Self-Restoring Cryogels Used for the Repair of Hemorrhagic Bone Defects by Modulating Blood Clots

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Abstract

Bleeding from severe traumatic bone defects poses a significant threat to patient survival, and conventional hemostatic agents prove inadequate for addressing bone defects when pressure application is not feasible. Therefore, the development of a bio-multifunctional material with outstanding hemostatic and osteogenic performances is highly desirable. In this study, chitosan/hydroxyapatite nanowires cryogels (CSHAn) were fabricated via Schiff base reaction and electrostatic interaction. These CSHAn cryogels demonstrate an impressive absorption capacity, reaching up to 5387%, and possess considerable mechanical strength, quantified at 167.92 KPa, along with significant compressive recovery capabilities. CSHAn cryogels demonstrate superior blood-clotting ability, enhanced blood cell and platelet adhesion, and activation compared to gelatin sponges. Moreover, in vitro cellular assessments indicate that the developed CSHAn2 exhibits favorable cytocompatibility and significantly promote osteogenesis by upregulating the formation of alkaline phosphatase and calcium nodules. Taken together, this multi-functional cryogels is potentially valuable for clinical applications towards hemorrhagic bone defect repair.

Keywords: Shape memory Cryogels; Chitosan; Hemostasis; Hydroxyapatite nanowires; Blood clot; Hemorrhagic bone defects

Introduction

Bone, as a highly organized and specialized connective tissue, possesses a unique structure and an abundant blood supply. In cases of trauma and surgical procedures, bone hemorrhage tends to be a formidable challenge especially when dealing with highly vascularized bones, such as the spine, pelvis, and sternum. Profound bone hemorrhage can compromise surgical visibility and trigger pathological responses, including tissue necrosis, hemorrhagic shock, and, in severe cases, mortality. Traditional surgical hemostatic methods, such as electrocautery, while effective, carry the risk of damage to the injured blood vessels, causing substantial thermal damage to surrounding tissues and impeding the natural healing of soft tissues. These factors often lead to delays in the bone regeneration. Since 1886, bone wax has been a reliable means of achieving robust physical sealing for hemostasis. However, its non-degradability poses a clinical challenge, as it provokes localized inflammatory responses and exacerbate intrabony infections, thus hindering bone repair process.
Consequently, absorbable hemostatic agents, such as oxidized regenerated cellulose (Surgicel), gelatin sponges (Gelfoam, Spongostan), and collagen fibers (Avitene), have emerged as alternatives in surgical hemostasis. However, these absorbable alternatives primarily rely on compressing the bleeding area, which may not be effective for incompressible bone bleeding. In addition, the degradation rates of these materials often do not match the rate of bone repair, posing a significant challenge. The situation underscores the urgent need to engineer a biofunctional material that combines potent hemostatic properties with osteogenic capabilities.

In contrast to conventional hydrogels, cryogels with water-triggered shape memory properties offer enhanced specific surface areas and more extensive porous architectures. These attributes are conducive to rapid water absorption and expansion, alongside promoting cell adhesion. The distinctive structural configuration of cryogels permits unimpeded water ingress and egress, facilitating shape retention through hydraulic pressure and shape recovery upon hydration. Such features are critically beneficial for managing large, non-compressible cavity wounds, a challenging aspect in tissue engineering and regenerative medicine. Meanwhile, cryogels have a positive impact on the stability of blood clots, enabling the material-clot complex formed after hemostasis to serve as a stable scaffold involved in tissue repair. Chitosan (CS), a natural cationic polysaccharide, boasts excellent biocompatibility, inherent antibacterial activity, and effective hemostatic capabilities. Under acidic conditions, protonated CS binds with negatively charged blood cells and proteins, facilitating coagulation and antibacterial activity. Meanwhile, the positively charged amino groups induce irregular blood cell aggregation, platelet activation via electrostatic interaction, and the formation of clots. Therefore, CS-based cryogels demonstrate superior performances compared to other hemostatic materials. However, the development of hemostatic materials for open bone defects should prioritize not only rapid hemostatic function but also their capacity to promote healing of bone defects, which is critical for patient recovery post-hemostasis.

Cryogels, characterized by their ultra-high porosity due to the formation of ice crystals, typically exhibit relatively weak mechanical strength. To address this
limitation, nanofibers are frequently incorporated into the material matrix to enhance its strength. Among these, Hydroxyapatite nanowires (HAn) have garnered significant interest in biomedical applications. Their popularity stems from their excellent biocompatibility, effective biodegradability, and osteoinductive properties, making them highly suitable for medical use.\textsuperscript{[17, 18]} These nanowires not only overcome the brittleness issue of conventional single-phase HAp, but also render the aerogel with excellent hemostatic properties.\textsuperscript{[16, 18-24]} Based on these findings, we hypothesize that by combining inorganic and organic materials, the cryogels would behave remarkable absorptive capacity and hemostatic and bone repair properties, presenting an excellent solution to address such complex bone hemorrhage problems. Furthermore, the study of blood clots to modulate their polarization and bone immunity by recruiting macrophages has received significant attention.\textsuperscript{[25, 26]} However, the potential of CS/HAn composite cryogels for hemostasis and bone repair has not been completely explored, which could have significant applications.

