells on CSS hydrogel, SEM is used to analyze blood cell adhesion (Figure 5A). A large number of blood cells adhered to the surface of the CSS hydrogel. The platelets on the hydrogel are activated with spiny pseudopods (Figure 5Ai). And the red blood cells adhered to the hydrogel gather together in an irregular

![Figure 4](image4.png)

Figure 4. (A) Hemolysis assay. Inset is the photograph of hemolytic red blood cells (RBCs) caused by the polymer and hydrogels. (B) Cell viability of L929 mouse fibroblasts after incubation with hydrogel extracts for 1, 2, 3 days. (C) Fluorescence images of LIVE/DEAD stained L929 cells after incubation with the hydrogel extracts for 1, 2, 3 days. (Scale bar: 100 µm. Cells cultured with DMEM as control).
shape. The normal red blood cells are biconcave disk-shaped, and most of the red blood cells are deformed after being incubated with the hydrogel (Figure 5Aii). Blood clotting index (BCI) is determined to evaluate the hemostatic performance of the hydrogel in the presence of recalcified blood (Figure 5B). Because low BCI indicates high clotting capability, The BCI of the CSS hydrogel system is much lower than that of control group, confirming the high hemostatic capability. Serotonin, a natural component of the body, activates platelets and releases clotting factors that cause blood to clot. CS forms a viscous substance in water-based solvents, thus playing the role of making blood become sticky and accelerating coagulation. As important components of CSS hydrogels, they play a key role in the adhesion of platelet and red blood cell experiments and BCI experiments.

To evaluate the hemostatic ability in vivo, a mouse liver hemorrhage model is set with 18 G needle puncture, and photographs of the bleeding site are taken every 30 s to monitor liver bleeding (Figure 5C-E). At the same time, the weight of blood absorbed on the filter paper is used to measure liver bleeding till complete hemostasis. In this study, the group without treatment is set as a negative control, and a commercially available chitosan-gelatin hemostatic agent (Cofoe) as a positive control group. Serotonin and CS as the main components of hydrogels play an important role, so they are also compared as controls. The bleeding amounts are calculated at the end of hemostasis after 120 s (Figure 5C). The bleeding amount for the CSS hydrogel is 14.2 ± 0.8 mg, whereas those of commercial glue are 31.0 ± 7.7 mg, and 69.2 ± 11.0 mg for the negative control group (*p<0.05, **p<0.01). With the same amount of hemostatic agent, the hemostatic performance of CSS hydrogels is much better than that of chitosan-gelatin hydrogel. Compared with the 120 s of the commercial glue to stop bleeding, the hemostatic time of the CSS hydrogel is about 30 s (Figure 5E). Only serotonin or CS cannot be prepared into hydrogels and the two components alone have only a slight effect on the hemostatic properties (Supporting Information, Figure S5). After the bleeding test, the liver tissues removed from each group are histologically analyzed with H&E staining. Compared with normal liver tissue or untreated group, CSS hydrogel treatment area does not show any abnormal immune response, confirming the safety and biocompatibility of medical CSS hydrogel (Supporting Information, Figure S6).

The above results are consistent with the previous studies on serotonin-induced hydrogel system.14 CSS hydrogel can prevent blood loss by quickly cross-linking and sealing the bleeding site on the wound. Because of its porous structure, it can capture the exudate from the wound site, gather the coagulation factors in the blood around the wound, and enhance the natural coagulation effect by activating platelets and red blood cells. In addition, the oxidized indole group on serotonin can further undergo Michael addition and Schiff base reaction with amine, thiol and imidazole residues in extracellular matrix proteins and carbohydrates, so that the hydrogel can firmly adhere to the wound to achieve sealing and hemostasis.46, 49

