

Research Article

Silk fibroin–gelatine haemostatic sponge loaded with thrombin for wound haemostasis and tissue regeneration

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Abstract

Background: Wound haemostasis is an important part of clinical treatments, especially treatments for patients with avulsion injury, destructive injury and large-scale soft tissue injury. Therefore, developing fast and effective haemostatic materials is critical. This study aimed to design a novel and efficient silk fibroin–gelatine composite haemostatic sponge loaded with thrombin (SFG@TB) to assist in wound haemostasis.

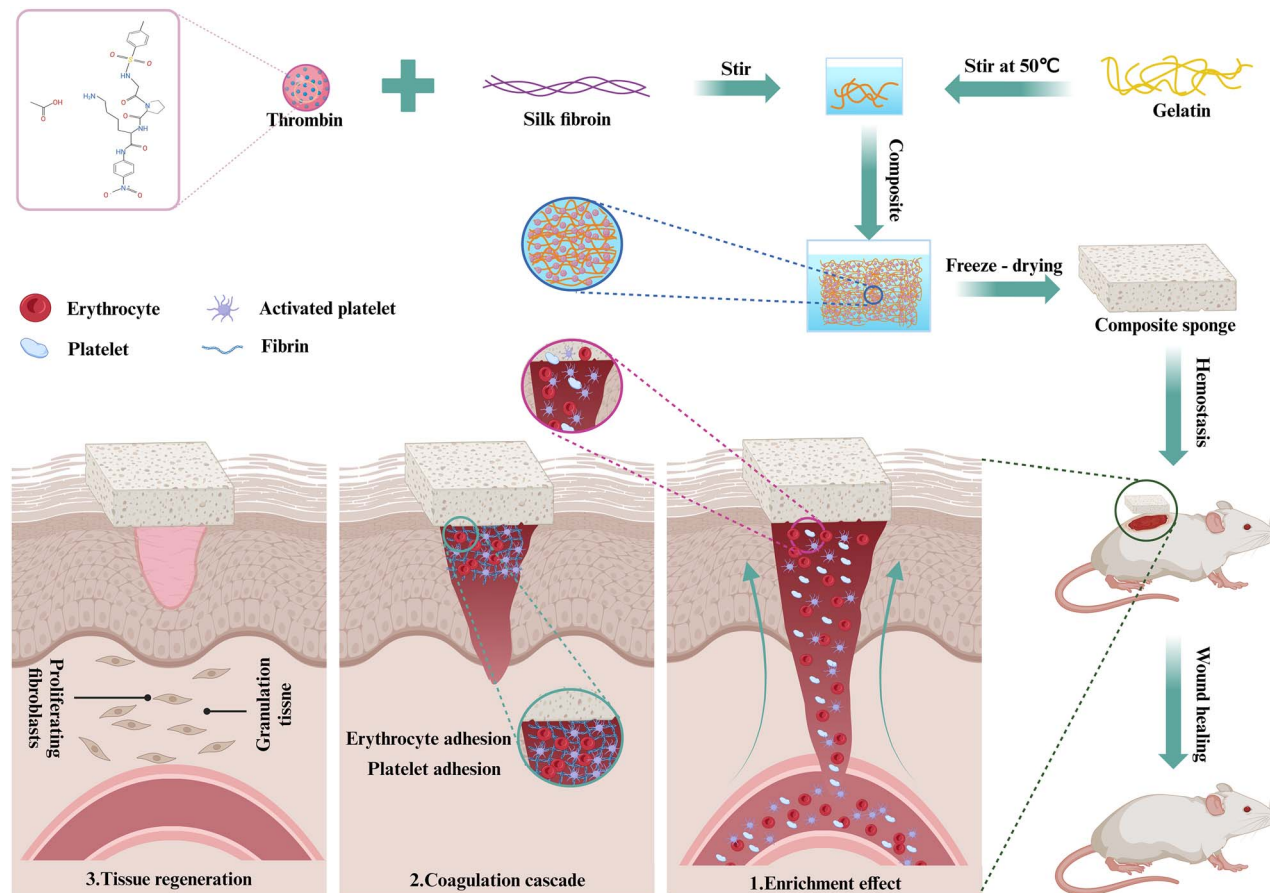
Methods: The SFG@TB composite haemostatic sponge was formed with gelatine, silk fibroin and thrombin through a freeze-drying technique. First, the material characteristics of SFG@TB were measured, including the elastic modulus, swelling rate and porosity. Second, *in vitro* cell coculture experiments, *in vivo* embedding experiments and haemolytic analyses were performed to evaluate the biocompatibility of SFG@TB. Then, coagulation experiments and femoral artery and liver bleeding models were used to evaluate the haemostatic performance of SFG@TB. Finally, the ability of SFG@TB to promote tissue healing was evaluated through experiments with Sprague–Dawley rat models of injury.

Results: Compared with gelatine sponges, SFG@TB exhibited outstanding mechanical properties and water absorption properties. In addition, the excellent biosafety of the composite haemostatic sponge was confirmed by cell experiments, subcutaneous embedding experiments and haemolytic analysis. Based on the *in vitro* coagulation test results, SFG@TB exhibited greater adhesion of red blood cells and platelets and a shorter dynamic coagulation time. Compared to the use

of silk fibroin–gelatine composite haemostatic sponges or gelatine sponges, the introduction of thrombin resulted in a shorter haemostasis time and a smaller bleeding volume, as revealed by *in vivo* coagulation tests. The experiments with Sprague–Dawley rat models of injury indicated that SFG@TB accelerated the wound healing process and reduced scar width, which was accompanied by thicker granulation tissue.

Conclusions: Overall, the SFG@TB composite haemostatic sponge, which exhibits outstanding mechanical properties, good haemostatic performance and high biosafety, promoted wound haemostasis and tissue repair. Therefore, the SFG@TB composite haemostatic sponge could be a promising material for wound haemostasis.

Graphical Abstract



Key words: Composite haemostatic sponge, Gelatine, Silk fibroin, Thrombin, Wound haemostasis, Tissue regeneration, Biomaterial

Highlights

- SFG composite haemostatic sponges were prepared using gelatine and silk fibroin, which can address the mechanical defects of gelatine and silk fibroin when used alone.
- Adding thrombin can further improved the haemostatic performance of the SFG@TB composite haemostatic sponge.
- The SFG@TB composite hemostatic sponge with outstanding mechanical properties, good hemostatic performance and high biosafety can promote wound hemostasis and tissue repair.

Background

Wound haemostasis is an important part of clinical practice. Rapid and effective haemostasis is essential for saving the lives of patients with trauma. Therefore, an increasing number of haemostatic materials are being developed and improved to better exert haemostatic effects. Excellent haemostatic materials should control bleeding quickly, have good biocompatibility and biodegradability, be suitable for various types of wounds, and be stored and used easily [1]. At present, commonly used haemostatic materials are mostly sponges, fibre membranes, powders, hydrogels, etc., which are processed from thrombin, gelatine, silk fibrin, chitosan and so on [2,3]. Composite materials have gradually gained attention because they can eliminate the drawbacks of the materials used and exhibit several advantages.

Gelatine is a macromolecular hydrophilic colloid obtained by partial hydrolysis or thermal denaturation of collagen. It is a major component of skin, bone and connective tissue and is widely used in medicine [4]. Gelatine exhibits excellent biocompatibility, biodegradability and nonimmunogenicity. For example, Ji *et al.* [5] used gelatine-methacryloyl hydrogels combined with metals to improve the accuracy of localization and resection of lung nodules. Zheng *et al.* [6] developed a gelatine-based bioelectronic hydrogel that is used to promote wound healing and monitor real-time human movement. In addition, gelatine contains RGD sequences, which can bind with integrin to promote cell adhesion, migration and proliferation [7–9]. As a haemostatic material, gelatine sponges can quickly absorb blood and form a gel, thereby blocking blood flow and preventing further bleeding. Simultaneously, the sponges can exert superior haemostatic ability by accumulating and activating platelets and other active substances in large quantities, accelerating the formation of platelet embolism and acting as a substrate during the formation of solid fibrin clots [10]. However, coagulation factors in the blood must be activated for gelatine sponges to function well. Thus, the haemostatic effect of these gels on patients with coagulation disorders is not ideal [11]. In addition, gelatine has a fast degradation rate, poor thermal stability and weak structural strength *in vivo*, so it easily breaks after absorbing blood. The structural strength of gelatine must usually be increased through crosslinking [12], but residual crosslinking agents in the body may cause cytotoxic effects, immune reactions and other side effects [11,13]. Therefore, compounding gelatine with other materials may be the best method to prepare a high-performance gelatine haemostatic material.

Silk fibroin is a natural polymer fibrous protein that is widely used in textiles, tissue engineering, imaging, sensing and other fields [14]. Silk fibroin contains 18 kinds of amino acids, of which glycine (Gly), alanine (Ala) and serine (Ser) account for >80%. Silk fibroin includes the repeated Gly-Ala-Gly-Ala-Gly-Ser repeat sequence, which can spontaneously form a β -sheet structure [15]. Through this structural property, researchers can regulate the degree of β -sheet formation and ultimately regulate the degradation rate of silk fibroin

[16]. An aqueous solution of regenerated silk fibroin is often used to prepare sponges, hydrogels, scaffolds, electrospinning films etc. Silk fibroin can exert haemostatic effects by physically adhering to and sealing wounds. At the same time, platelets are activated to aggregate and adhere [15]. In addition, silk fibroin can be used to load and deliver active substances, such as enzymes and antibodies, due to its mild processing conditions, superior mechanical properties and stable effects [14]. Moreover, similar to gelatine, silk fibroin is a natural macromolecular material that can promote the migration, adhesion and proliferation of cells and the healing process of tissue [17]. Brown *et al.* designed a shaped-memory silk fibroin sponge for promoting the regeneration of soft tissue [16,18]. Gomes *et al.* developed a sponge with an anti-inflammatory effect using silk fibroin and choline gallate [19], but compared to natural silk fibroin, the regenerated silk fibroin exhibited poorer mechanical properties. However, blending some polymers (such as gelatine) with silk fibroin can improve its mechanical properties [20].

