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A cysteine enzyme hemostat for efficient heparin-tolerant blood coagulation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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It is challenging to stop bleeding effectively in patients treated with heparin which leads to enhancive risk of uncontrolled bleeding during operation. Herein, we report an easy-to-use and heparin-tolerant hemostatic agent based on a thrombin-like cysteine enzyme (papain), which catalyzes hydrolyzation of fibrinogen and cross-linking of fibrin clot. Papain-based hemostat with increased procoagulant activity is developed through immobilizing papain on the cellulose carrier, which displays short clotting time in both of normal and heparinized plasma. The excellent hemostatic performance of papain-based hemostat is further confirmed with reduced hemostatic time and limited blood loss in mice tail amputation model, rabbit auricular artery injury model and rat liver injury model, in which natural coagulation system fails to function on account of heparin. This bio-hemostat has great potential to reverse the effect of heparin and stop topical hemorrhage rapidly in surgical procedures.

1. Introduction

Anticoagulant therapy based on heparin has been developed to prevent thrombotic disorders in cardiovascular surgery, extracorporeal circulation, mechanical valve prosthesis and hemodialysis.[1-3] Heparin is an indirect anticoagulant;[3,4] it exhibits anticoagulant function through potentiating the activity of antithrombin (AT), which is an endogenous serpin inactivating coagulation factor Xa (FXa) and thrombin.[5,6] Given its physiological nature and unpredictable dose requirement in individual patients, the dose of heparin injection must be closely monitored to achieve the required anticoagulant activity while avoiding bleeding.[7] Nevertheless, there is still a risk of bleeding complication which has an impact on prolonged hospitalization and potential deaths.[8,9] Therefore, the development of an efficient hemostatic material is of great importance to reduce bleeding in the anticoagulant system, where natural coagulation mechanism is inhibited.

Although hemostatic agents aimed at massive hemorrhage have developed quickly for varies application in different occasions,[10] the progress of efficient and easy-to-use hemostat to stop bleeding in anticoagulant situation is still limited. Nowadays, neutralization of anticoagulant activity is still the first choice to prevent the risk of fatal hemorrhage in clinical practice. Protamine is clinically approved to neutralize heparin after major surgical procedures and for patients who suffer bleeding. However, protamine can induce serious side effects such as several toxicities or severe decrease in blood pressure and heart rate.[11-13] Furthermore, protamine is largely ineffective in patients treated with low-molecular-weight heparin. These concerns with protamine treatment are motivating the development of improved anticoagulation-reversal strategies. Several heparin antidotes have been reported, including cationic peptides, cationic polymers and small molecules.[14-18] Most of these strategies still have disadvantages of ineffectiveness and toxicities like protamine, and all have experienced limited clinical success. In addition, they are incapable of promoting blood coagulation. Other strategies using coagulation factors have been tested, but might not be effective or economic.[19,20] In natural hemostatic process, the essence of coagulation process to form fibrin network is the activation of fibrinogen by thrombin,[5,21,22] as shown in Scheme 1. However, while the procoagulant activity of thrombin and FXa is inhibited by heparin, the extra coagulation factors may have limited therapeutic functions. Thus, a high-efficiency, low-cost and safe hemostat to reverse anticoagulant effect of heparin and promote blood coagulation is of high value for clinical need.

The fact that papain can convert fibrinogen into fibrin has been known since 1930s. The procoagulant mechanism is similar to what occurs with thrombin and snake venom enzymes.[23-25] However, the procoagulant effect of papain in anticoagulant system is still unexplored, and its application in anticoagulation condition is vacant. Herein, we first demonstrate the high procoagulant ability of papain on fibrinogen hydrolyzation in heparin-inhibited coagulation system (Scheme 1). Papain acts like thrombin, and accelerates the fibrin clotting in the heparinized fibrinogen solution and

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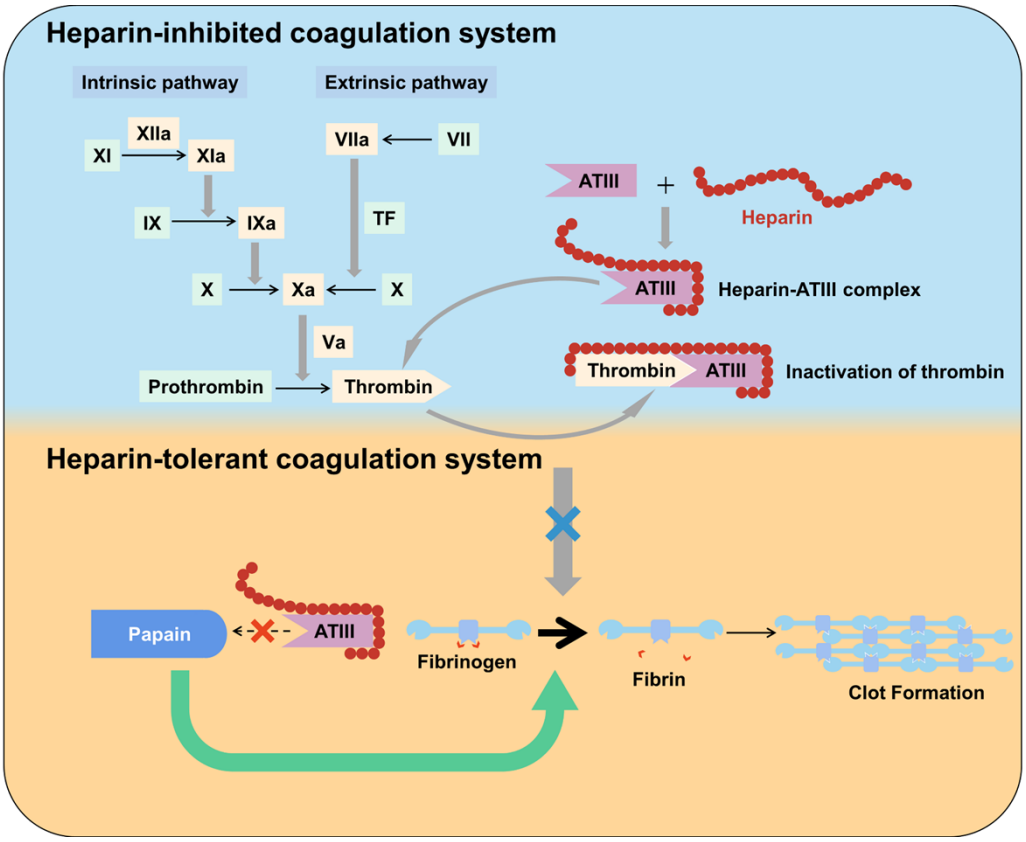
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

plasma. For practical application, a papain-based hemostat was developed via immobilizing papain on the cellulose, which was easy-to-use and presented an enhanced procoagulant activity in heparin-treated bleeding animal models.