The excellent biocompatibility, hemostasis and cell regulation render the CSS hydrogels available for wound healing. To prove the versatility of CSS hydrogel and further improve the wound healing capability, the aloe vera powder (AVP) is added to the hydrogel system to obtain CSS-AVP hydrogels (The mass ratio of aloe vera powder to CSS polymer was 1:5). The structure of the hydrogel is analyzed by infrared spectroscopy. The typical peaks of C=C group and C-N group are observed at ~1732 cm⁻¹ and ~1029 cm⁻¹ in the infrared spectroscopy (Supporting Information, Figure S7), confirming the successful doping of AVP. As shown in Figure 6A, compared with the untreated group and control group (commercial dressings), CSS and CSS-AVP absorb wound exudate, prevent wound dehydration, and moisturize the wound at the same time to promote healing. On the fifth day after the operation, the naked eye image shows that the CSS and CSS-AVP hydrogel-treated wound has obvious regeneration of epidermal cell, while the wound area of the control group reduces slightly. The wound area of CSS-AVP hydrogel, control group and untreated group are 43%, 40% and 51%, respectively (Figure 6B). The defects of the CSS hydrogel treatment basically recover on the tenth day. The new skin tissues after treatment are stained with the H&E and observed (Figure 6C). Granulation tissue proliferation without inflammation (red arrow), larger blood vessel formation (yellow arrow) and higher collagen content appear in the wounds after treatment with CSS and CSS-AVP hydrogels, which is consistent with the morphology of normal tissue. In contrast, the untreated group and the commercial adhesive group show some degrees of inflammation. Thus, compared with control group, CSS and CSS-AVP hydrogels exhibit reduced inflammation and enhanced number of blood vessels for repaired tissues.

This difference may be due to the perfect match and integration of the CSS hydrogel and tissue. After dropping the pre-gel solution into the injury site, the abundant indole groups in the polymer network possess strong binding affinity to various nucleophiles (for example, amino bonds, thios and amines) on the tissue surface. CSS and CSS-AVP hydrogels provide the bionic microenvironments for cell proliferation and migration, and accelerating the growth of new epidermis.27 In addition, aloe vera powder has been reported to contain many physiological active substances, which have anti-inflammatory, immunomodulatory and promoting wound healing, etc.30

Conclusions
Inspired by a platelet coagulation mediator, a new type of in-situ cross-linked injectable hemostatic hydrogel was developed based on chondroitin sulfate and serotonin. The structure, physical and biological and hemostatic properties of the hydrogel were systematically characterized. It was demonstrated that serotonin acted as a crosslinker to form adhesive hydrogels and a blood clotting mediator for rapid hemostasis. CS adjusted cell behaviors and fates to facilitate wound healing. The serotonin-conjugated chondroitin sulfate hydrogel exhibited improved hemostatic capability in vivo and rapid wound healing after hemostasis. In addition, the wound healing capability of the hydrogel were further improved with aloe vera powder, confirming the versatility of the hydrogel system. Therefore, the chondroitin sulfate-serotonin hydrogels show the potential for effective hemostasis and wound healing.

### Experimental

#### Materials.

Chondroitin sulfate (CS) was obtained from Hua Xia Chemical Reagent Co., Ltd (Chengdu, China). Hydrogen peroxide (H$_2$O$_2$, 30 wt%), horseradish peroxidase (HRP), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were all purchased from Aladdin. Serotonin hydrochloride was purchased from Energy Chemical. Aloe Vera Powder (AVP) was purchased from Yunnan Wanly Biological Co., LTD. All the chemicals were of analytical grade and were used without further purification.

#### Characterization.

The chemical structure of the newly synthesized CSS polymer was measured using proton nuclear magnetic resonance (1H NMR, Bruker Avance 500 MHz), Fourier transform infra-red (FT-IR) and ultraviolet-visible (UV-visible) light spectrophotometer at 280 nm. CSS with different substitutions were dissolved in deuterated water and characterized using 1H NMR spectra. The degree of substitution (DS) was determined by comparing the integral area ratio of the peak of serotonin to that of the methyl groups of the CS backbone. The degree of substitution of CSS and their preparation parameters are illustrated in Table 1.

#### Synthesis of CSS polymer.

Chondroitin sulfate-serotonin (CSS) conjugates were synthesized by modifying serotonin on the CS backbone using EDC and NHS via a simple carbodiimide coupling reaction. Briefly, CS was dissolved in distilled water at a concentration of 1 mg/ml. EDC and NHS were added to the CS solution at an equal molar ratio to CS and stirred for a few minutes at PH 5.0-6.0, after which a predetermined amount of serotonin hydrochloride was added. The reaction mixture was protected with nitrogen and stirred overnight at room temperature at the same PH. After the reaction, the resultant mixture was dialyzed using a dialysis membrane with a MW cut-off of 7 kDa in phosphate buffer solution (PBS) for 2 days. The synthesized product was collected by lyophilization thereafter, and the polymer was stored at 4 ºC until use.