In the current study, we have synthesized a multifunctional shape memory cryogel CSHAn by combining CS with HAn and facilitating slow cross-linking at low temperatures. The robust cryogels were successfully produced due to abundant amine groups in CS and aldehyde groups in crosslinker, which form Schiff-base linkages, as along with the reinforcing effect of HAn (Scheme 1). The CSHAn retained the shape memory behavior and exhibited excellent mechanical properties, enabling rapid blood absorption and the formation of a strong physical barrier at the bleeding site to prevent secondary hemorrhage. Compared to commercial gelatin hemostatic sponges, CSHAn demonstrate superior coagulability, blood cell and platelet adhesion, and activation in vitro. Moreover, the in vivo hemostatic ability of CSHAn was evaluated using rat liver defect incompressible hemorrhage model and a rat femoral artery hemorrhage model, confirming its enhanced hemostatic performance. It is particularly noteworthy that in vitro osteogenesis tests revealed that when CSHAn is combined with blood clots, it has the ability to promote osteoblast differentiation. Collectively, these findings highlight the potential of CSHAn as a hemostatic matrix material for managing bleeding in bone defects.
Scheme. 1. Preparation of CSHAn cryogels and its managing bleeding in bone defects.

2. Materials & methods

2.1 Preparation of HAn (Hydroxyapatite nanowires)

HAn were synthesized by the calcium oleate precursor solvothermal method, with minor adaptations based on previous work.[27] In brief, 12 mL methanol and 21 mL oleic acid was mixed and dissolved in 27 mL deionized (DI) water under continuous stirring for 10 min. 30 mL NaOH (2.1 g) was introduced into the above mixture and stirred for an additional 30 min. 24 mL CaCl$_2$ (0.66 g) and 36 mL NaH$_2$PO$_4$·2H$_2$O (1.872 g) were added dropwise and stirred for another 30 min respectively. The reaction mixture was then transferred to a Teflon container with a stainless steel autoclave and subjected to treatment at 180 °C for 24 h. After cooling to room temperature, the
resulting slurry was collected and washed thrice with ethanol and DI water. The HAn were separated and dried at 60 °C for further use.

2.2 Preparation of CSHAn

CSHAn cryogels were fabricated by a one-pot method. Briefly, CS solution (2%, w/v) was obtained by dissolving CS in a 5 mL solution of hydrochloric acid (0.1 M) in DI water. 0.1%, 0.2%, and 0.3 % (w/v) of HAn were then respectively dispersed in 3 mL DI water, each forming a uniform mixed solution upon the addition of 2 mL of glutaraldehyde (0.05%, w/v). The mixed precursor solution was shaken for 30 s and then injected into a mold, where it was frozen at -20 °C for 24 h. The synthesized cryogels were categorized and labeled as CSHAn0, CSHAn1, CSHAn2, and CSHAn3, respectively. Detailed information regarding the composition of each CSHAn is provided in Table S1.

2.3 Characterizations of CSHAn

The surface morphology and elements distribution of HAn and CSHAn were observed by a field-emission scanning electron microscope (FE-SEM; S-4800; Hitachi; Japan). To improve the electrical conductivity of the samples for observation, a thin layer of gold was sputtered on the surface for 30 s. Transmission electron microscopy (TEM) images of HAn were obtained using a field-emission transmission electron microscope (JEOL JEM-F200; JEOL JEM-1011, Japan). X-ray diffraction (XRD) patterns of HAn were obtained by an X-ray diffractometer (Rigaku D/max 2550 V, Cu Kα radiation, λ = 1.54178 Å).

Pore size of the CSHAn was determined by analyzing SEM images with NIH Image J software. Measurements were taken from over 100 pores across different SEM images to calculate the average pore size. Porosity of CSHAn was assessed using the ethanol replacement method. The porosity of CSHAn was computed using the equation:

\[ \text{Porosity (\%)} = \frac{(W_1 - W_0)}{(\rho V)} \times 100\% \]

Where \( W_0 \) and \( W_1 \) represent the mass of CSHAn before and after ethanol immersion, respectively, \( \rho \) corresponds to the density of ethanol (0.785 g cm\(^{-3}\)), and \( V \) is the sample volume.

The functional groups of HAn, CS, CSHAn0, CSHAn1, CSHAn2, CSHAn3 were
characterized by Fourier Transform Infrared Spectrometer (FTIR, Nicolet 6700, Waltham, USA) in the wavelength range of 4000-400 cm\(^{-1}\). The CSHAn were subjected to compressive mechanical tests using an Electronic Universal Testing Machine (CMT2503, Mester Industrial, China) at a loading rate of 5 mm/min. Young’s modulus was calculated by the stress-strain curve.

The zeta potential of CSHAn was determined by a Nano Particle Size and Zeta Potential Analyzer (DLS, Malvern, Zetasizer Nano ZS90, UK); Thermogravimetric analysis (TGA) was performed on the CSHAn using a thermogravimetric analyzer (TGA, METTLER TOLEDO, Switzerland) under nitrogen protection (30~500°C, 10K/min).

Ions release measurements of CSHAn were performed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Thermo Fisher Scientific, USA) to investigate the concentration of calcium ions (Ca\(^{2+}\)) in the soaking solution.