This study used gelatin and silk fibroin to prepare haemostatic sponges. First, the composite material eliminates the disadvantages of gelatine and silk fibroin when these materials are applied individually. On the one hand, using a mixture of silk fibroin and gelatine can improve the degradation ability of gelatine and enhance its mechanical properties without the use of toxic cross-linking methods [21]. For example, Shi *et al.* [21] mixed gelatine with silk fibroin to optimize the internal structure and prepare a 3D scaffold that meets the needs of tissue engineering scaffolds. On the other hand, gelatine can improve the internal structure of silk fibroin, regulate its mechanical properties and enhance the adhesion of cells [20]. Second, the composite material combines the advantages of the raw materials, as both gelatine and silk fibroin can play a haemostatic role through adsorption, physical closure and other methods.

To further improve the haemostatic ability of the composite haemostatic sponge, we selected a special haemostatic drug, thrombin. Thrombin is a serine protease that is activated by sodium ions and plays an important role in the coagulation cascade, which can activate coagulation factors and promote the conversion of fibrinogen to fibrin [22]. As a haemostatic agent, thrombin can accelerate blood coagulation and shorten wound healing time; due to these properties, thrombin is widely used in clinical practice for the haemostasis of small blood vessels, capillaries and major organs [23]. In previous studies, we found that thrombin exhibits good haemostatic properties [24]. Ali-Mohamad *et al.* also demonstrated its good haemostatic performance through a live pig gastrointestinal bleeding model [25]. However, thrombin has weak adhesion ability and is easily washed away by blood flow, resulting in a decrease in its haemostasis [24,26]. This problem can be effectively solved by loading thrombin into SFG.

In addition, both silk fibroin and gelatine are natural biological macromolecules that can simulate the microenvironment of the extracellular matrix (ECM) and

promote its synthesis [4]. Moreover, gelatine contains RGD sequences, which play important roles in promoting cell adhesion, proliferation and differentiation [8]. Therefore, the composite haemostatic sponge prepared in our study can also promote the healing of skin tissue and accelerate tissue regeneration.

In this study, we prepared an SFG@TB composite haemostatic sponge by freeze-drying. First, we cocultured fibroblasts with a composite haemostatic sponge to evaluate its biological safety *in vitro*. Then we used a Sprague–Dawley (SD) rat model of subcutaneous tissue embedding to evaluate the *in vivo* biosafety of the sponge. Furthermore, to evaluate its coagulation ability, we performed dynamic coagulation experiments and erythrocyte and platelet adhesion experiments *in vitro*. Next, SD rat models of femoral artery haemorrhage and liver haemorrhage were used to evaluate the coagulation ability of the sponge *in vivo*. Finally, we used an SD rat model of full skin injury to evaluate the tissue repair ability of the composite haemostatic sponge.

Methods

Preparation of the sponge

A total of 1 g of gelatine (Zhengzhou Tianshun Food Additives Co., Ltd, Zhengzhou, China) powder was dissolved in 10 ml of phosphate buffer (PBS, purchased from Thermo Fisher Science & Technology Co., Ltd, Shanghai, China) and stirred in a water bath at 50°C for 20 min until it completely dissolved. After the gelatine solution with a concentration of 10% was obtained, the mixture was left at room temperature. A total of 0.2 g of silk fibroin (purchased from Simet Biotechnology Co., Ltd, Suzhou, China) was dissolved in 10 ml of PBS to generate a silk fibroin solution (2%). Thrombin (purchased from Hanhai New Enzyme Biotechnology Co., Ltd, Wuhan, China) was dissolved in sterile PBS (concentration of 1 µg/ml) for use.

Next, 10 ml of gelatine solution (10%), 10 ml of deionized water and 1 ml of PBS solution were added, and the mixture was stirred overnight. Then, the solution was frozen at –80°C for 12 h and dried in a vacuum freeze-dryer for 2 days to generate a gelatine sponge (GE). Similarly, 1 ml of gelatine solution (10%), 1 ml of 2% silk fibroin solution and 1 ml of PBS solution were stirred and lyophilized to generate a silk fibroin–gelatine complex haemostatic sponge (SFG). To form an SFG@TB composite haemostatic sponge, 1 ml of gelatine solution (10%), 1 ml of 2% silk fibroin solution and 1 ml of thrombin solution (1 µg/ml) were mixed, stirred and freeze-dried.

Morphological observations

After immersing the vacuum freeze-dried sponge sample in liquid nitrogen, cut it off quickly to get the sample cross-section. Adhere the sample to the sample stage coated with conductive adhesive and make the cross-section of the sample face upwards. After gold spraying, the internal pore structure was observed by field emission scanning electron microscopy

(SEM, HITACHI SU8010, Japan) at an accelerated voltage of 5 kV.

Fourier-transform infrared spectroscopy

The freeze-dried sponge sample was placed on a Fourier-transform infrared (FTIR) spectrometer (Thermo Scientific, Nicolet 6700, USA) sample station, and the probe was lowered to closely contact the sample for measurements. In total, 128 scans were performed, and the scanning range was 4000–400 cm^{–1}.

Water absorption capacity

The vacuum freeze-dried sponge sample was placed on a 0.45 µm filter membrane in a container filled with PBS buffer at pH = 7.4 so that the liquid could freely pass through the filter membrane to simulate wound seepage, and the swelling properties of the three sponges could be measured. The whole system was placed at 37°C and removed at 0 s, 30 s, 1 min, 5 min, 10 min, 30 min and 60 min. The excess water on the surface was removed with filter paper and the samples were weighed. The swelling rate was calculated as follows:

$$\text{Swelling ratio (\%)} = (m_t - m_0) / m_0 \times 100\%$$

where m_0 is the initial sample weight and m_t is the sample weight at different time points.

Porosity

The porosity of the sponge was determined by the solution volume displacement method. First, the dry sponge was immersed in an ethanol solution with a known volume (V_1) for 12 h. After immersing the sponge in the ethanol solution, record the total volume of the solution as V_2 . The volume of residual ethanol after removing the sponge was denoted V_3 . The porosity calculation formula is as follows:

$$\text{Porosity (\%)} = (V_1 - V_3) / (V_2 - V_3) \times 100\%$$

Mechanical measurements

A sponge sample with a height of 10 mm and a radius of 8 mm was prepared and placed on the operating platform of an electronic universal testing machine (MTS Metus Industrial System, CMT6103 manufacturer) to test the compressive properties of the sponge. The compression speed was 1 mm/min. After the measurements, the compression moduli of the three sponge samples (GE, SFG and SFG@TB) were recorded, and the operation was repeated three times for each group.

Cytotoxicity assay

GE, SFG and SFG@TB samples of different qualities were weighed and soaked in medium for 24 h to obtain extraction solutions with concentrations of 25, 50, 75 and 100 mg/ml for subsequent use.

Cell Counting Kit-8 (CCK-8 (Dojindo, Tokyo, Japan) was used to determine the cytotoxicity of different concentrations of extracts. L929 cells at a density of $10^5/\text{ml}$ were inoculated into a 96-well plate, and different concentrations of extracts from each group of samples were added. After 1 day, the sample was washed with PBS and incubated in 10% CCK-8 (Dojindo, Tokyo, Japan) solution. After incubation for 2 h, the absorbance of the solution at 450 nm was measured using an enzyme reader (SpectraMaxM2, Molecular Devices, Sunnyvale, CA, USA).

Cell proliferation assay

L929 cells at a density of $10^5/\text{ml}$ were inoculated into a 96-well plate, and an extract at a concentration of 100 mg/ml was added to each group of samples. After 1, 3 and 5 days of culture, the samples were washed with PBS and incubated in 10% CCK-8 solution. After incubation for 2 h, the absorbance of the solution at 450 nm was measured using an enzyme reader (SpectraMaxM2, Molecular Devices, Sunnyvale, CA, USA).

Cell live/dead staining and cytoskeletal staining

To further evaluate cell compatibility, we cocultured L929 cells with 100 mg/ml of each group of extraction solutions at 38°C for 1 day. Then, the cells were washed with PBS and stained with calcein-AM/propidium iodide in a dark environment for 30 min. After the cells were cleaned, they were observed by confocal microscopy (Nikon A1, Tokyo, Japan). Additionally, the cells were stained with 4,6-diamino-2-phenylindole (Sigma, USA) and F-actin (Solarbio, China) and observed by confocal laser microscopy (OLYMPUS FV1000, Tokyo, Japan) on the third day.

Cell migration assay

L929 cells at a density of $10^5/\text{ml}$ were inoculated into a 96-well plate and cultured in a constant temperature incubator. After the cells had fully grown, a sterile gun was used to evenly scratch the wells. The old culture medium was discarded, and the cells were washed with PBS two to three times until the scratched cells were washed clean. The extraction solution was added according to the grouping, and photos were taken as the 0 h control. The cells were incubated at 37°C in a 5% CO_2 incubator. The scratch width was observed, and images were taken at the same position after 24 and 48 h.