Scheme 1 Mechanism of heparin-inhibited coagulation system and papain-based heparin-tolerant coagulation system.

2. Materials and methods

2.1. Materials

Papain (origin from papaya), heparin sodium salt, D-(+)-trehalose dihydrate and silicon oxide were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The porcine plasma (platelet poor plasma, anticoagulant with sodium citrate) was purchased from Shinuoda Biological Technology Co., Ltd (Chuzhou, China). The rabbit blood (anticoagulant with sodium citrate) was obtained from Laboratory Animal Center, Zhejiang university. Fibrinogen and chitosan (from shrimp shells, deacetylated ≥ 75%) were purchased from Sigma-Aldrich (Shanghai, China). α-cellulose (particle size: 90 mm) was obtained from Aladdin. Collagen (90%) was purchased from Macklin. Agarose was purchased from BIOWEST. Cell counting Kit-8 was obtained from Sangon Biotech.

2.2. Conversion of fibrinogen to cross-linked fibrin

Fibrinogen was dissolved in PBS buffer (pH 7.4) with storage concentration of 50 mg/mL. Then the solution was diluted into working concentration of 10 mg/mL and mixed with a series of concentrations (0.05-8 mg/mL) of papain at a fixed total volume (200 μL) at 37 °C. The standard of cross-linking was observation of white clot which stuck to the wall of a polystyrene tube without liquid flowing. The corresponding cross-linking time was recorded. In conversion process of fibrinogen to cross-linked fibrin by papain, fibrinogen is a substrate and papain is an enzyme. When the concentration of substrate is much more than that of enzyme, the reaction rate has a liner correlation with concentration and catalytic activity of enzyme. The reciprocal of cross-linking time is positively correlated with concentration of papain, and the slope reflects catalytic activity of papain.[26]

2.3. Heparinization of plasma

In a typical assay, 500 μL sodium citrate anticoagulated plasma (porcine) was mixed with calcium chloride (0.2 M) to recover normal clotting time (9-11 min). Calculated volume of stock heparin solution (100 U/mL) was added to achieve working concentration of 2 U/mL. In this case, heparinized plasma was ready for subsequent experiments.

2.4. In vitro plasma/blood clotting assay

An in vitro plasma/blood clotting assay was used to assess the procoagulant activity of hemostatic agent. The assay evaluated

coagulant response in terms of clotting time, defined as the time required from activation of coagulation cascade to appearance of a firm clot which stuck to the wall of a polystyrene tube. This assay has been described in previous reports.[27,28] In a typical assay, 500 µL sodium citrate anticoagulated plasma (porcine) or full blood (rabbit) with/without addition of heparin was mixed with calcium chloride (0.2 M) and calculated mass of the hemostatic agent in a 2 mL polystyrene tube at 37 °C. The mixture was oscillated quickly and the corresponding clotting time was recorded. In plasma clotting assay in vitro, fibrinogen is still a substrate, and papain is an enzyme. The plasma clotting time is represented as cross-linking time of fibrin. When the concentration of substrate is much more than that of enzyme, the reaction rate has a liner correlation with concentration and catalytic activity of enzyme. The reciprocal of clotting time is positively correlated with the concentration of papain, and the slope reflects catalytic activity of papain.

2.5. Thermal stability

The protease (papain or thrombin) with different mass (0.01-0.2 mg) was preheated at 25, 37 and 50 °C for 20 min. Then 500 µL of recalcified plasma was mixed with 50 µL of preheated enzyme in a 2 mL polystyrene tube at corresponding temperature. In vitro plasma clotting assay as mentioned above was used to evaluate the thermal stability of papain or thrombin. The mixture was oscillated quickly and the corresponding clotting time was recorded.

2.6. Fabrication of papain/supporter composite hemostat

Immobilization of papain on suitable supporters was prepared via the impregnation method. 6 mg of supporter powders (cellulose, chitosan, SiO_2, Ca-Y zeolite, collagen, mycose and agarose) were dispersed with ultrasound in a 1.5 mL polystyrene tube, and 40 µL papain solution with different concentrations (0-1 mg/mL) was added. After oscillation, the mixture was incubated at 37 °C for 30 min and finally freeze-dried at -50 °C to obtain the composite powder. In the in vitro plasma clotting assay and in vivo heparin-treated animal bleeding models, the final mass ratio of papain-to-cellulose for papain/cellulose composite hemostat was 1:2.

2.7. Zeta potential of different supporters with papain

Above mentioned supporters (cellulose, chitosan, SiO_2, Ca-Y zeolite, collagen, mycose and agarose) were dispersed by ultrasound to form a uniform suspension. Then 0.05 mg/mL papain was incubated with 0.1 mg/mL supporters in 1 mM KCl solution at 37 °C for 30 min. Next, zeta potential was measured by Malvern Zetasizer Nano (Marlvern, UK) for three times.

2.8. Microstructure of papain/cellulose composite

In order to evaluate the microscopic structure of papain/cellulose composite, Alexa Fluor 488 protein labelling kits (purchased from Sigma) was used to label the papain. Firstly, papain was dissolved to 2 mg/mL in PBS. Then 50 µL of 1 M sodium bicarbonate was added into 0.5 mL of papain solution mentioned above. The mixture was added into a vial of reactive dye and inverted a few times to fully dissolve the dye. Stir the reaction mixture for 1 h at room temperature. To remove of non-conjugated fluorescent dyes, the labelled protein solution (about 0.5 mL) was slowly added to the settled resin and centrifuged at 1000 g for 2 min. The Alexa Fluor 488 labelled papain solution was collected in the filtrate. The labelled papain was used to prepare the papain/cellulose composite which was followed to be monitered by fluorescence imaging.