2.4 Water absorption properties of CSHAn

The freeze-dried CSHAn were immersed in DI water until they reached swelling equilibrium. The weight of full-water CSHAn were measured (\(M_2\)). Followed that, the original weight (\(M_1\)) of the freeze-dried CSHAn were obtained. Water absorption was calculated by the following equation:

\[
\text{Water absorption} = \frac{(M_2-M_1)}{M_1} \times 100\%
\]

2.5 Shape recovery and cyclic compression properties of CSHAn

The shape recovery properties of CSHAn were evaluated by measuring shape recovery ratio (%) and shape recovery time (s). Briefly, CSHAn were molded into uniform cylinders with a diameter of 5 mm and a height of 10 mm. The initial height of the material \(H_1\) was recorded. Then, the lyophilized CSHAn were compressed to their minimum possible height, \(H_2\), and keep in this state for 5 min. Next, CSHAn cylinders were immersed in water or fresh whole blood (obtained from rats) to recover its shape. The height after recovery \(H_3\) was measured. The shape recovery process was visually documented with a digital camera, and the shape recovery time was recorded with a timer. The shape recovery rate was calculated according to the following equation:

\[
\text{Shape recovery rate} = \frac{(H_3-H_2)}{(H_1-H_2)} \times 100\%
\]
The cyclic compression properties of CSHAn were tested using an electronic universal testing machine. In the cyclic compression test, a drop of water was applied around the CSHAn on the platform. The sample was then compressed to 80% of its compressive strain at a constant speed of 5 mm/min and then restored to 0% compressive strain at the same rate. The cycle was repeated 50 times to record the relationship between stress (MPa) and strain (%). Compression curves were plotted for samples at cycles 1st, 25th, and 50th cycles.

2.6 Biocompatibility of CSHAn

Hemocompatibility of CSHAn

In vitro hemocompatibility of CSHAn were evaluated by hemolysis assay following previously established method. Red blood cells (RBCs) were obtained from centrifuging sodium citrate anticoagulated rabbit blood at 1500 rpm for 10 min. Then the RBCs were subsequently diluted to form a 5% (v/v) suspension in PBS. Subsequently, 500 µL of the RBCs suspension was added dropwise to CSHAn (5mg) and then incubated at 37°C for 1 h. Triton X-100 and PBS served as positive and negative controls.

After incubation, all samples were centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 540 nm by a Microplate Reader (SpectraMax M2, Molecular Devices, USA). The hemolysis rate was calculated by the following formula:

\[
\text{Hemolysis rate} \, \% = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{negative}}}{\text{OD}_{\text{positive}} - \text{OD}_{\text{negative}}} \times 100\%.
\]

Where OD_{Sample} represented the absorbance of the CSHAn, OD_{PBS} represented the absorbance of the negative control (PBS), OD_{Triton} represented the absorbance of the positive control (Triton X-100).

Cytocompatibility of CSHAn

The in vitro cytocompatibility of CSHAn was evaluated by direct contact with bone marrow mesenchymal stem cells (BMSCs). BMSCs were cultivated at 37°C, 5% CO₂, 100% humidity in complete growth medium consisting of modified eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1.0 × 10⁵ U/L penicillin (Hyclone) and 100 mg/L streptomycin (Hyclone). BMSCs were seeded in a
48-well plate at a density of 10000 cells/well and allowed to adhere for 12 h. Subsequently, all the CSHAn with 5mm diameter and 1mm length were immersed in the culture medium. The viability of the BMSCs in contact with CSHAn were quantitatively analyzed by CCK-8 assay (Beyotime Biotechnology, China) after 1, 3, and 5 days.

OD values at 450 nm were detected by a Microplate Reader. Cells seeded on plates without any materials served as the control group. In addition, the cytotoxicity of the CSHAn on BMSCs were evaluated using LIVE/DEAD® Viability/Cytotoxicity Kit. Similar to the CCK-8 assessment, the cells were cultured for 1, 3, and 5 days. After 3 days, the Live/Dead staining assay was performed to observe cell viability and morphology with confocal laser scanning microscopy (CLSM, Leica-SP8, Germany).

In vivo biocompatibility of CSHAn

The in vivo biocompatibility of all CSHAn samples was examined by subcutaneous implantation in Sprague-Dawley (SD) rats. The rats were anesthetized with an injection of 3% pentobarbital sodium. Four 1-cm incisions were made on the backs of the rats, and different CSHAn samples were implanted into these incisions. After 7 and 14 days, the rats were euthanized, and the tissues in contact with the implants were collected. The collected samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Potential inflammatory reactions were analyzed using H&E staining and toluidine blue staining. This study adhered strictly to “Regulations for the administration of affairs concerning experimental animals” issued by the Chinese Ministry of Science and Technology and was approved by the ethical committee of the National Tissue Engineering Research Center (Shanghai, China).

2.7 In Vitro Hemostatic Performance of CSHAn

2.7.1 Blood-clotting index (BCI)

The in vitro blood coagulation performance of CSHAn was evaluated using the BCI. CSHAn samples were molded into cylindrical shapes, each with a height of 5 mm and a diameter of 5 mm, and then placed in 24-well plates pre-warmed to 37 °C. A mixture consisting of 50 µL of citrated blood and 5 µL of 0.2 M calcium chloride was rapidly added onto the surface of both CSHAn and Gelatin Sponges® (GS). After incubation
at 37 °C for 1, 2, 3, and 4 min respectively, 3 mL of DI water was added to each well to lyse the unbound red blood cells and release hemoglobin. The supernatant, containing hemoglobin, was then transferred into a 96-well plate, and its absorbance was measured at 540 nm. The BCI was calculated using the following formula:

\[ BCI = \left( \frac{OD_{sample}}{OD_{negative}} \right) \times 100\% . \]

Where \( OD_{sample} \) represented the absorbance of CSHAn and GS, \( OD_{negative} \) represented the absorbance of 50 \( \mu L \) recalcified blood without materials dropped directly in 3 mL of DI water.