Genome expression analysis

After 5 d of culture, total RNA was extracted from L929 cells grown on sponges using an RNA purification kit (ES Science, Shanghai, China) and then reverse transcribed into cDNA using a reverse transcription kit (ABM, Vancouver, Canada). Then, quantitative real-time PCR was performed on a CFX96TM Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). The relative gene expression was

calculated via the $2^{-\Delta\Delta\text{Ct}}$ method and normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in this study were as follows: Col I a1: F1: 5'-TAG AGG CTC TGA AGG TCC CC-3', R1: 5'-CAC CAG CAA TAC CAG GAG CA-3'; Col III a1: F1: 5'-CAG CCA GGT CGA GAT GGA TC-3', R1: 5'-CAG GGC CAG TTT CTC CTC TG-3', F2: 5'-ATG CCC ACA GCC TTC TAC AC-3', R2: 5'-GGG TCA CCA TTT CTC CCA GG-3'; GAPDH: F1: 5'-GAA GGG CAT CTT GGG CTA CAC-3', R1: 5'-GTT GTC ATT GAG AGC AAT GCC A-3'.

In vivo biosafety of the sponge

To evaluate the biocompatibility and degradability of the sponges *in vivo*, SD rats were anaesthetized with pentobarbital sodium and the hair on the back was removed. An incision was created on the back skin and 20 mg of sample was implanted. In the blank control group, only skin incisions and sutures were created without embedding any material. The positive control group was implanted with filter paper. Skin reactions in each group were observed on days 0, 3, 7 and 14. After 14 days, the rats in each group were euthanized and the whole skin tissue of each group was stained with haematoxylin-eosin (HE). Then, pathological changes in the skin, especially foreign body reactions and inflammatory reactions, were observed. The degradation performance of the material was also evaluated. In addition, the shape and size of the heart, lung, stomach, liver, spleen and kidney in the blank group and the SFG@TB group were observed. Then, pathological changes in these organs were observed by HE staining to assess whether SFG@TB caused substantial damage to various organs after degradation and absorption.

Measurement of the whole blood coagulation index

Fresh rat blood was extracted and mixed with anticoagulant citrate dextrose solution (20 mM citric acid, 110 mM sodium citrate, 5 mM D-glucose) at a ratio of 9 : 1 (v/v) to generate anticoagulant whole blood for later use. The GE, SFG and SFG@TB samples were cut into squares of the same size (0.5×0.5 cm) and placed into 5 ml centrifuge tubes. An orifice plate without samples was used as the control group. Then, 50 μl of anticoagulant whole blood was added to the surface of each group of samples and 10 μl of 0.2 mol/l CaCl_2 solution was added to initiate coagulation. Then, the samples were placed in a constant temperature oscillating incubator at 37°C for 30, 60, 90 and 120 s. Afterwards, 3 ml of deionized water was added to each sample, which was gently shaken and incubated for 5 min. Finally, the absorbance of the supernatant was measured at 545 nm by an enzymograph. The whole blood coagulation index (BCI) was calculated as follows:

$$\text{BCI} = [(A_s - A_b) / (A_c - A_b)] \times 100\%$$

A_s is the optical density (OD value of the experimental group. A_c is the OD value of the control group. A_b is the OD

value of the blank well. Each group of samples was tested three times.

Erythrocyte and platelet adhesion *in vitro*

To measure erythrocyte adhesion, the GE, SFG and SFG@TB samples were placed in 24-well perforated plates. Then, 1 ml of anticoagulant whole blood was added to each sample individually and the 24-well plates were incubated in a 37°C incubator for 5 min to ensure that the samples fully interacted with the blood. Next, deionized water was slowly dripped onto the surface of each sample to wash away the erythrocytes that were not trapped by the material. The samples were fixed with 2.5% glutaraldehyde for 12 h and dehydrated with gradient alcohol. Then, after gold spraying, the morphology and number of erythrocytes adhering to the surface of the samples were observed by SEM.

The procedure for platelet adhesion was similar to that used for the adhesion of erythrocytes, but the anticoagulant whole blood was centrifuged at 800 rpm for 10 min. The supernatant was collected with a pipette to obtain platelet-rich plasma, which was then dripped onto the surface of the sample. After the samples were incubated for 2 h, SEM was used to observe the number and activation degree of platelets adhering to the surface of the sample.

Haemolytic analysis

Fresh anticoagulant blood was diluted five times with NaCl solution (0.9%). Fresh blood diluted with deionized water served as a positive control, while fresh blood diluted with NaCl solution served as a negative control. Fresh blood was cultivated with different samples at 37°C for 30 min. The absorbance was measured at 541 nm and the haemolysis rate was calculated according to the following formula:

$$\text{Haemolysis ratio (\%)} = (\text{OD}_1 - \text{OD}_2) / \text{OD}_3 \times 100\%$$

OD₁ represents the experimental group, OD₂ represents the control group and OD₃ represents the deionized water group.

In vivo haemostatic effect of the sponge

All experimental procedures in this study were carried out in accordance with ethical guidelines and approved by the Animal Ethics Committee of Peking University People's Hospital (approval number: 2021PHE067). Thirty SD rats purchased from Beijing Weitonglihua Laboratory Animal Technology Co., Ltd (Licence No. SCXK (JING) 2021-0011), were randomly divided into the following groups, with six animals in each group: the control group, the GE group, the SFG group, the SFG@TB group and the commercial group (with absorbable soluble haemostatic materials purchased from Qingdao Zhonghui Shengxi Biological Engineering Co., Ltd, Qingdao, China).

Femoral artery haemostasis experiment The SD rats were anaesthetized and fixed in the supine position. The skin and

muscle were cut layer by layer from the inner thigh to expose the femoral artery. After the femoral artery was punctured with the needle of a 1 ml syringe, materials from each group were placed at the wound and fully fitted to the wound through appropriate pressure. The time at which the bleeding completely stopped was recorded, as well as the amount of bleeding.

Liver haemostasis experiment SD rats were anaesthetized, fixed in the supine position, shaved and disinfected; a median abdominal incision ~5 cm long was created, and the skin, muscle and peritoneum were cut layer by layer. The liver was exposed into the abdomen. After the right lobe of the liver was fully exposed, a scalpel was used to make a cutting wound ~0.5 cm long and 0.2 cm deep. The prepared materials of each group were placed into the wound, which was fully covered and attached to the liver tissue. A dry cotton ball was applied to the wound on the outside of the material. The time and amount of bleeding were recorded.

In vivo wound healing effect of the sponge

Twenty-four SD rats purchased from Beijing Weitonglihua Laboratory Animal Technology Co., Ltd [Licence No. SCXK (JING) 2021-0011], were randomly divided into the following groups with six animals in each group: the control group, the GE group, the SFG group and the SFG@TB group.

After anaesthesia, the back hair of the rats was removed and a skin defect wound with a diameter of 1 cm was created in the middle of the dorsal side with a skin punch. Skin defects were covered with corresponding sponges in each group except for the control group. The wounds were photographed on the 3rd, 7th and 14th days after treatment to record the healing process of the wounds. The following formula was used to calculate the wound healing rate:

$$\text{Wound healing rate} = (A_0 - A) / (A) \times 100\%$$

A₀ was the initial wound area, and A was the current wound area.

Histological assessment

The rats were killed on the 14th day after treatment and the tissues were fixed with phosphate-buffered formalin for 24 h, dehydrated and embedded in paraffin. The paraffin-embedded samples were cut into 5 μm slices, dewaxed with xylene and dehydrated with gradient alcohol. The sections were then stained with HE according to the established protocol. A pathologist blinded to the groups performed the histological analysis to assess wound healing. Then, collagen deposition was observed using Masson three-colour staining and the number of newly formed hair follicles in each group was quantified. The sections were incubated with collagen I primary antibodies and collagen III primary antibodies, and the positive staining results were quantified.

In addition, the sections were incubated with CD31 primary antibodies on the 7th and 14th days and the positive results were quantified. Furthermore, the expression levels of vascular endothelial growth factor (VEGF) and fibronectin (FN) in the wound tissue were measured using an enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Statistical analysis

All the data are expressed as the mean $\pm$ standard deviation. SPSS 13.0 statistical software and one-way analysis of variance were used to analyse the data. Differences between multiple groups were analysed using one-way analysis of variance followed by Tukey's *post hoc* test. * $P < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered to indicate statistical significance. ns indicates $p > 0.05$, defined as no significant difference in the results.

Results

Characterization of the composite haemostatic sponge

To evaluate the material properties of the composite haemostatic sponge, the pore diameter, porosity, swelling rate and elastic modulus of GE, SFG and SFG@TB were measured, and stress curves and FTIR spectra were obtained (Figure 1). The SEM scanning images and diameter distributions of GE, SFG and SFG@TB are shown in Figure 1a, and they all had smooth surfaces and uniformly arranged pore structures. The pore diameter was also measured, and compared to GE ($50.47 \pm 14.10 \mu\text{m}$), SFG ($145.43 \pm 61.52 \mu\text{m}$) and SFG@TB ($119.66 \pm 38.92 \mu\text{m}$) had larger pores. In addition, the porosity results in Figure 1g show that the porosities of SFG ($32.41 \pm 4.10\%$) and SFG@TB ($32.66 \pm 2.58\%$) were significantly greater than that of GE ($16.20 \pm 4.08\%$, $p < 0.05$).

FTIR spectroscopy was performed to characterize the interaction between the functional groups and molecules of the composite haemostatic sponges. The results are shown in Figure 1b. The peaks near 1516 and 531 cm^{-1} indicated that the characteristic functional groups, such as nitrocellulose and halogen groups, did not change during the preparation process. To further evaluate the swelling properties of the composite haemostatic sponges, we tested their swelling rates. The results in Figure 1c and d show that after ~ 10 min, the swelling rates of the three sponges almost reached saturation. At all points, the swelling rates of SFG and SFG@TB were greater than that of GE, indicating that the liquid absorption speed and capacity of SFG and SFG@TB were greater than those of GE. Figure 1e and f show the stress-strain curves and elastic modulus results, indicating that SFG and SFG@TB exhibited better mechanical properties than GE.