#### Enzymatic crosslinking of CSS hydrogels and gelation kinetics.

As a typical example, the CSS hydrogels were prepared in situ in the presence of H$_2$O$_2$ via HRP crosslinking. The concentration of enzyme was selected based on gel time. Briefly, a series of CSS polymer solution was prepared by dissolving CSS in phosphate buffer solution (PBS: 0.01 M, pH 7.4), which was then divided into two equal parts. The polymer solution was mixed with equal volume H$_2$O$_2$ and HRP respectively, and then gently mix the two ingredients with a double-tube syringe to form an in-situ hydrogel. To evaluate the effect of H$_2$O$_2$ and HRP on gel time, 5 wt% of polymer solution was mixed with different concentrations of H$_2$O$_2$ (0-8.0 mM) and HRP (6.0-24.0 U/ml) in glass vials containing a stirring bar. The gelation time of CSS hydrogels was determined when the stirring bar speed was rapidly reduced and stopped due to rheological changes.

### Rheological experiments and adhesive force measurement.

Rheological properties of the hydrogels were characterized by a model MCR 702 rheometer. The pre-gel solution containing CSS, H$_2$O$_2$, and HRP were in situ injected into parallel plate (plate diameter = 25 mm, gap = 0.5 mm) with a double- tube syringe, and silicone oil was dripped around the mixture to prevent evaporation of water from the hydrogel. The storage modulus (G’) and loss modulus (G") of the hydrogel were measured in a frequency sweep mode, and the linear viscoelastic zone of the hydrogel was determined by observing the changes of the two. The strain value (1 %) in the linear viscoelastic region of hydrogel was selected as the strain in a time sweep mode, and the rheometer test frequency was 1 Hz. The elastic modulus of the hydrogel was determined by calculating the average storage modulus of each hydrogel at 1 Hz.

Adhesive force of the hydrogel was measured in a tack-separation mode by recording the detachment force of the hydrogel between probe and base plate while pulling the probe at -10 μm/s. All rheological measurements were performed in triplicate.

### Morphology of the hydrogels.

The microstructures of CSS hydrogels were observed using a scanning electron microscopy (SEM) at an acceleration voltage 20.0 kV. Before examination, the hydrogel specimens were freeze-dried. Then, the dried hydrogel was cut into thin sections using a sharp blade in liquid nitrogen. The cross-section of dried sample was gold-coated and viewed using a microscope.

#### Swelling and Degradation profiles of CSS hydrogel.

To test the swelling behavior of the crosslinked CSS gel, the hydrogel sample were prepared by enzymatic crosslinking at 37 ºC for 24 h to ensure complete crosslinking, and the initial weight (W$_i$) of the dry hydrogel was measured after being freeze-dried. The dried hydrogel samples were immersed in PBS solution at 37 ºC and the weight of the hydrogel at each time point was measured. In order to reduce errors brought about by PBS solution left on the gel surfaces, the residual PBS solution was roughly wiped off, and the sample were weighed again (W$_d$). The swelling ratio was calculated using the following equation:

$$\text{Swelling ratio (\%) = } \frac{W_d - W_i}{W_i} \times 100\%$$

To investigate the in vitro enzymatic degradation of the crosslinked CSS gel, the hydrogel was incubated at 37 ºC in PBS solution for 1 day to reach their swelling equilibrium. The mass of the hydrogel after swelling is recorded as the initial mass (W$_i$). After that, the CSS gel samples were immersed in 10 ml of PBS solution with and without 0.01 U/ml of chondroitin enzyme. The media were removed and changes in the weight of remaining hydrogel (W$_d$) was measured at each time point (2, 4, 8, 12, 24, 36 hours after incubation).