2.7.2 Clotting test

The CSHAn samples were fabricated into cylindrical shapes, each 5 mm in height and 5 mm in diameter, and were placed into 1.5 mL EP tubes. These tubes were preheated at 37°C for 10 min. 1 mL of citrated rabbit whole blood were mixed with each sample, followed by the immediate addition of 100 \( \mu L \) of 0.2 M CaCl\(_2\) to each EP tube. The centrifuge tubes were inverted every 10 s to observe the blood flow. The clotting time was recorded when the blood flow ceased. The GS and blank group were respectively served as the positive control and negative control.

2.7.3 Clot strength

The blood rheological properties of CSHAn were evaluated by a rheometer (RS600, Thermo, USA). CSHAn were fully swollen in DI water or fresh rat whole blood for 5 min. An amplitude sweep test was conducted, ranging from 0.1 to 1% at a fixed frequency of 1 Hz, to measure the rheological change related to shear strain. Frequency sweep testing was performed from 0.1 to 10 Hz at a constant strain of 0.1% to analyze the variations in storage and loss modulus based on oscillatory frequency.

2.7.4 Blood cells and platelets adhesion

The adhesion performance of blood cells and platelets on CSHAn was evaluated according to the previous references.\[28, 30\] A 50 \( \mu L \) suspension of RBCs was dropped onto the surface of the sample, which had dimensions of 5 mm in diameter and 5 mm in height. After incubation at 37°C for 5 min, the sample's surface was washed with PBS to remove non-adherent RBCs. Subsequently, the sample was transferred to 5 mL of DI water and allowed to remain for 1 h to fully lyse the erythrocytes adhered to the
sample. The absorbance of each sample solution was measured at 540 nm using a Microplate Reader. The absorbance value of 50 µL of RBC suspension mixed with 5 mL of DI water served as a control value. The percentage of adherent RBCs was calculated by the following formula.

\[
\text{Percentage of RBCs} = \left( \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100\%
\]

Meanwhile, the adherent RBCs were fixed with 2.5% (v/v) glutaraldehyde for 2 h, followed by dehydration with ethanol solutions of 50%, 60%, 70%, 80%, 90%, and 100% at 5-min intervals. Isoamyl acetate was added for 1-h replacement reaction. The erythrocyte adhesion on the dried samples was observed by FE-SEM.

For the assessment of platelet adhesion, platelet-rich plasma (PRP) was obtained from citrated whole blood by centrifugation at 1500 rpm for 10 min. Subsequently, 50 µL of the separated PRP was dropped onto the samples of CSHAn and GS. The samples were then incubated at 37°C for 1 h. All samples were rinsed three times with PBS to remove physically adherent platelets and then immersed in 1% Triton X-100 to lyse platelets and release lactate dehydrogenase (LDH). The concentration of LDH was measured using a Lactate Dehydrogenase Assay Kit.

Similarly, the samples were fixed in 2.5% glutaraldehyde and dehydrated with ethanol solutions. The morphology of platelet adhesion on the dried samples was observed using FE-SEM. The steps were identical to those in the erythrocyte adhesion assay and would not be repeated here.

**2.7.5 Platelet activation**

Platelet activation was assessed by evaluating the expression of P-selectin. CSHAn and GS (5mg) were placed in a 1.5mL EP Tube, followed by the addition of 200 µL of PRP to each sample. After incubating for 15 min at 37 °C, each sample was stained with platelet-activation-dependent monoclonal antibodies (FITC anti-rat CD61 for platelet activation, PE anti-rat CD62p for activation identification). The percentage of platelets in different activation states was calculated using flow cytometry (CytoFLEX S, Beckman Coulter, USA). The soluble P-selectin expression in PRP was determined by enzyme-linked immunosorbent assay (ELISA).

**2.7.6 PT and APTT t**

The effect of the material on the intrinsic and extrinsic coagulation pathway was
determined by assaying activated partial thromboplastin time (APTT) and prothrombin time (PT). Platelet-poor plasma (PPP) was obtained by centrifuging citrated PRP at 3500 rpm for 10 min. The CSHAn sample and GS were mixed with PPP and pre-warmed at 37°C for 5 min. APTT and PT assays were performed using a fully automated coagulometer (RAC-030, Rayto, China). PPP containing CaCl$_2$ was utilized as a control.

2.7.7 TEG test

Thromboelastometry (TEG) was adopted to evaluate the whole dynamic process of blood coagulation. CSHAn sample and GS (1 mg) was incubated with anticoagulated whole blood (340 μL) in a centrifuge tube for 5 min. The mixture was combined with 20μL of 0.2 M CaCl$_2$, and the TEG analysis was started at 37°C in a TEG device (JJ-8000, Guizhou Jinjiu Biotch, China) immediately. Anticoagulated whole blood operated under the same conditions was selected as a control.

2.8 In vivo hemostasis

Rat liver defect hemorrhage model and rat femoral artery hemostatic bleeding model were employed to evaluate the hemostatic abilities of CSHAn and GS. The study strictly adhered to “Regulations for the administration of affairs concerning experimental animals” issued by the Chinese Ministry of Science and Technology and was approved by the ethical committee of the National Tissue Engineering R&D Center (Shanghai, China). Rats were used for the experiment after one week of feeding, and they were fasted for 24 hours with water before the experiment. The CSHAn and GS samples were shaped into cylinders with a diameter of 5 mm and a height of 5 mm. All experimental materials were sterilized in advance. For the rat hepatic defect hemorrhage model, rats were randomly assigned to four groups. Rats were deeply anesthetized, and the abdomen was incised to expose the middle lobe of the liver. Pre-weighed filter paper was positioned beneath the liver. Circular perforated wounds with a diameter of 4 mm were created in the liver to induce bleeding. These wounds were then filled with CSHAn or GS, respectively. Total blood loss and clotting time were recorded. Livers without any treatment served as the blank control group. For the rat femoral artery hemorrhage model, rats were randomly divided into four groups. All
animals were anesthetized, and a 3-mm-long longitudinal incision was created at the root of the left thigh to expose the blood vessel. The blood vessel was cut with a scalpel, and after natural bleeding for 3 s, CSHAn or GS was pressed to the wound and held in place for 5 s. The amount of blood loss and bleeding time were recorded. A no-treatment group served as the blank control.