Biosafety of the composite haemostatic sponge *in vitro*

Cell experiments were conducted to evaluate the biosafety of sponges and the ability to promote wound healing (Figure 2a). The CCK-8 assay results are shown in Figure 2b

and c. As shown in Figure 2b, the survival rates of cells cultured on GE, SFG and SFG@TB at different concentrations for 1 day were all $>90\%$. After the cells were cultured on GE, SFG or SFG@TB for 1, 2 or 3 days, the absorbance of the CCK-8 solution gradually increased, as shown in Figure 2b, indicating

that the number of cells gradually increased. The above results indicate that all samples exhibited good biocompatibility. The results of calcein-AM/propidium iodide staining are shown in Figure 2d. Living cells were stained green by calcein-AM and dead cells were stained red by propidium iodide. In Figure 2d, the amount of red fluorescence was very small, indicating that the GE, SFG and SFG@TB composite haemostatic sponges exhibited good cytocompatibility. Furthermore, cytoskeleton staining revealed that the cells exhibited a typical spindle shape and had good morphology (Figure 2e). The results and image of the cell migration experiment showed that the healing rate of the SFG@TB group was the highest (Figure 2f and i). Therefore, the ability of SFG@TB to promote cell migration was greater than that of GE and SFG. The PCR results are shown in Figure 2g and h. The relative mRNA expression levels of collagen I and collagen III genes in the SFG@TB group were significantly greater than those in the GE and SFG groups.

Biosafety of the composite haemostatic sponge *in vivo*

To further evaluate the biosafety of the samples, filter paper (control group), GE, SFG and SFG@TB sponges were implanted into the subcutaneous tissue of the backs of the rats (Figure 3a). Wound images were recorded on days 0, 3, 7 and 14 after surgery, as shown in Figure 3b. On the 7th day, the wounds in the control group showed obvious redness and swelling. After 14 days, all wounds recovered completely and the SFG@TB group exhibited the greatest degree of healing. In addition, the HE results showed more serious foreign body granulomas and inflammation in the control group, while the other groups remained normal, indicating that GE, SFG and SFG@TB all had good biosafety.

To further investigate the biotoxic effects of SFG@TB and its degradation products on other organs, including the spleen, kidney, stomach, heart, liver and lung, we compared various organs in SD rats with and without subcutaneous implantation of SFG@TB. As shown in Figure 3c and d, the shape and size of organs in the SFG@TB group did not change significantly and were similar to those of normal rats. HE staining revealed no obvious damage to the organs of the rats implanted with SFG@TB, indicating that SFG@TB and its metabolites exerted no obvious adverse effects on the circulatory, respiratory, digestive or metabolic systems.

Haemostatic property and haemocompatibility evaluation of the composite haemostatic sponge *in vitro*

To evaluate the coagulation function of the sponge, we performed coagulation tests *in vitro* Figure 4f. After calcium ions were added, images and BCIs of the supernatants were

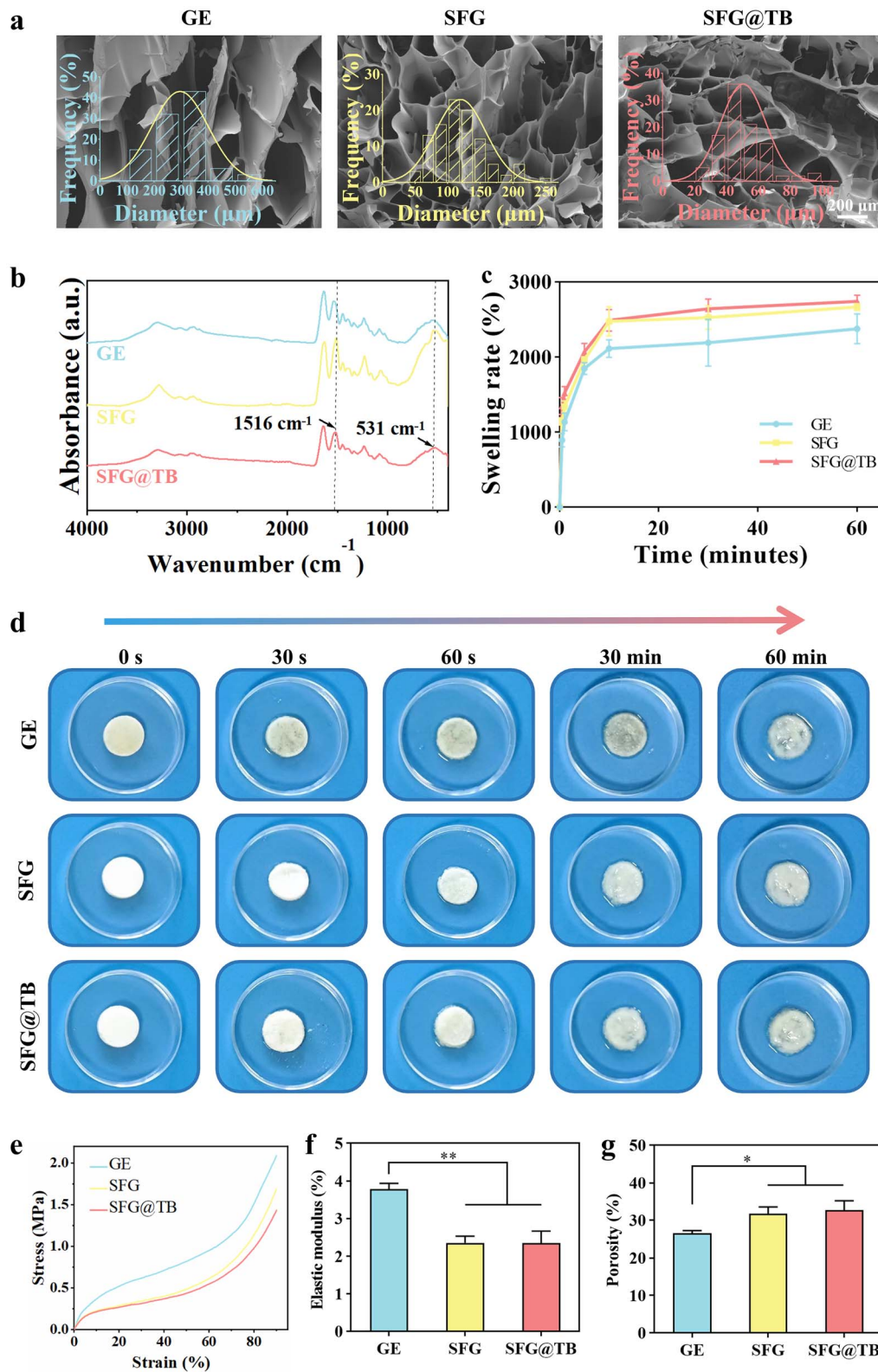


Figure 1. Material characterization of the composite haemostatic sponge. (a) SEM images and diameter distribution of GE, SFG and SFG@TB (scale bar: 200 μm). (b) FTIR spectra of GE, SFG and SFG@TB in the scanning range of 4000–400 cm⁻¹. (c) Swelling rate and (d) photos of GE, SFG and SFG@TB during the swelling process. (e) Strain curves, (f) elastic modulus and (g) porosity of GE, SFG and SFG@TB. **p* < 0.05, ***p* < 0.01. GE gelatine sponge, SFG silk fibroin-gelatin sponge, SFG@TB silk fibroin-gelatin sponge loaded with thrombin