2.9. Hemostasis in heparin-treated mice tail amputation model

In order to assess the hemostatic effect of papain/cellulose composite in vivo, a bleeding model of tail amputation in ICR mice (female, 8 weeks old, 25-30 g; Shanghai SLAC Laboratory Animal Co., Ltd) was designed. The test conformed to the Guide for the Care and Use of Laboratory animals and the animal welfare guidelines of the laboratory animal center of Zhejiang University. The test received ethical approval from Zhejiang University Experimental Animal Ethics Committee. Eighteen female mice were randomly divided into three groups and each group contained 6 mice. Each group was treated with corresponding hemostatic management (blank, cellulose and papain/cellulose composite).

Before processing, all mice were injected intraperitoneally with heparin (1 U/g). 15 min later, 1% pentobarbital sodium were injected intraperitoneally for general anesthesia (0.01 mL/g). When mice were fixed, the tail was cut off with 3.3 mm diameters of cross section. After free bleeding for 1 min, 60 mg of hemostatic powder was applied to the injured site without pressure for 2 min. Subsequently, the unattached hemostatic material was removed and hemostatic time was recorded. Mice were finally executed with injection of excess pentobarbital sodium after observation for 60 min.

2.10. Hemostasis in heparin-treated rabbit auricular artery injury model

An auricular artery injury model in female rabbits was performed to evaluate the hemostatic effect of papain/cellulose composite. The study obeyed the Guide for the Care and Use of Laboratory animals and animal welfare guidelines of the Laboratory animal center of Zhejiang University. The study was approved by Zhejiang University Experimental Animal Ethics Committee. Six female New Zealand White rabbits with weight of about 2.5 kg were obtained from Laboratory Animal Center of Zhejiang University. All rabbits were fasted overnight before experiments, but allowed free access to water. Left and right auricular artery of the rabbits were randomly treated with papain/cellulose composite or cellulose alone, and each group contained six results.

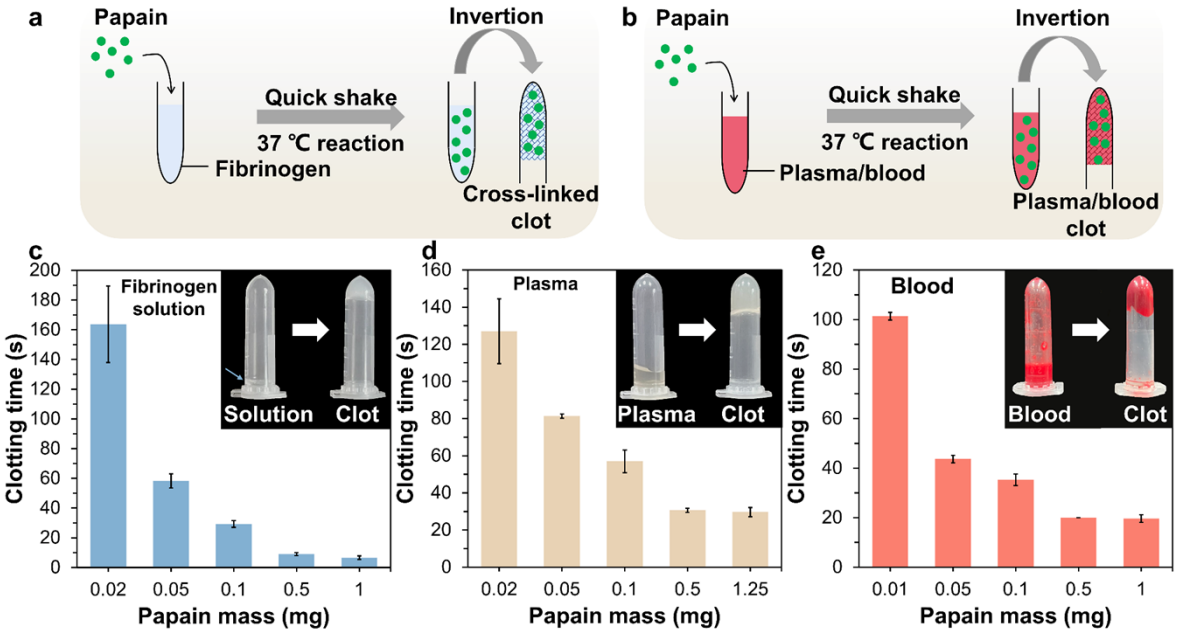


Fig. 1 Procoagulant performance of papain. Schematic diagram of (a) fibrinogen cross-linking assay and (b) plasma/blood clotting assay in vitro. (c) Hydrolytic and cross-linking time of papain in the fibrinogen solution. Clotting time of papain in the (d) normal plasma and (e) whole blood. Data values corresponded to mean ± SD, n = 3. Error bars represent SD.

Rabbits were anesthetized with 40 mg/kg pentobarbital sodium. Then an anticoagulant model was constructed through ear vein injection of heparin sodium with concentration of 1000 U/kg. The middle auricular artery was shaved and severed transversely with a wound length of 1 cm at the site of 3 cm distant from thick branch. After free bleeding for 30 s, 4 g of hemostatic powder was applied to the wound with constant weight of 60 g. The compression was removed every 1 min to check the hemostasis. When hemostasis was achieved, the hemostatic time was recorded. The blood loss was recorded through calculation of weight difference between blank and hemophoric gauze. Rabbits were finally euthanized with an overdose of pentobarbital sodium after observation for 10 min.

2.11. Hemostasis in heparin-treated rat liver injury model

The hemostatic effect of papain/cellulose composite in vivo was further evaluated through liver injury model in heparin-treated rats. The study obeyed the Guide for the Care and Use of Laboratory animals and animal welfare guidelines of the Laboratory animal center of Zhejiang University. The study was approved by Zhejiang University Experimental Animal Ethics Committee. Eighteen Wistar rats (male, 180-250 g; Shanghai SLAC Laboratory Animal Co., Ltd) were randomly divided into three groups (blank, cellulose and papain/cellulose composite) and each group contained six rats. All rats were fasting overnight before experiments.

Firstly, 1% of pentobarbital sodium (5 mL/kg) was injected intraperitoneally for general anesthesia. Then the heparinized model was constructed through intraperitoneal injection of heparin sodium with concentration of 1250 U/kg. 30 min later, the liver of rat was exposed through midline abnormal incision. The serous fluid covering liver was meticulously removed to prevent imprecise measurement of blood loss. A pre-weighted filter paper on a paraffin film was placed beneath the liver to weigh blood loss. Two perpendicular wounds with 2 cm long and 5 mm deep were created on the exposed liver. After free bleeding for 10 s, 0.8 g of cellulose gauze or papain/cellulose gauze was applied to the injured site without pressure. The gauze was removed every 3 min to check for hemostasis. When hemostasis was achieved, the hemostatic time was recorded and blood loss was calculated. Rats were finally executed with injection of excess pentobarbital sodium after observation for 50 min.