2.9 Interaction of blood pre-incubation CSHAn with BMSCs

To determine the effect of CSHAn-blood interaction on osteoblasts, BMSCs were seeded in the lower chamber of each transwell at a density of $1 \times 10^4$ cells/well. The CSHAn samples, pre-incubated with 50 μL of whole blood at 37 °C for 30 min, were placed in the upper chamber of the transwell. Cell activity was measured on days 1, 3, and 5 using the CCK-8 kit. The cellular morphology was fixed using 4% paraformaldehyde and the cytoskeleton was stained with Alexa Fluor 488-labeled phalloidin (1:40, Invitrogen, Switzerland) and DAPI after 3 days of culture.

Similarly, BMSCs at a density of $1 \times 10^4$ cells/well were co-cultured with CSHAn for 1 d, and then the medium was changed to osteoblastic medium (BLDM) containing dexamethasone (10 mg/mL), sodium β-glycerophosphate (10 μg/mL), and ascorbic acid (50 μg/mL) for 7 d. Cells were fixed with 4% paraformaldehyde and subsequently stained with an alkaline phosphatase (ALP) staining kit (Solarbio, China). The images were captured using an inverted microscope. After 14 days of culture in BLDM, cells were fixed with 4% paraformaldehyde, rinsed with PBS and stained with alizarin red staining (ARS) for observation under a microscope. The semi-quantitative analysis of ALP activity and ARS was analysis by using image J software to count the stained area.

Statistical analysis

All tests were carried out at least three times. Statistical results were expressed as mean ± standard deviation (mean ± SD), and all data were statistically analyzed using Origin or GraphPad Prism9 software, and the results were plotted using GraphPad Prism9. All data were analyzed by one-way ANOVA. p < 0.05 was considered statistically significant. Statistical significance was considered at *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
3 Results & Discussion.

3.1 Synthesis and characterization of HAn

SEM image showed that the HAn possessed excellent flexibility, with lengths ranging from 10 to 100 μm and diameters of only 10 nm (Fig. S1). TEM results confirmed that the synthesized HAn had a one-dimensional fiber structure with a high aspect ratio, and elemental mapping showed that the elements of Ca and P were uniformly distributed throughout the HAn nanofibers. The high-resolution TEM image revealed an interplanar spacing of 0.34 nm, which matched the spacing of the d002 crystal plane (Fig. S2). HRTEM results confirmed that HAn growth was preferentially oriented along the c-axis. XRD patterns showed that the synthesized HAn exhibited lower crystallinity, indicating a higher degradation rate (Fig. S3). In the FTIR spectra, typical PO$_4^{3-}$ asymmetric and symmetric stretching vibration peaks were found at 1097, 1028, and 962 cm$^{-1}$ The peak at 3448 cm$^{-1}$ corresponded to the hydroxyl stretching vibration peaks of hydroxyapatite, while the absorption band at 2884 cm$^{-1}$ was attributed to the symmetric stretching vibration of CH$_2$. These results indicated that the flexible HAn with high aspect ratio were successfully prepared.

3.2 Preparation and characterization of CSHAn

Scheme. 1 illustrated the synthesis process of CSHAn. Initially, the -NH$_2$ groups from CS precursor react with the -CHO groups of the cross-linker to form the primary cross-linking network. Simultaneously, under weak acidic conditions, HAn underwent dissociation to release Ca$^{2+}$, which then form a secondary cross-linking network through a complexation reaction with CS. Finally, the macroporous structure was constructed by freeze-drying. These stable dual cross-linked polymer networks endowed the cryogels with enhanced mechanical strength and excellent mechanical stability, enabling them to exhibit rapid water-triggered shape memory properties and high-water absorption ability. The presence of abundant positively charged protonated-chitosan confers the inherent hemostatic properties to CSHAn, while the HAn in the cryogels contributes to improved mechanical strength and the release of calcium ions to promote hemostasis$^{[31]}$. Nevertheless, it was observed that an excessive concentration
of HAn led to a decrease in the energy storage modulus of CSHAn. Additionally, the
hemostatic effectiveness of CSHAn was compromised due to the over-release of Ca$^{2+}$
ions. To further investigate these effects, four cryogels with varying HAn
concentrations (0, 10, 20, and 30 wt%) were prepared in this study.

SEM images revealed the interconnected porous structures of CSHAn (Fig. 1A),
signifying the formation of a uniform 3D network during the gradual cross-linking
process at low temperatures. The magnified image of CSHAn showed that the HAn
fibers had been successfully embedded in the CSHAn matrix, rather than merely
adhering to the surface, thus providing a hierarchical structure ranging from nanometer
to micrometer scales. Cryogels, characterized by their high porosity, offered a larger
3D space and ample surface area, which is conducive to the aggregation and adhesion
of blood components, such as blood cells and proteins involved in coagulation.[32] The
average pore size of CSHAn was quantified using SEM images. Fig. 1B indicated that
CSHAn0, CSHAn1, CSHAn2, and CSHAn3 had pore structures with average
diameters of 111.63 ± 33.65, 95.70 ± 21.67, 97.31 ± 21.36, and 85.81 ± 20.38 μm,
respectively.