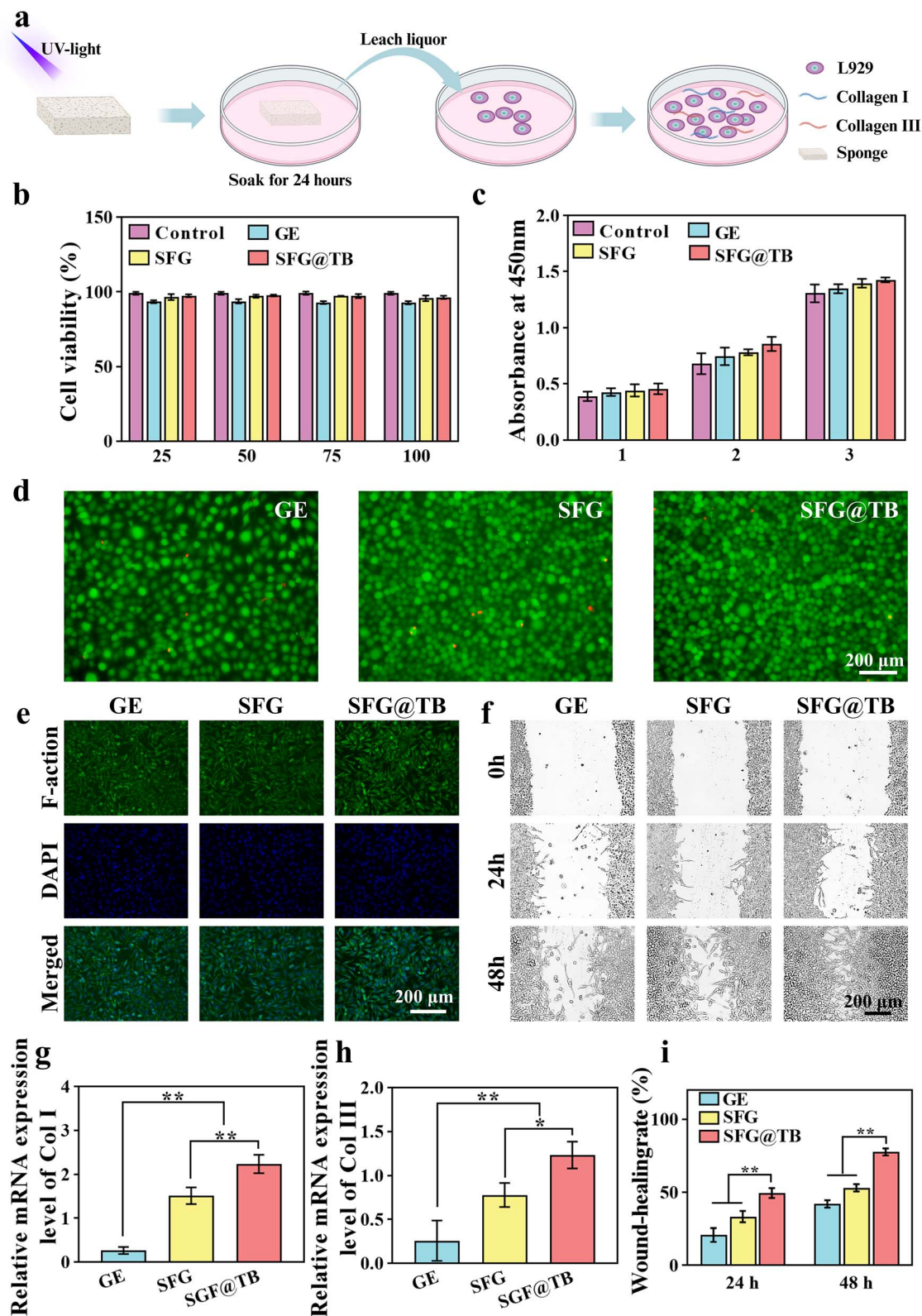


Figure 2. The biosafety and ability of sponges to promote cell migration in vitro. (a) Schematic diagram of cell culture. (b) Cell viability of fibroblasts cultured with different concentrations of the control, GE, SFG, or SFG@TB for 24 h. (c) Absorbance of fibroblasts cultured on 100 mg/mL control medium, GE, SFG or SFG@TB for 1 day, 2 days, or 3 days. (d) Calcein-am/propidium iodide staining image (scale bar: 200 μ m). (e) Cytoskeletal staining image at 3 days. Cells were stained with F-actin and DAPI (scale bar: 200 μ m). (f) Image of the cell migration experiment (scale bar: 200 μ m) and (i) the wound healing rate. Relative mRNA expression levels of (g) collagen I and (h) collagen III. ** $p < 0.01$. GE gelatine sponge, SFG silk fibroin-gelatin sponge, SFG@TB silk fibroin-gelatin sponge loaded with thrombin, *Col I* collagen I, *Col III* collagen III

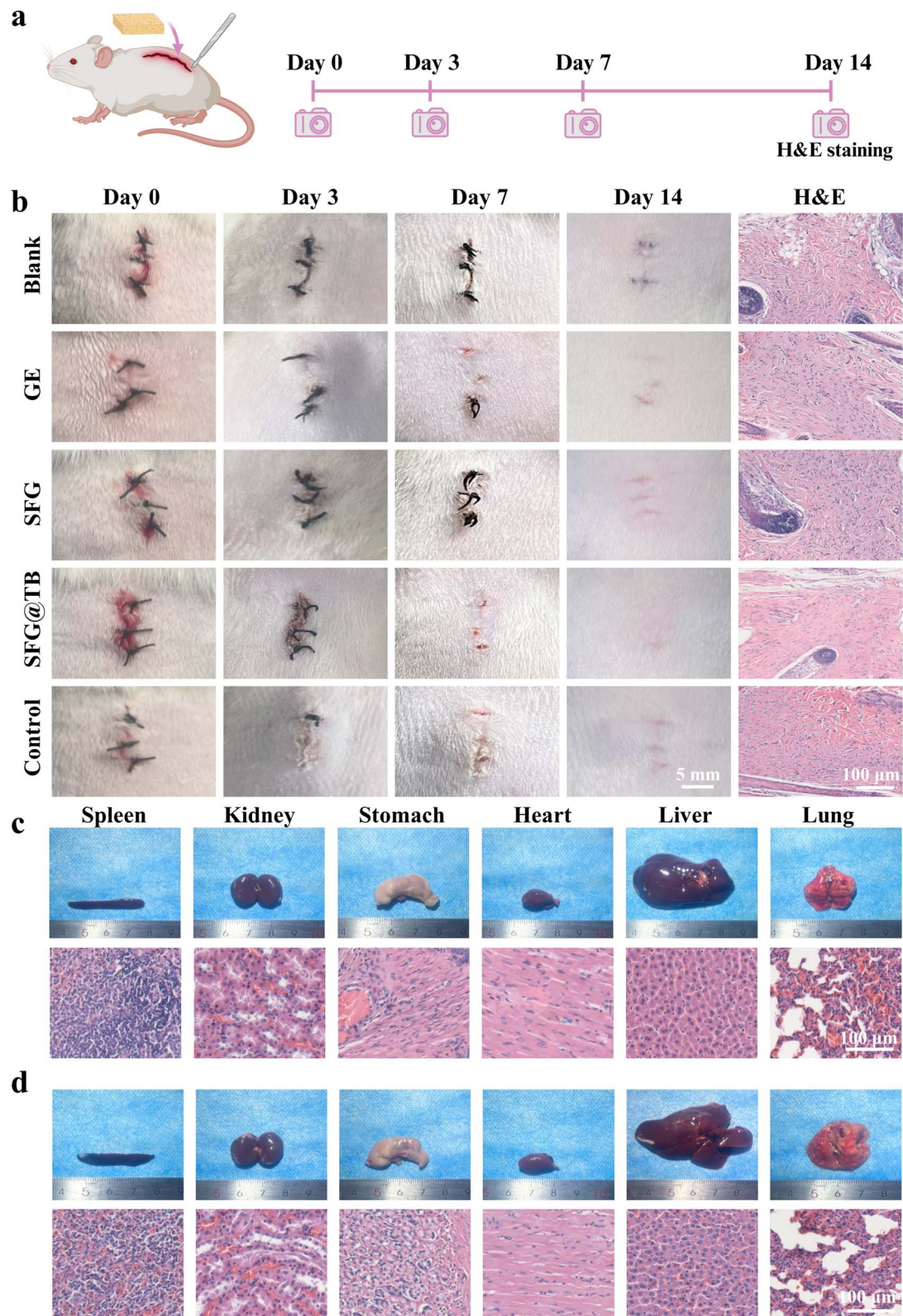


Figure 3. The biosafety of the sponges in vivo. (a) Schematic diagram of the embedding experiment. (b) Wound photos of the GE, SFG, SFG@TB, control and blank groups on the 0th day, 3rd day, 7th day and 14th day after surgery (scale bar: 5 mm), and H&E staining images (scale bar: 300 μ m) of the subcutaneous tissue of the wound on the 14th day after the embedding surgery. Images and HE staining images of rat internal organs (spleen, stomach, kidney, heart, liver and lung) after embedding in (c) the SFG@TB group and (d) the blank control group for 14 days (scale bar: 100 μ m). *GE* gelatine sponge, *SFG* silk fibroin-gelatine sponge, *SFG@TB* silk fibroin-gelatine sponge loaded with thrombin

recorded at different times, as shown in Figure 4a and b. At each time point, the supernatant clarity of the SFG@TB group was always higher than that of the other groups, which is consistent with the results of BCI. At 120 s, the BCI of the SFG@TB group ($7.34 \pm 1.17\%$) was significantly lower than that of the SFG ($30.45 \pm 3.56\%$, $p < 0.01$) and GE ($61.23 \pm 10.23\%$, $p < 0.01$) groups. The results showed that the *in vitro* coagulation ability and rate of SFG@TB were significantly improved after thrombin was added. All the above results indicate that compared to the other groups, SFG@TB had better coagulation ability *in vitro*.

We also carried out erythrocyte and platelet adhesion experiments, as shown in Figure 4c and d. Compared to GE and SFG, SFG@TB had more activated and attached platelets and erythrocytes due to the excellent haemostatic function of thrombin and the high porosity of SFG@TB. At the same time, these findings indicate that thrombin can remain active in SFG@TB and play a role in promoting fibrin formation and the adhesion of erythrocytes and platelets. In addition, all the samples showed a low haemolysis effect (Figure 4e), indicating that the samples exhibited good blood compatibility.

Haemostatic properties of the composite haemostatic sponge *in vivo*

We tested the haemostatic performance of GE, SFG and SFG@TB in liver and femoral artery haemorrhage models. The photographs are shown in Figure 5a and d. The amount of blood loss and haemostatic time in the liver haemorrhage model are shown in Figure 5b and c. As shown, the blood loss (241.32 ± 31.57 mg) and haemostatic time (83.67 ± 13.72 s) of the SFG@TB group were significantly lower than those of the GE (949.87 ± 130.09 mg, 129.50 ± 8.22 s, $p < 0.01$), SFG (615.05 ± 92.17 mg, 109.33 ± 7.63 s, $p < 0.01$) and control (1261.72 ± 147.76 mg, 149.17 ± 13.67 s, $p < 0.01$) groups. There was no significant difference between SFG@TB and commercial haemostatic materials (231.77 ± 28.90 mg, 71.17 ± 7.22 s, $p > 0.05$).

The results were similar in the femoral artery haemorrhage model. The amount of blood loss and haemostatic time are shown in Figure 5e and f. SFG@TB blood loss (89.73 ± 18.64 mg) and haemostatic time (41.83 ± 6.68 s) were also significantly lower than those of the GE (210.75 ± 14.75 mg, 96.00 ± 11.90 s, $p < 0.01$), SFG (159.10 ± 17.24 mg, 78.17 ± 5.42 s, $p < 0.01$) and control (261.53 ± 20.68 mg, 122.83 ± 13.04 s, $p < 0.01$) groups. Additionally, SFG@TB was not significantly different from commercial haemostatic materials (113.32 ± 19.97 mg, 37.50 ± 4.40 s, $p > 0.05$).