2.12. Cytotoxicity of papain/cellulose composite

The CCK-8 assay was conducted to evaluate cytotoxicity of papain/cellulose composite with macrophage (Raw264.7) and myoblasts (C2C12). All materials were sterilized by ultraviolet for 2 h. Raw264.7 cells were incubated in DMEM with 10% inactivated serum at 37 °C in 5% CO_2. 100 μL of cell suspension was added into 96 wells with a cell density of about 2×10^4 cells/cm^2 and incubated for 24 h to form a full layer. 10 μL of cellulose or papain/cellulose composite with different cellulose concentrations (0.3-3.3 mg/mL) was added into cells and incubated for 0.5 h. Then materials were removed and 10 μL of CCK-8 reagent with 100 μL of DMEM was added to each well for 30 min at 37 °C in 5% CO_2. The absorbance was recorded at 450 nm through a microplate reader and the cytotoxicity was presented as the percentage of viable cells compared with that of untreated control. The assay was also conducted with myoblasts (C2C12), 100 μL cell suspension was added into 96

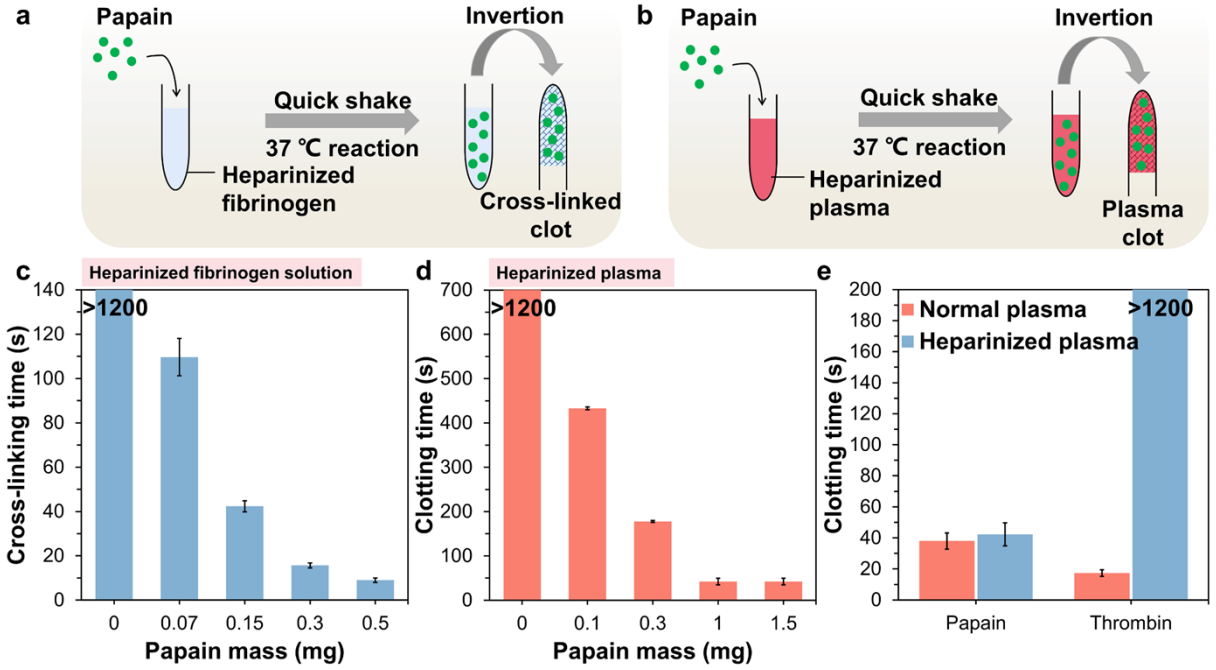


Fig. 2 Procoagulant performance of papain in heparinized system. Schematic diagram of (a) heparinized fibrinogen cross-linking assay and (b) heparinized plasma/blood clotting assay in vitro. Clotting time of papain in the (c) heparinized fibrinogen solution and (d) heparinized plasma. (e) Compared with thrombin, papain can promote clot formation in heparinized plasma. Data values corresponded to mean ± SD, n = 3. Error bars represent SD.

wells with a cell density of about 5×10^3 cells/cm^2 and incubated for 24 h to form a full layer. The remaining protocol was the same as that with Raw264.7 cells.

3. Results and discussion

3.1. Procoagulant activity of papain in normal system

In the coagulation process, enzymatic hydrolysis of fibrinogen by thrombin is a key reaction in coagulation cascade. In detail, thrombin catalyzes and removes N-terminal fibrinopeptides from Aα chain and Bβ chain of fibrinogen, which promotes formation of mature fibrin monomers.[22] Fibrin monomers derived from hydrolyzation of fibrinogen in high concentration can crosslink spontaneously to form an insoluble clot.[29] Herein, papain which functions as thrombin to hydrolyze fibrinogen was investigated. Papain was added into the fibrinogen solution, then cross-linking time was recorded according to observation of a white flocculent clot sticking to the tube, which can evaluate the rate of accelerated proteolysis and crosslinking (Fig. 1a). When the papain mass was 1 mg at a fixed total volume (200 µL), the fibrinogen solution was quickly transformed into a white flocculent clot sticking to the tube (7 ± 1 s, Fig. 1c), which suggested that the hydrolysate of fibrinogen catalyzed by papain were active fragments (fibrin) instead of non-functional product.[30,31] Thus, the thrombin-like function of papain has been proved.

To further illustrate procoagulant performance of papain, it was added into normal platelet-poor plasma and whole blood to observe clot formation (Fig. 1b). The clotting time of normal plasma and blood without papain were 560 ± 11 s and 514 ± 5 s, respectively. Papain exhibited excellent procoagulant activity in terms of rapid formation of blood clots with a short clotting time. As the concentration of papain in the plasma and blood increased, the clotting time was further shortened. The shortest clotting time in the plasma and blood were 30 ± 3 s and 20 ± 2 s, respectively (Fig. 1d and e). The clotting time of blood was shorter than that of plasma under the same concentration of papain, owing to procoagulant effect of platelets in blood. It participates in the formation of large amounts of thrombin and accelerates the coagulation process.[32] According to fibrinogen cross-linking assay and plasma/blood clotting assay in vitro, we have confirmed that papain with thrombin-like function can effectively hydrolyze fibrinogen to form active fibrin monomers and quickly promote blood coagulation. Thus, papain with wide availability and low cost (~$300/kg) represents superiority for procoagulant treatment.