Additionally, the porosity decreased with increasing HAn content CSHAn, with
values of 94.26%, 90.07%, 91.76% and 88.83% for CSHAn0, CSHAn1, CSHAn2, and
CSHAn3, respectively (Fig. 1C).

Fig. 1D confirmed the cross-linking reaction of the CSHAn by FT-IR analysis.
Characteristic peaks at 1097, 1028, and 962 cm⁻¹, corresponding to the asymmetric and
symmetric stretching vibrations of PO₄³⁻ groups, indicated the successful incorporation
of HAn into CSHAn. The absorption peaks at 1650 cm⁻¹ and 1550 cm⁻¹ represented the
vibrational absorption peaks of the amide group in CS. After the addition of the
crosslinker, the characteristic peak at 1650 cm⁻¹ diminishes, and a new absorption peak
appeared near 1650 cm⁻¹, indicating the formation of the C=N Schiff base bond.[31, 33]
The zeta potential data (Fig. 1E) showed that CSHAn0(40.9 ± 1.54 mV), CSHAn1(25.9
± 1.82 mV), CSHAn2(33.6 ± 1.38 mV) and CSHAn3(18.3 ± 0.78 mV) all exhibited
positively charges at pH 7.4. ICP results (Fig. 1F) indicate that after immersion in DI
water for 240 min, the Ca$^{2+}$ concentrations for CSHAn1, CSHAn2, and CSHAn3 were
41.32±1.27, 75.55±1.73, and 84.13±2.05 mg/L, respectively, confirming that increased HAn content leads to a higher release of Ca$^{2+}$ to promote chelation.

The compressive properties of the dry CSHAn were evaluated at 80% strain. CSHAn0 exhibited a compressive strength of 42.57 ± 1.72 Kpa, comparable to chitosan sponges reported in the literature\textsuperscript{35}. The addition of HAn improved the compressive strengths of CSHAn1, CSHAn2, and CSHAn3 to 124.93±2.43 KPa, 167.67±1.78 KPa, and 167.92±2.43 KPa, respectively. Concurrently, the Young's modulus of the CSHAn increased (Fig. 1G), indicating that HAn incorporation enhanced the cross-linking degree through Ca$^{2+}$ chelation and strengthens the polymer system through the fiber structure, which is consistent with SEM results.\textsuperscript{34, 35}

Differential scanning calorimetry (DSC) was conducted to assess the thermal stability. Fig. 1H displayed the decomposition temperatures of 236.84 °C for CSHAn0, 238.03 °C for CSHAn1, 243.02 °C for CSHAn3, and 236.35 °C for CSHAn3, indicating the improved thermal stability with higher HAn content due to the chemical interaction between HAn and CS.\textsuperscript{34} The lower decomposition temperature of CSHAn3 (236.35 °C) may be attributed to excessive cross-linking. TGA indicated no additional weight loss phases for CSHAn1, CSHAn2, and CSHAn3 compared to CSHAn0 (Fig. 1I).

Furthermore, the HAn content in CSHAn2 was determined to be 16.88 % (w/w), matching with theoretical additions.
3.3 Shape recovery and cyclic compression properties of CSHAn

The shape-restoring properties of cryogels enabled them to expand upon blood absorption, effectively filling the wound and creating compressive hemostasis. Simultaneously, the rapid water absorption capability concentrates clotting factors
within the cryogels, accelerating the hemostasis process\textsuperscript{[33]}. In this study, the shape recovery time and rate of CSHAn upon water and blood absorption were evaluated. The water and blood absorption ability of the CSHAn was shown in Fig. 2A. After water absorption, the shape recovery time for CSHAn0, CSHAn1, CSHAn2, CSHAn3 and GS was \((1.53 \pm 0.20)\) s, \((2.56 \pm 0.13)\) s, \((2.74 \pm 0.21)\) s, \((2.93\pm 0.42)\) s, and \((107 \pm 2.23)\) s, respectively. After blood absorption, the shape recovery time for CSHAn0, CSHAn1, CSHAn2, and CSHAn3 was \((1.8 \pm 0.14)\) s, \((2.8 \pm 0.21)\) s, \((2.6 \pm 0.18)\) s, and \((2.8\pm 0.22)\) s respectively, which were significantly faster than GS (Fig. 2B). The maximum swelling of CSHAn0 in water and blood was 5627% and 5485%, respectively. With the increased HAn content in CSHAn, the swelling rate gradually decreased to 4650% (in water) and 4710% (in blood). In contrast, the solubility of GS in water and blood was 816% and 1126%, respectively (Fig. 2C, D). Moreover, the swelling capacity of different components of the cryogels and GS was compared (Fig. 2E, F).