Promotion of tissue healing by the composite haemostatic sponge

To evaluate the ability of GE, SFG and SFG@TB to promote tissue healing, we conducted a full-layer skin injury healing experiment in SD rats Figure 6a. The images and results of the

quantification are shown in Figure 6b and c. The wound healing rate in the SFG@TB group was significantly greater than that in the other groups. On the 7th day, the width and area of the wounds in the SFG@TB group were significantly smaller than those in the other groups, and the wounds were completely healed on the 14th day. To further evaluate the degree of healing, HE staining was performed on the skin tissue on the 14th day, and the results are shown in Figure 6d. Compared to the control, GE and SFG groups, SFG@TB had thicker granulation tissue and narrower scar width. The scar width and granulation tissue thickness recorded in the experiment are shown in Figure 6e and f. As shown, the granulation tissue thickness of the SFG@TB group (407.17 ± 13.39 μ m) was significantly greater than that of the GE (293.30 ± 28.14 μ m, $p < 0.01$), SFG (340.03 ± 16.63 μ m, $p < 0.01$) and control (279.62 ± 18.56 μ m, $p < 0.01$) groups. Moreover, the scar width in the SFG@TB group (275.47 ± 20.95 μ m) was significantly smaller than that in the GE (643.02 ± 80.10 μ m, $p < 0.01$), SFG (371.99 ± 38.32 μ m, $p < 0.01$) and control (679.65 ± 58.03 μ m, $p < 0.01$) groups. This finding demonstrated that SFG@TB was significantly superior to GE, SFG and the control group at promoting tissue healing.

In addition, other histological assessment methods have also been used (Figure 7a). Masson staining revealed that collagen deposition in the SFG@TB group was more extensive and abundant than in the other groups (Figure 7b). The number of new hair follicles in the SFG@TB group (70.51 ± 11.29 n/mm²) was significantly greater than that in the GE (40.16 ± 12.76 n/mm², $p < 0.05$), SFG (52.35 ± 10.87 n/mm², $p < 0.05$) and control (19.46 ± 6.15 n/mm², $p < 0.05$) groups (Figure 7d). This result indicates that compared to the other groups, the SFG@TB group had a greater ability to promote ECM reconstruction and wound healing. The results of immunohistochemical staining for collagen I and collagen III are shown in Figure 7c. The sedimentation areas of collagen I and collagen III in the SFG@TB group were greater than those in the GE, SFG and control groups ($p < 0.01$) (Figure 7e and f). These results indicate that SFG@TB can promote wound healing by promoting the deposition and remodelling of two types of collagen. CD31 is an important indicator for evaluating the generation of new blood vessels during wound healing. Compared with that of the GE, SFG and control groups, the wound site treated with SFG@TB showed greater expression of CD31 on day 7 ($p < 0.05$). In addition, on the 14th day, the expression levels of CD31 in the SFG@TB and SFG groups were similar and significantly greater than those in the GE and control groups ($p < 0.05$) (Figure 7g and h). These results indicate that SFG@TB has the greatest effect on promoting angiogenesis and can thus accelerate wound healing. The concentration of VEGF and FN showed that the expression of VEGF and FN in the SFG@TB group was significantly higher than those in the other groups (Figure 7i and j). Therefore, SFG@TB can promote the expression of VEGF and FN and thus promote vascular proliferation and tissue regeneration.

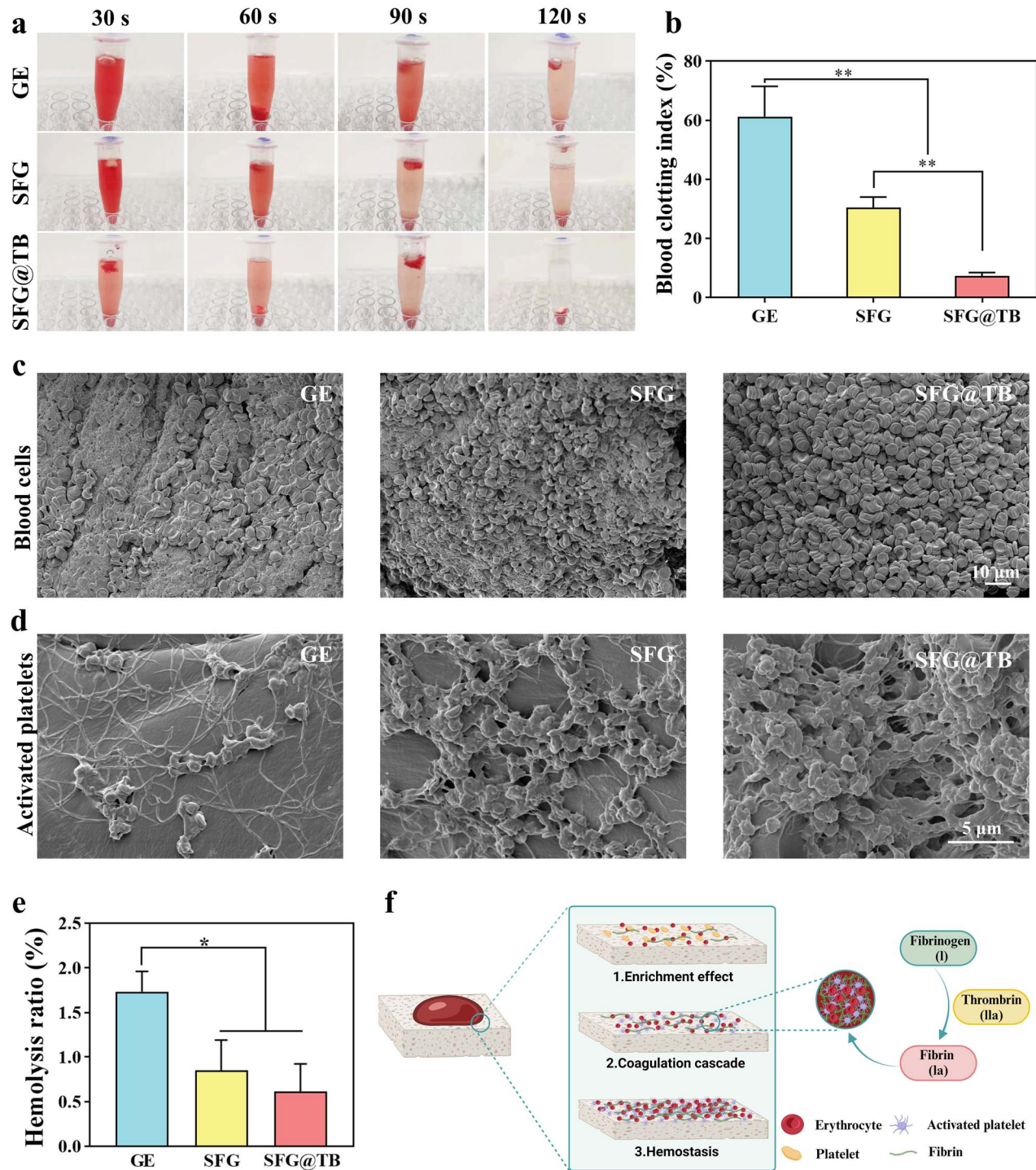


Figure 4. The haemostatic properties and haemocompatibility of the sponges were evaluated in vitro. **(a)** Photos of the supernatant after adding deionized water for anticoagulation at 30 s, 60 s, 90 s, and 120 s, and **(b)** BCI value at 120 s. **(c)** SEM images of the erythrocyte adhesion test (scale bar: 10 μ m) and **(d)** platelet adhesion test on GE, SFG and SFG@TB (scale bar: 5 μ m). **(e)** Haemolysis ratio of GE, SFG and SFG@TB. **(f)** Schematic diagram of coagulation in vitro. * $p < 0.05$, ** $p < 0.01$. GE gelatine sponge, SFG silk fibroin-gelatine sponge, SFG@TB silk fibroin-gelatine sponge loaded with thrombin