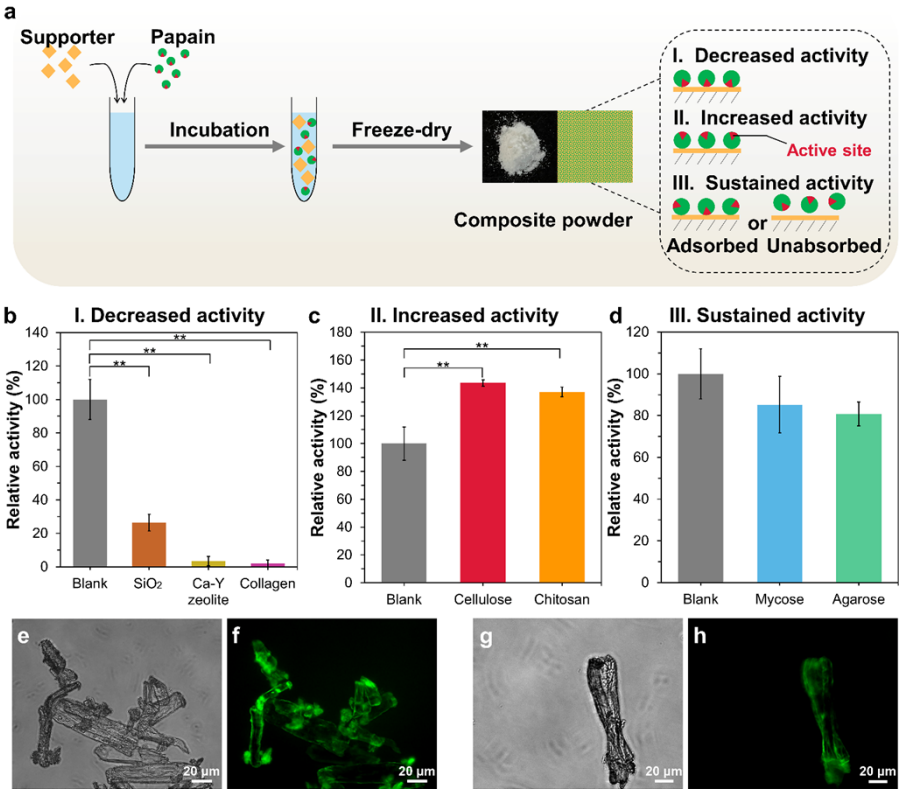


Fig. 3 Immobilization of papain on supporters. (a) Schematic diagram of preparation of papain/supporters and three possibilities of enzymatic activity. Relative procoagulant activity of papain immobilized on (b) SiO_2, Ca-Y zeolite, collagen, (c) chitosan, cellulose, (d) mycose and agarose according to the clotting time. (e,g) Bright field and (f,h) corresbonding fluorescence images of cellulose with Alexa Fluor 488-labeled papain. Data values corresponded to mean ± SD, n = 3. Error bars represent SD.

3.2. Procoagulant performance of papain in heparinized system

In the anticoagulant therapy, heparin binds to antithrombin III (ATIII) and promotes inactivation of FXa and thrombin.[33,34] It is worth noting that papain belongs to cysteine protease family while thrombin is a serine protease, we speculate that the inhibition mechanism of heparin and ATIII complex to thrombin will not apply to papain. To verify this hypothesis, we evaluated procoagulant performance of papain in heparinized fibrinogen solution in vitro (Fig. 2a). Papain could still transform fibrinogen into fibrin which formed a cross-linked clot sticking to the tube. When the concentration of papain was 2.5 mg/mL (0.5 mg in 200 μL), the cross-linking time was 9 ± 1 s which was about the same as in normal fibrinogen solution (Fig. 1c and Fig. 2c). Then we assessed the procoagulant ability of papain in heparinized plasma (Fig. 2b). As expected, the heparinized plasma lost ability to form a stable clot due to lack of activated thrombin. Even the experiment was extended to more than 1200 s, there was no plasma clot observed in the control group (Fig. 2d). With the addition of papain, clotting ability of heparinized plasma gradually recovered. By contrast, thrombin totally lost its procoagulant activity in heparinized plasma (Fig. 2e). Thus, the procoagulant ability of papain in hydrolyzing fibrinogen and accelerating clotting can be maintained in the plasma pretreated with heparin. Papain can play an alternative physiological role of thrombin in the heparin-intervened blood coagulation.

3.3. Fabrication of papain/supporter composite hemostat

To facilitate practicability of proteases, the technology of immobilizing enzyme has been widely used. In the process of enzyme binding to supporters, enzyme not only regulates its orientation to approach supporter in an appropriate area, but also adjusts its own conformation to enhance its binding stability and energy. On the other hand, proteases with specific adsorption orientation and reconstructed conformation still have diverse enzymatic activity and biological function due to supporters with different properties.[35,36] Actually, the enzymatic activity of immobilized proteases has three possibilities, including decreased, increased and sustained activity (Fig. 3a). Then, we have examined procoagulant performance of papain on seven types of supporters. In detail, procoagulant activity of immobilized papain was evaluated via in vitro plasma clotting assay. The clotting time has been recorded. According to the linear correlation between the reciprocal of clotting time and the amount of papain when the substrate was excessive, the procoagulant activity of papain was revealed in enzyme kinetic curves, which reflects the rate of enzymatic reaction (Fig. S1).