When fully saturated with water or blood, the CSHAn achieved maximum shape recovery rate of 94.66% and 88.8%, surpassing GS at 52.46% and 73.49%. The notable shape recovery capability of CSHAn is likely due to the abundance of hydrophilic groups, such as hydroxyl, within CS molecular chain. In contrast, GS, despite their high porosity, had poor water absorption and shape recovery abilities, primarily due to its low hydrophilicity, which results in shape recovery driven mainly by air tension, thereby hindering its rapid liquid absorption capability\textsuperscript{[15]}

Cyclic stress-strain tests were conducted to assess the mechanical stability of CSHAn. CSHAn0, CSHAn1, CSHAn2, and CSHAn3 exhibited maximum compressive stresses of 33 kPa, 37 kPa, 142 kPa, and 148 kPa, respectively (Fig. 2G). Even after 50 cycles of compression-absorption-shape recovery at 80% maximum strain, CSHAn maintained its shape and elasticity. As the of HAn content in CSHAn increased, the recovery loss slightly increased from 5.79% for CS to 11.1% for CSHAn1, 7.97% for CSHAn2, and 6.46% for CSHAn3. Overall, all the CSHAn exhibited favorable compression properties, confirming that HAn significantly enhances their mechanical strength without compromising their high resilience and rapid recovery behavior.
Figure 2. Swelling and shape recovery properties of CSHAn under water and blood. (A) Photographs of CSHAn and GS before and after compression and their shape recovery behavior after absorbing water or blood. (B) Shape recovery speed of CSHAn and GS. (C-D) Swelling rate of CSHAn and GS after water or blood absorption. (E-F) Shape recovery rate of CSHAn and GS after water or blood absorption. (G) Compressive stress-strain curves after 1, 25, and 50 cycles of CSHAn0, CSHAn1, CSHAn2 and CSHAn3. (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ****, p < 0.0001) using Student's t-test (two-sided), Error bars indicate SD. n = 3.

3.4 Biocompatibility of CSHAn

To evaluate the cytocompatibility of CSHAn, live-dead cell staining and CCK-8 proliferation assays were performed. As shown in Fig. 3A, after 24 hours of co-culture with CSHAn and BMSCs, the cells predominantly exhibited green fluorescence, indicating high viability, with minimal red-stained dead cells observed. The cell
morphology resembled that of the blank control group, suggesting healthy cell growth. The CCK8 assay results staining showed a cell survival rate exceeding 90% in all groups (P > 0.05), indicating that CSHAn was non-cytotoxic (Fig. 3B). The blood compatibility of CSHAn was further evaluated. Fig. 3C showed that CSHAn displayed a yellowish color similar to the negative control (PBS), indicating minimal erythrocyte destruction and hemoglobin release, which is indicative of good blood compatibility. The maximum hemolysis rate of CSHAn was only 0.4%, much lower than the international threshold of 5%\cite{36}, further confirming its blood compatibility.

To investigate the inflammatory response and in vivo biocompatibility, CSHAn was implanted subcutaneously in SD rats. Fig. 3D illustrated that after 7 days post-implantation, the CSHAn were easily separated from the tissue margins with only a few cells infiltrating with the implant site. By the 14th day, increased cellular infiltration, neovascularization and collagen deposition were observed in the CSHAn, and all samples showed a weak inflammatory response with nearly complete healing of the traumatic wounds, comparable to the positive control group (Blank). Overall, the result suggested that CSHAn possessed excellent biocompatibility, highlighting its potential for application in hemostasis and tissue repair.
Figure 3. In vivo and in vitro biocompatibility evaluation of CSHAn. (A) Live/Dead staining of CSHAn after 24 hours of contact with BMSCs. (B) Cell viability of BMSCs after 3 days of co-culture with CSHAn. (C) Hemolysis rate and hemolysis photographs of CSHAn and GS. (D) H&E staining and TB staining images of CSHAn-treated of subcutaneous implant on days 7 and 14.

3.5 In vitro hemostatic effect and blood cell adhesion properties of CSHAn
In vitro BCI depicted the coagulation effect of CSHAn in Fig. 4A. At predetermined time intervals (1, 2, 3, and 4 min), non-crosslinked blood cells were lysed in DI water, and coagulation capacity of the material was determined by measuring the absorbance of the hemoglobin in solution. The CSHAn group exhibited a lighter color compared to the blank and GS groups, indicating denser blood cell coagulation on the surface of samples. Remarkably, within 2 min, the BCI values for all CSHAn samples dropped below 20%, while the BCI for GS remained above 40% (Fig. 4B). The enhanced coagulation capability of CSHAn was likely attributed to its macroporous structure, which rapidly absorbed and concentrated blood cells. Furthermore, the chemical composition of CSHAn, rich in hydroxyl and amino groups, significantly contributes to its procoagulant properties.\textsuperscript{[37]} Interestingly, as the increases, the BCI initially decreased and then increased with HAn content in CSHAn, suggesting that the increased Ca\textsuperscript{2+} concentration influences coagulation. However, this procoagulant effect diminishes when the Ca\textsuperscript{2+} concentration exceeds a certain threshold. The coagulation ability was further supported by Fig. 4C, where the final clotting times for GS, CSHAn0, CSHAn1, CSHAn2, and CSHAn3 were 483s, 450s, 230s, 90s, 70s, and 90s, respectively, which was consistent with the BCI results.

Adhesion and aggregation of RBCs and platelets are essential stages in blood coagulation. Thus, we conducted a detailed investigation into the blood adhesion capacity of CSHAn. SEM observation (Fig. 4F) revealed substantial RBC adhesion onto the CSHAn pores, forming irregular aggregates, with the majority of adhered RBCs maintaining their characteristic discoidal shape. After a 5-min incubation, the RBCs adhesion rate for CSHAn reached a maximum of 62.11\pm2.86% (CSHAn1), compared to only 20.85\pm0.86% for GS under the same conditions (Fig. 4D). At the same time, a large number of platelets adhered to the CSHAn surface and formed aggregated states. The morphological transformation of e platelets from spherical to irregular shapes suggested that CSHAn stimulated platelet activation.\textsuperscript{[38]} Platelet adhesion was quantified through LDH measurement (Fig. 4E), with CSHAn1 showing the highest platelet adhesion rate of 67.1\pm5.74%, while GS was only 21\pm1.73%. These results can be attributed to the positively charged CS within the CSHAn, which aids in
the adsorption of negatively charged blood cells\cite{39}. Although the partial neutralization of positive charges by HAn, CSHAn’s ability to adhere to blood cells remained strong, with the RBC adhesion capability even surpassing that of CSHAn0.