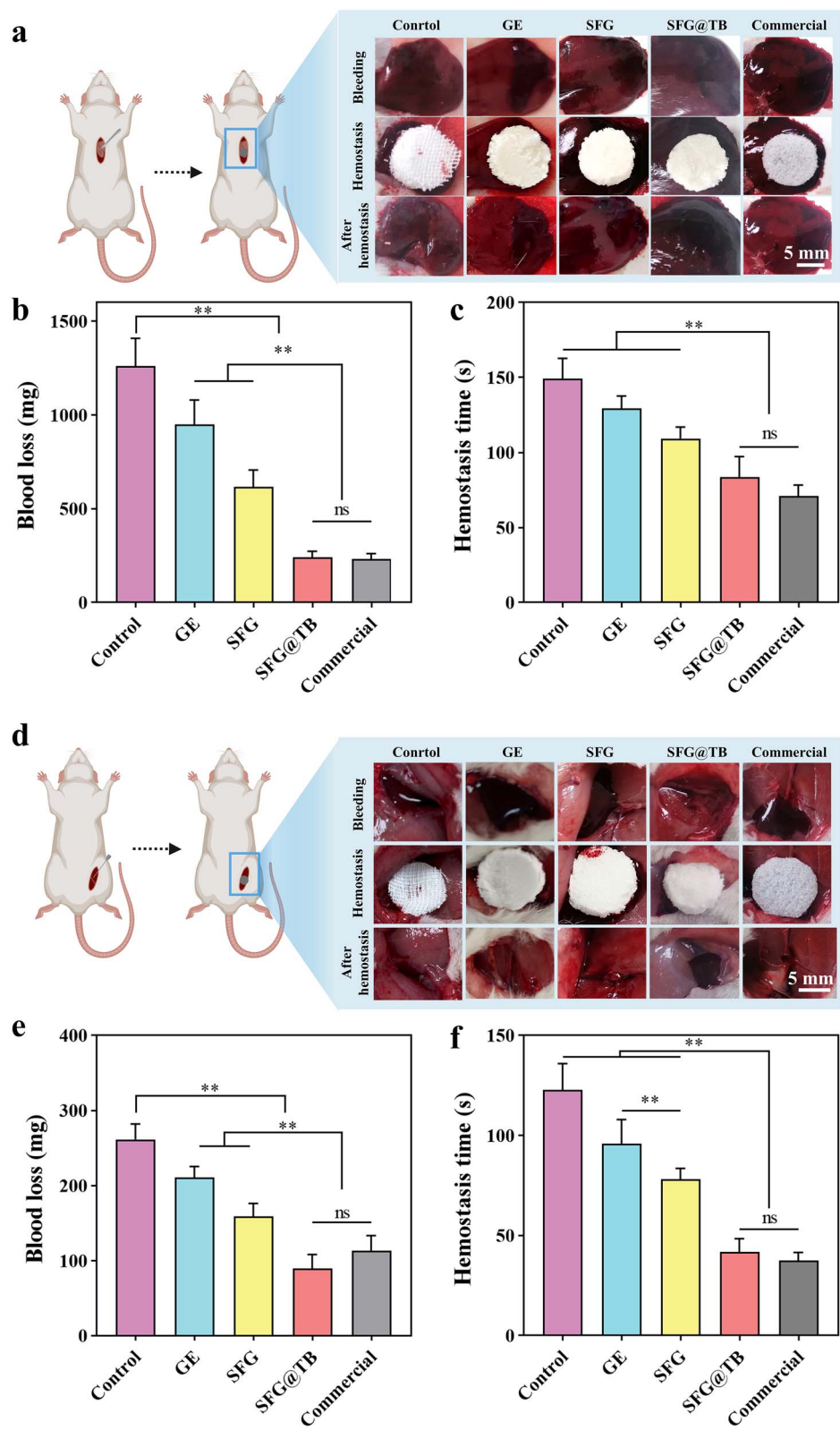


Figure 5. The haemostatic properties of the sponges were evaluated *in vitro*. (a) Schematic diagram and images of the rat model for liver haemorrhage (scale bar: 5 mm). (b) Blood loss and (c) haemostatic time in the rat model for liver haemorrhage. (d) Schematic diagram and images of the rat model for femoral artery haemorrhage (scale bar: 5 mm). (e) Blood loss and (f) haemostatic time in the rat model for femoral artery haemorrhage. $n=6$, $**p < 0.01$, ns means $p > 0.05$. GE gelatine sponge, SFG silk fibroin-gelatine sponge, SFG@TB silk fibroin-gelatine sponge loaded with thrombin

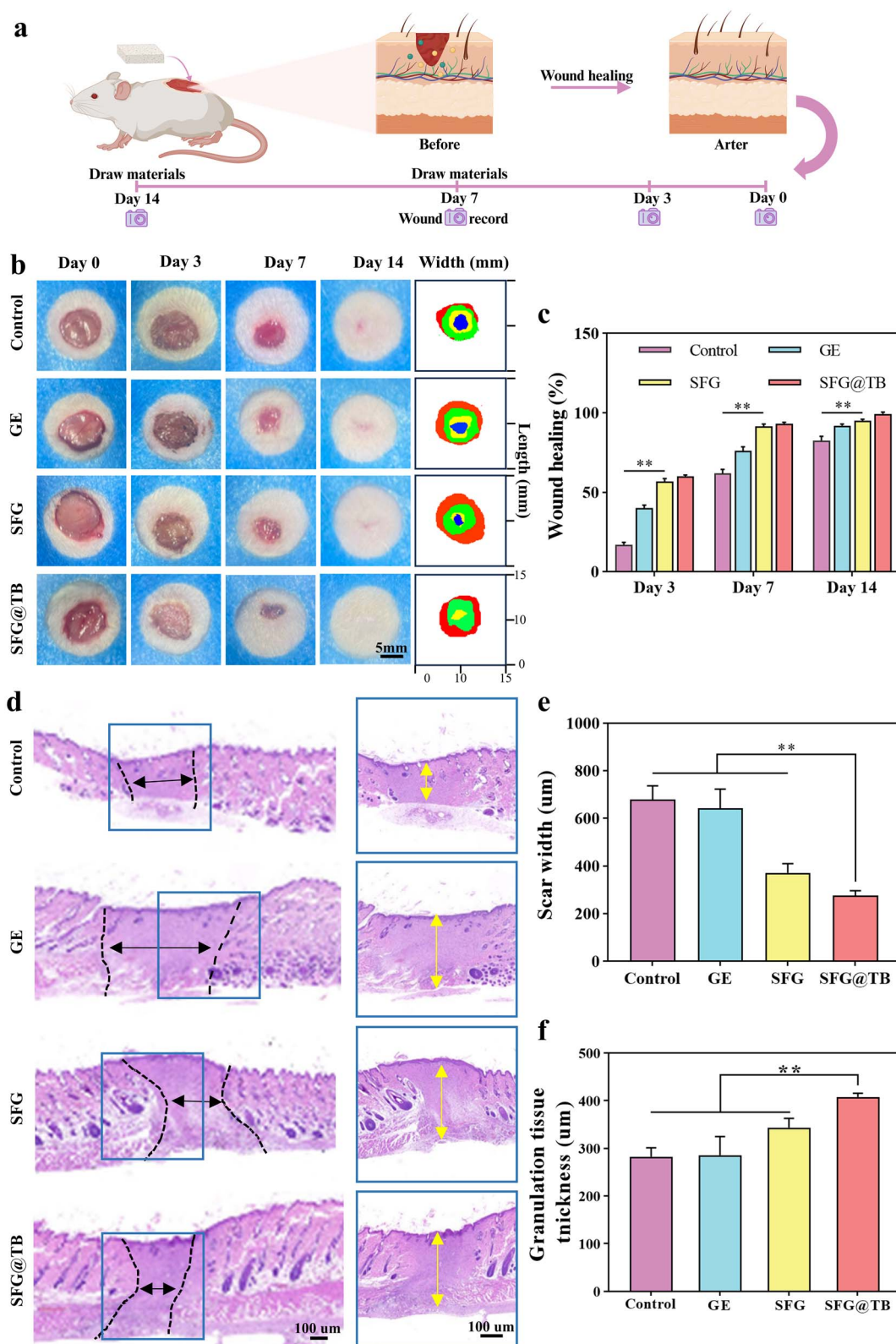


Figure 6. The ability of sponges to promote tissue healing. (a) Schematic diagram of wound healing. (b) Photos and quantified images (scale bar: 5 mm) and (c) the wound healing rate of the full-layer skin injury model rats on Days 0, 3, 7 and 14. (d) H&E staining images of the rat full-layer skin injury model on the 14th day (scale bar: 100 μ m). The dashed line represents the outline of the regenerated dermis and granulation tissue, as well as the width of the scar. The distance between the two triangles represents the thickness of granulation tissue. (e) The scar width and (f) granulation tissue thickness of the rat full-layer skin injury model on the 14th day. $n=6$, $**p < 0.01$. GE gelatine sponge, SFG silk fibroin-gelatine sponge, SFG@TB silk fibroin-gelatine sponge loaded with thrombin