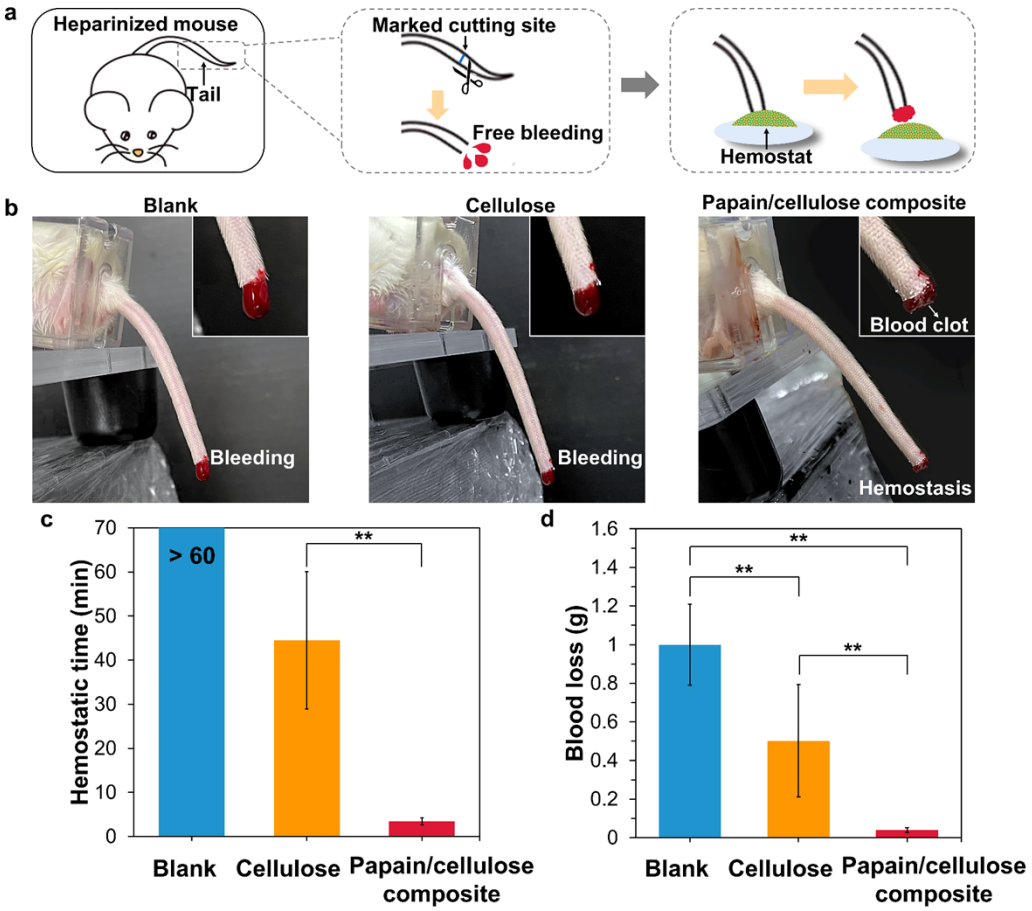


Fig. 4 Hemostatic superiority of papain/cellulose composite in heparin-treated mice tail amputation model. (a) Schematic diagram of the mice tail amputation model. (b) Bleeding state of mice at 10 min after blank, cellulose and papain/cellulose composite treatment, respectively. Quantitative analysis of (c) hemostatic time and (d) blood loss after treatment of blank, cellulose and papain/cellulose composite in the heparin-treated mice tail amputation model. Data values corresponded to mean ± SD, n = 6. Error bars represent SD. **P < 0.01, one-way analysis of variance (ANOVA).

In terms of the slope of kinetic curve, papain immobilized on agarose and mycose had slightly lower activity, while anchored on SiO_2, Ca-Y zeolite and collagen lost most of the enzymatic activity (Fig. 3b and d, Fig. S2a and c). Noteworthily, the catalytic activity of papain was enhanced on the surface of cellulose and chitosan (Fig. 3c and Fig. S2b). To understand the interaction between papain and supporters, zeta potential of different supporters before and after immobilizing papain was measured. The activity of papain decreased may due to strong interaction between papain and supporters (SiO_2 and Ca-Y zeolite) with change of zeta potential more than 30 mV (Fig. S3a). The activity of papain increased when immobilized on cellulose and chitosan owing to appropriate strength of interaction with change of zeta potential between a suitable range (Fig. S3b). As for an efficient hemostat, cellulose and chitosan are suitable substrates for anchoring papain. Actually, a lot of studies have reported that cellulose and chitosan are great supporters for their hemostatic capacity and biocompatibility.[37-39]

Further, cellulose-based dressing is a clinically used hemostatic device with irreplaceable advantages, such as low cost, reliable safety, great water absorption and easy manufacturability by weaving and knotting.[40-44] In this way, cellulose could be a suitable choice to load papain for further hemostatic application. The microscopic structure of papain/cellulose composite was observed through fluorescence imaging (Fig. 3e,f,g,h). Papain was labelled with Alexa Fluor 488, then was immobilized on cellulose via impregnation method. The green fluorescent signal was detected on the surface of cellulose uniformly, suggesting successful loading of papain on the cellulose. The procoagulant performance of papain immobilized on cellulose was evaluated via in vitro plasma clotting assay. The plasma clot was observed in normal plasma after 633 ± 8 s, while heparinized plasma lost ability to coagulate (Fig. S4). Cellulose can promote the clot formation to some extent in normal plasma, but cannot function in heparinized plasma. Notably, papain/cellulose composite displayed an excellent procoagulant activity in both normal and heparinized plasma (Fig. S4). Thus, the papain/cellulose composite is expected to be a good hemostatic agent in the heparinized situation.