The remaining weights was calculated using the following equation:

$$\text{Remaining weight (\%) = } \frac{W_p}{W_d} \times 100\%$$

#### In vitro biocompatibility evaluation.

In vitro hemolysis assay is a universal method to evaluate the blood compatibility of materials. According to previous reports, the method for hemolytic activity assay is as follows: Centrifuge the fresh blood at a speed of 1500 r/min for 10 minutes, remove the supernatant, wash the precipitated red blood cells repeatedly with PBS solution according to the above method until the supernatant does not show red, collect the purified red blood cells and further dilute to the final concentration 5% (v/v). Lay the CSS hydrogel on the bottom of the test tube and add drop wise the red blood cell suspension, mix gently, and incubate in a 37 ºC constant temperature shaker for 1 h. After incubated, all the samples were centrifuged at 2000 r/min for 5 min. The obtained supernatants were transferred into a 96 well clear plate. The
absorbance of the solutions at 540 nm was read using an enzyme standard instrument. Water served as the positive control and PBS served as the negative control. The hemolysis percentage of the hydrogel was calculated using the equation (3):

\[
\text{Hemolysis (\%)} = \frac{A_b - A_c}{A_b} \times 100\% \tag{3}
\]

Here, \(A_b\) represents the absorbance of the sample, \(A_c\) represents the absorbance of the positive control, \(A_p\) represents the absorbance of the negative control.

The cytotoxicity of CSS hydrogel in vitro was evaluated by an indirect contact method, according to ISO10993 standard test that the L929 mouse fibroblast were cultivated with the hydrogel extracts. All the pre-gel solutions were sterilized by filtration via 0.22 µm syringe filters in advance. After the hydrogels were formed in situ for 24 h, the gel surfaces were washed with sterile PBS solution three times. Subsequently, the disinfected hydrogel samples were extracted in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) at a leaching ratio of 1 cm³/ml for 1 day. The fibroblasts were seeded at a density of 1.0×10⁵ cells well⁻¹ in 200 µL of medium containing 100 µL DMEM (10 vol% fetal bovine serum and 1.0 wt% penicillin-streptomycin) and 100 µL sample extract and cultured at 37 °C in an incubator with 5% CO₂. The cell viabilities were tested by means of a CCK-8 assay on day-1, day-2 and day-3.

To visually observe the viability of cells cultured with CSS gels, L929 cultured in medium with and without CSS gel-immersing medium were stained using the Live/Dead viability kit on day-1, day-2 and day-3, respectively. Viabilities of the staining cells were examined by fluorescence microscope, and the ratio of viable cells (green) to dead cells (red) was quantified by manual counting from the acquired images.

Whole blood clotting performance.

On the basis of previous reports, whole blood clotting experiment were studied to analyze the coagulation ability of hydrogel. To eliminate the effect of blood clotting itself, fresh rabbit blood was collected using vacuum tube containing a certain amount of anticoagulant. Following that, samples were washed with PBS and successively dehydrated in neutral paraformaldehyde for H&E staining. Image analysis software Image J was selected to analyze the data. Finally, all sections were analyzed and photographed by fluorescence microscopy.

Blood cell and platelet adhesive performance.

The blood cell and platelet adhesive tests were conducted according to the literature. The whole blood was dropped on the hydrogel and then incubated at 37 °C for 30 min. After incubation, the sample was washed with PBS (pH 7.4) solution for 3 times followed by fixation in glutaraldehyde (2.5 %) for one night. Following that, samples were washed with PBS and successively dehydrated in 20/40/60/80/100 % ethanol aqueous solution and dried in air. Red blood cells adhered to the surface of hydrogel were observed on a scanning electron microscope (SEM). A similar protocol was followed to demonstrate cell adhesion on dressings.

Histological analysis.

To investigate the inflammation on the wound surface, mice were sacrificed and liver tissues were taken for histological analysis 3 days after treatment of liver bleeding. Similarly, the mice were sacrificed 15 days after the skin healing treatment and the new skin tissue was taken for histological analysis. The collected samples were fixed with 4% paraformaldehyde and treated with tissue processor, then embedded with paraffin, sliced into 5 µm thick slices and stained with haematoxylin and eosin. Finally, all sections were analyzed and photographed by fluorescence microscopy.

Conflicts of interest

The authors declare no competing financial interest.

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