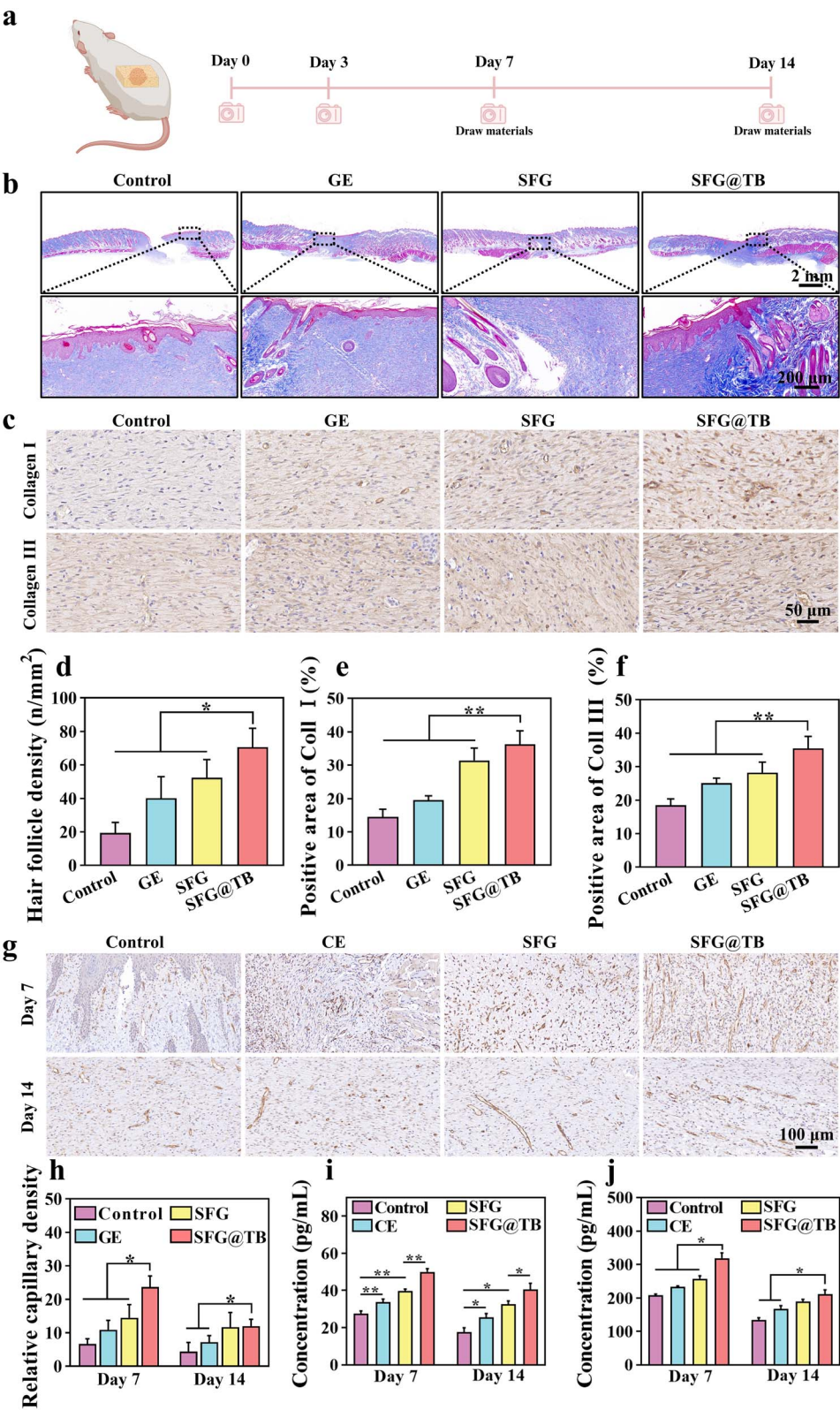


Figure 7. Evaluation of the ability of sponges to promote tissue healing from a histological perspective. **(a)** Schematic diagram of the staining test. **(b)** Masson staining image (scale bar: 200 μm). **(c)** Immunohistochemical staining of collagen I and collagen III in wound tissues on Day 14 (scale bar: 50 μm). Quantification of **(d)** hair follicles, **(e)** collagen I and **(f)** collagen III. **(g)** Immunohistochemical staining of CD31 on Days 7 and 14 (scale bar: 100 μm) and **(h)** quantification of the CD31-positive area. The concentrations of **(i)** VEGF and **(j)** FN in the wound tissue were measured by ELISA on Days 7 and 14. **p* < 0.05, ***p* < 0.01. GE gelatine sponge, SFG silk fibroin-gelatine sponge, SFG@TB silk fibroin-gelatine sponge loaded with thrombin, Coll I collagen I, Coll III collagen III

Discussion

Haemostasis is very important for treating patients with trauma. As mentioned above, good haemostatic materials should exhibit biocompatibility and biodegradability, achieve fast and effective haemostatic performance, and be easy to store and use [27]. However, few haemostatic materials currently used in clinical practice meet the above standards. On this basis, we prepared an SFG@TB composite haemostatic sponge for wound haemostasis and tissue regeneration using freeze-drying technology.

The performance of haemostatic materials is affected by the raw materials and the processing form [28]. Sponge, a common form of haemostatic material, mainly achieves haemostatic effects by absorbing water through its porous structure and aggregating platelets and coagulation factors [29]. The physicochemical properties of sponges, such as porosity, swelling rate and mechanical properties, greatly influence their haemostatic performance [30]. A high porosity and swelling rate enable sponges to rapidly absorb liquid and agglutinate platelets and clotting factors [31], and appropriate mechanical properties can alleviate the discomfort and pain of patients during the use of haemostatic sponges [32]. Gelatine sponges are the most commonly used haemostatic material in the clinic [12]. However, gelatine sponges exhibit poor mechanical properties, and they easily break after absorbing blood; thus, the application scope of gelatine sponges is relatively limited [33]. In this study, GE, SFG and SFG@TB haemostatic sponges were prepared and their material properties were tested. The experimental results showed that the porosity, elastic modulus and swelling rate of SFG and SFG@TB were greater than those of GE, indicating that the mechanical strength and water absorption properties of the haemostatic sponge were effectively improved after silk fibroin was added. Additionally, through repeated experiments, the appropriate temperature and conditions for the chemical reaction were determined, providing a basis for possible industrial production in the future; furthermore, these results have important practical value. Moreover, in the SFG@TB composite haemostatic sponge, the three raw materials have good biocompatibility. Thrombin is a key enzyme in the clotting cascade and can be extracted from human or animal blood [34]. Both gelatine and silk fibroin are natural macromolecular materials. Furthermore, coculture of the composite haemostatic sponge with fibroblasts, and subcutaneous tissue embedding of SD rats, confirmed that the SFG@TB haemostatic sponge had good biosafety.

Thrombin occurs in the human body as prothrombin, which is activated under specific conditions to produce thrombin [35]. Thrombin, which is located at the end of the coagulation cascade, can promote the transformation of fibrinogen into fibrin, activate clotting factor XIII, accelerate the formation of blood clots and activate platelets to promote platelet aggregation [36]. Thrombin is widely used in clinical practice because it has a rapid haemostatic process, significant effect, wide application range and does not destroy the normal coagulation mechanism [27,37]. At the same

time, gelatine and silk fibroin can have haemostatic effects through blood adsorption, blocking the wound surface, and enriching platelets and coagulation factors [26]. In conclusion, the SFG@TB composite haemostatic sponge can exert haemostatic effects in two main ways. On the one hand, the material is a sponge that can exert haemostatic effects by absorbing water, and aggregating platelets and coagulation factors; on the other hand, thrombin can remain active in the composite and have a haemostatic effect. In the *in vitro* coagulation experiment, the results showed that the SFG@TB composite haemostatic sponge promoted blood coagulation quickly and effectively. In the femoral artery and liver haemorrhage models, SFG@TB quickly infiltrated and absorbed blood, and the haemostatic effect was not significantly different from that of commercial haemostatic dressings. These results indicate that the SFG@TB composite haemostatic sponge has excellent haemostatic performance. However, thrombosis may occur when thrombin enters the circulatory system. Although the probability of this event occurring is low, it cannot be ignored. In the future, this problem may be solved with the use of other new haemostatic drugs.

The process of tissue healing includes the following stages: haemostasis, inflammation, hyperplasia and remodelling [38,39]. Rapid adhesion, migration and proliferation of cells are essential for wound healing [40]. Gelatine contains RGD sequences and matrix metalloproteinase-sensitive degradation sites [7]. Cell migration, proliferation, differentiation and other activities can be achieved through integrin-mediated cell adhesion and cell-mediated enzyme degradation [41]. Silk fibroin, which is widely used in tissue regeneration engineering, has good physical and mechanical properties [42] and has been demonstrated to promote the attachment of fibroblasts and human keratinocytes [16]. In addition, both gelatine and silk fibroin are natural macromolecular materials that can simulate the microenvironment of the ECM, provide a good matrix platform, and promote the transfer of growth factors and cell activities [43]. Therefore, SFG@TB also greatly influences the regeneration of tissue. In summary, thrombin in SFG@TB can promote platelet aggregation, accelerate the formation of prothrombin complexes and help convert fibrinogen into fibrin. Thus, the use of thrombin can promote haemostasis, reduce the formation of blood scabs and haematomas, and reduce the occurrence of related inflammation. Additionally, SFG@TB provides space for epidermal cells and fibroblasts to participate and play a key role in promoting wound healing. On the other hand, SFG@TB can promote cell migration, adhesion and proliferation and further promote tissue regeneration. This result was confirmed through a full-layer skin injury healing experiment in rats. Therefore, the SFG@TB composite haemostatic sponge can also play an active role in tissue healing.

However, our study can be improved in several areas, including the following. (1) Due to research limitations, we could not explore further mechanisms related to haemostasis and tissue regeneration, resulting in a lack of theoretical

support. Determining the mechanism will be an important research goal in the future. (2) The number of animal samples in this study is relatively small, which may cause some bias in this study. (3) We used SD rats as the traumatic bleeding model in this study and did not use large animals, such as pigs. The application of more advanced trauma bleeding models and human experiments is an important future research direction.

Conclusions

In this study, an SFG@TB composite haemostatic sponge was prepared through freeze-drying technology. SFG composite haemostatic sponges were prepared using gelatine and silk fibroin to address the mechanical defects of gelatine and silk fibroin when used alone. Adding thrombin further improved the haemostatic performance of the composite haemostatic sponge. Cell and subcutaneous embedding experiments suggested that the SFG@TB composite haemostatic sponge exhibits great biological safety. The results of coagulation tests *in vitro* and *in vivo* demonstrated that the SFG@TB composite haemostatic sponge had excellent haemostatic function. In addition, it was demonstrated that SFG@TB composite haemostatic sponges play a positive role in tissue healing through SD rat injury model experiments. In short, the SFG@TB composite haemostatic sponge had excellent haemostatic ability and promoted tissue healing through the joint action of gelatine, silk fibroin and thrombin. Overall, the SFG@TB composite haemostatic sponge prepared in this study exhibits high biological safety and good haemostatic performance and can promote tissue healing; therefore, this material has great application potential in the clinic.

Abbreviations

BCI: Blood coagulation index; ECM: Extracellular matrix; FN: Fibronectin; FTIR: Fourier-transform infrared; HE: Hematoxylin-eosin; PBS: Phosphate buffer; SEM: Scanning electron microscopy; SFG@TB: Silk fibroin-gelatine composite haemostatic sponge loaded with thrombin.

Authors' contributions

Conceptualization, Y.Z., M.L., Y.W. and W.X.; Methodology, Y.Z., M.L. and J.C.; Software, M.L.; Validation, C.L. and Y.H.; Formal Analysis, M.L. and J.C.; Investigation, Y.Z., M.L. and J.C.; Resources, M.L.; Data Curation, M.L. and J.C.; Writing - Original Draft Preparation, Y.Z., M.L. and J.C.; Writing-Review & Editing, J.C., C.L. and Y.H.; Visualization, Y.Z., M.L. and J.C.; Supervision, Y.W. and W.X.; Project Administration, Y.W. and W.X.; Funding Acquisition, Y.Z., M.L., Y.W. and W.X.

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Data availability

Data collected and analyzed for the study are available from the corresponding author upon reasonable request.

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