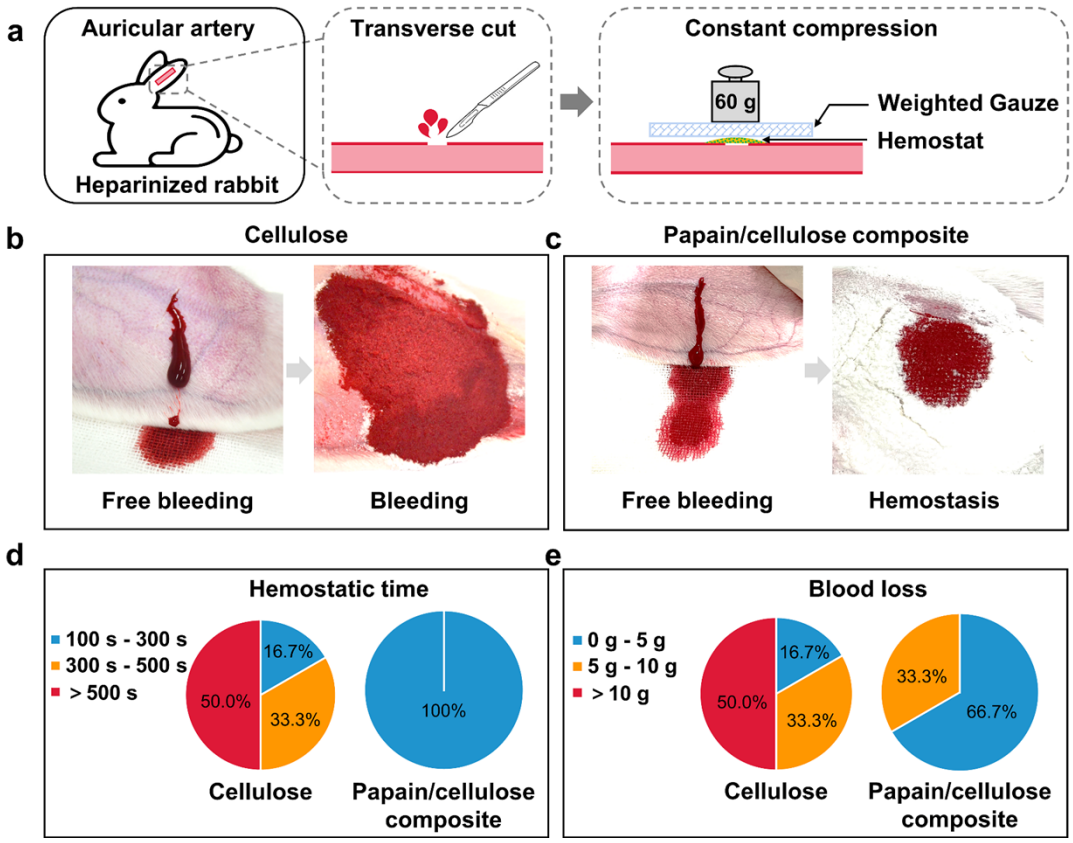


Fig. 5 Hemostatic performance of papain/cellulose composite in the heparin-treated rabbit auricular artery injury model. (a) Schematic diagram of a rabbit auricular artery injury model. Hemostatic agent was applied with constant pressure to stop bleeding. (b) Bleeding was not stopped when cellulose alone was applied with compression in 10 min. (c) Hemostasis was achieved with the application of papain/cellulose composite within 4 min. Quantitative and statistical analysis of (d) hemostatic time and (e) blood loss (n = 6 independent injuries).

3.4. In vivo hemostatic efficiency of papain/cellulose composite hemostat

The hemostatic performance of papain/cellulose composite in vivo was firstly investigated in mice tail bleeding model. Heparin sodium was injected intraperitoneally into mice to establish heparinized model (Fig. S5). Then, excessive bleeding was induced by amputation of mice tail (Fig. 4a). All mice failed in response to bleeding event, because heparin successfully inhibited the formation of fibrin clot (Fig. 4b). In the blank group, all the mice cannot stop bleeding within 60 min, leading to the severe blood loss (1.00 ± 0.21 g). In consideration of great solubility of papain, the practicability and safety of papain were two important problems. Fixing a soluble powder at the injured site is difficult, and a high dose of papain is needed to seal the wound. According to heparin-treated mice tail amputation model and mice liver injury model (Fig. S6 and S7), when the blood flow was quick or the bleeding volume was large, most of papain dissolved into the blood and little of papain sticked around the edges of wound. As soon as papain dissolved, the wound began to bleed again. In the heparin-treated mice tail amputation model, nearly 40% of the injured sites rebled with hemostatic time more than 1800 s and blood loss larger than 0.5 g (Table S1). Also, papain may flow into the blood circulation and cause terminal thrombus. Thus, experimental group of papain alone was not included in the in vivo animal experiments. In the cellulose group, it took 44 ± 16 min to form a blood clot at the injured site, showing relatively large blood loss (0.50 ± 0.29 g). Importantly, the papain/cellulose composite group exhibited significant procoagulant effect with an impressively shorter hemostatic time (3.5 ± 0.8 min) and ten-fold less blood loss (0.04 ± 0.01 g) as compared to cellulose group (Fig. 4c and d). In addition to rapid formation of blood clot, we found that the mechanical strength of blood clot in the papain/cellulose composite group was robust enough to seal the injured site (Fig. 4b).

Rabbit auricular artery injury model in heparinized system was also used to evaluate in vivo hemostatic effect. Bleeding model was constructed through transverse cut of middle auricular artery. Then hemostatic agent was applied to the wound with constant compression (Fig. 5a and Fig. S8). In the cellulose group, half of the rabbits failed to stop bleeding with hemostatic time more than 500 s (Fig. 5b and d). While in the papain/cellulose composite group, hemostasis was achieved with time between 100 s and 300 s (Fig. 5c and d), which suggested a superior hemostatic effect of papain/cellulose composite in the heparinized system. Other than rapid formation of blood clot, papain/cellulose composite group had

blood loss less than 10 g, while cellulose group had larger proportion of blood loss more than 10 g (Fig. 5e and Table S2).

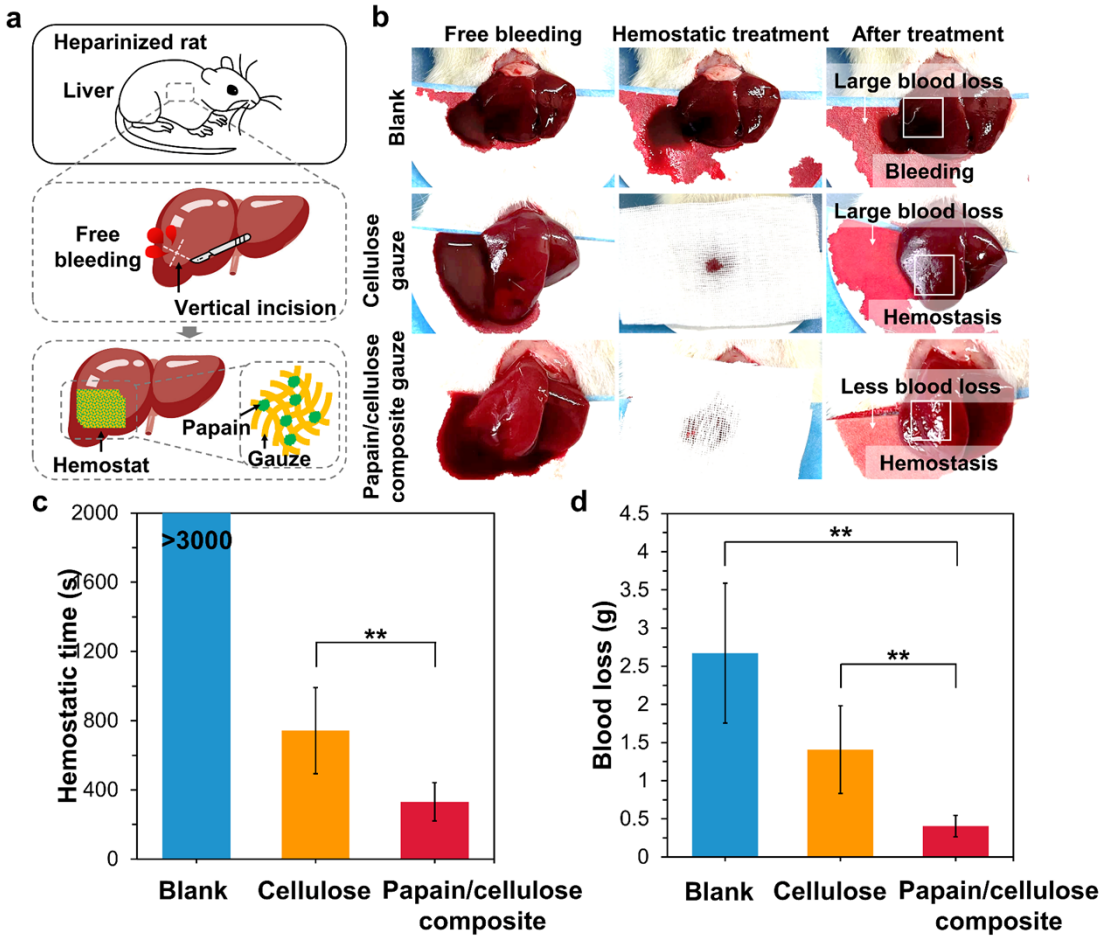


Fig. 6 Hemostatic effect of papain/cellulose gauze in the heparin-treated rat liver injury model. (a) Scheme presentation of the rat liver injury model. (b) Representative pictures of hemostatic states at 10 min after blank, cellulose gauze and papain/cellulose gauze treatment, respectively. Quantitative analysis of (c) hemostatic time and (d) blood loss after treatment with blank, cellulose and papain/cellulose gauze in the heparin-treated rat liver injury model. Data values corresponded to mean ± SD, n = 6. Error bars represent SD. **P < 0.01, one-way ANOVA.

The hemostatic effect of papain/cellulose composite in vivo was further assessed in rat liver injury model in heparinized system (Fig. S9). The bleeding was induced through vertical incision on the exposed liver (Fig. 6a). Rats in blank group failed to stop bleeding within 50 min with severe blood loss of 2.67 ± 0.92 g, suggesting a successful anticoagulant model (Fig. 6b). In the cellulose gauze group, it took 743 ± 249 s to stop bleeding at the wound with relatively large blood loss of 1.41 ± 0.57 g. While in the papain/cellulose gauze group, significant hemostatic effect was presented with shorter hemostatic time (332 ± 111 s) and less blood loss (0.40 ± 0.14 g, Fig. 6c and d).

In this way, the excellent hemostatic performance of papain/cellulose composite in heparinized system was shown in mice tail amputation model, rabbit auricular artery injury model and rat liver injury model. In summary, the papain/cellulose composite promotes hemostasis mainly through three mechanisms. Firstly, as a cysteine protease different from thrombin, papain can function in the presence of heparin and hydrolyze fibrinogen into fibrin which is a key reaction during coagulation process. Secondly, cellulose can not only serve as a supporter that improves the activity and stability of papain, but also promote hemostatic process through water absorption and platelet aggregation. Thirdly, the blood clot formed with cellulose can function as a physical barrier to stop blood flow in situ. Among the above three points, most important of all is rapid and successful formation of cross-linked network of fibrin monomers catalyzed by papain in heparin-inhibited blood coagulation system.

Also, the papain/cellulose composite hemostat almost had no obvious cytotoxicity, which was confirmed by CCK-8 assay on macrophages and myoblasts (Fig. S10). The superior hemostatic performance and biological safety of artificial

material based on papain is of great significance to control bleeding in patients with heparin treatment, such as during cardiovascular surgery and extracorporeal circulation.

3.5. High thermostability of papain/cellulose composite hemostat

Papain is well-known for its excellent heat resistance.[45] We examined the procoagulant activity of papain at high temperature through in vitro plasma clotting assay. Thrombin can promote clot formation quickly with a short clotting time of 17.3 ± 2.1 s at 37 °C, but partly lost its procoagulant activity at 50 °C, and totally lost its activity at 60 °C (Fig. S11a). However, papain could maintain active at a wide temperature range between 25 and 90 °C. More significantly, papain can present maximum activity at 50 °C with a clotting time of 18.0 ± 1.0 s, which verified the high thermostability of papain compared with thrombin (Fig. S11a).

In heparinized plasma, thrombin also lost its ability to promote clot formation regardless of temperature with a clotting time of more than 1200 s. However, papain/cellulose composite can still keep procoagulant activity at 25, 37 and 50 °C. Predictably, 50 °C is the best temperature for papain/cellulose composite with a clotting time of 64.0 ± 4.6 s (Fig. S11b). In this way, the heat-tolerant papain hemostat makes it a promising hemostatic agent to meet practical transportation, storage and application.

4. Conclusions

Herein, we firstly developed papain/cellulose composite with thrombin-like function to promote hemostasis regardless of anticoagulation effect of heparin. The main hemostatic mechanism of composite is that papain with thrombin-like activity can promote hydrolyzation and cross-linking of fibrinogen in presence of heparin. Another hemostatic advantage is that cellulose functions as a supporter to improve the activity and stability of papain and a concentrator to accelerate platelet aggregation. We apply papain as the form of papain/cellulose composite on heparin-intervened system including heparin-treated mice tail amputation model, rabbit auricular artery injury model and rat liver injury model. We envision this composite could be an economically viable device to provide effective protection for patients with massive bleeding in the heparin treatment and reduce the risk of deaths caused by surgical bleeding.

Author Contributions

Mengchi Lin: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Lisha Yu: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing. Liping Xiao: Conceptualization, Resources, Validation, Writing – review & editing. Jie Fan: Conceptualization, Resources, Validation, Supervision, Funding acquisition, Writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work is financially supported by National Natural Science Foundation of China (Nos. 91545113 and 91845203).

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