To verify the interaction between blood and CSHAn, amplitude scans and frequency scans were performed using a rheometer. The storage modulus (G’) of all CSHAn samples was higher than the loss modulus (G’’), characteristic of viscoelastic solids (Fig. 4G, H). The storage modulus and loss modulus increased with the increasing HAn concentration in CSHAn. CSHAn groups remained stable in the shear strain range of 0.1% to 100% (Fig. 4G). However, when the CSHAn were bound to blood, the storage and loss moduli began to decrease after about 10% shear strain, possibly due to the disintegration of the blood clot structure when the strain exceeds this threshold, resulting in a reduced elastic response of the material-blood complex (Fig.5H). In contrast, CSHAn interacting with water did not exhibit this phenomenon. Frequency scan tests (Fig. 4I, J) showed similar tendencies, with the storage modulus and loss modulus increasing with HAn concentration, and all CSHAn groups exhibiting high stiffness and stability in the oscillation frequency range of 0.1-10 Hz. In summary, these results indicated that CSHAn possessed high elasticity and rapid recovery characteristics, and its ability to enhance the strength of the blood clot is further strengthened with increasing HAn content.
Figure 4. In vitro hemostatic effect and hemocyte adhesion capacity of CSHAn. (A) Photographs of blood cell binding capacity of CSHAn and GS in 1-4 min. (B) BCI index of CSHAn and GS at 1-4 min. (C) Total clotting time of CSHAn and GS. (D, E) Adhesion rates of CSHAn and GS to erythrocytes and platelets. (F) SEM images of erythrocytes and platelets attached to the surface of CSHAn and GS. (G-J) Energy storage modulus ($G'$) and loss modulus ($G''$) of CSHAn after water (G, I) or blood (H, J) absorption, as determined by (G, H) frequency scan, (I, J) amplitude scan tests. (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ****, p < 0.0001) using Student's t-test (two-sided). Error bars indicate SD. n = 3.

3.6 Coagulation Mechanism of CSHAn

Blood coagulation is a complex process, and the hemostatic ability of materials is
closely related to their physical structure and chemical properties. To investigate the
hemostatic mechanism, APTT and PT tests were utilized to evaluate the material's
impact on the intrinsic and extrinsic coagulation pathways. As depicted in Fig. 5A, the
APTT range for normal blood is typically between 20-24 s. Interestingly, Both GS and
CSHAn0 exhibited APTT values within this physiologically normal range. However,
the introduction of HAn led to a significant reduction in APTT, suggesting that the
negatively charged HAn initiated the factor XII (FXII) self-activation pathway, which
promotes the intrinsic coagulation cascade reaction. Notably, the PT remained
consistent across all experimental groups, indicating that the material did not affect the
extrinsic coagulation pathway (Fig. 5B).

To elucidate the coagulation dynamics of CSHAn, an in vitro analysis using TEG
was performed. Four key parameters—R, K, A, and MA—play pivotal roles in the
thrombus formation process. Specifically, R denotes the time required for clot initiation,
K represents the rate at which a clot achieves a specific strength, A relates to the
coaagulation rate, and MA signifies the maximum clot strength. As depicted in the Fig.
5E, the blank group initiated that clot formation at the 9.8±0.4 min mark, with clot
maturation occurring in 2.3±0.2 min.

The resulting clot exhibited a strength of 62±4.3 mm, accompanied by a coagulation
angle of 60±1.8°. When hemostatic materials were introduced, both R and K values
decreased. Remarkably, CSHAn2 showed the best comprehensive coagulation
capability among these samples, with R and K values of 5.9 ± 0.4 min and 1.3 ± 0.1 min
respectively, and MA values of 72.5 ± 1.74 mm and 71.96 ± 2.34°.

The adhesion and activation of platelet on a material are direct indicators of its
hemostatic potential. Positively charged chitosan accelerates platelet adhesion and
activation through direct electrostatic interactions. Platelets activation was evaluated
by rat P-selectin detection kit and flow cytometry. The results (Fig. 5C) showed that
CSHAn enhanced platelet activation in blood, with CSHAn2 recording the maximum
platelet activation ability of 52.43±1.32 ng/mg, demonstrating that the incorporation of
HAn enhances platelet activation. Significantly, our investigations revealed that the
optimal amount of HAp addition is not necessarily "the more, the better." Specifically,
an HAn addition of 20% (w/w) was found to yield the highest levels of platelet adhesion and activation. This result further supported by flow cytometry, which confirmed optimal platelet activation at a 20% (w/w) HAn addition (Fig. 5D). In summary, our research underscored the exceptional suitability of CSHAn for hemostatic applications, attributed to the integration of the hemostatic properties of CS, the superhydrophilicity of Han, the activation of the endogenous coagulation cascade, and the inherent microporous network structure of the cryogels. Subsequent experiments focused on CSHAn2 due to its excellent in vitro hemostatic ability.
Figure 5. In vitro coagulation characterization of CSHAn. (A, B) APTT and PT values of CSHAn and GS. (C) Quantitative analysis of P-selectin expression on CSHAn and GS. (D) Flow cytometry results of CSHAn. (E) Coagulation dynamics of CSHAn and GS. (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ****, p < 0.0001) using Student's t-test (two-sided). Error bars indicate SD. n = 3.
3.7 *In vivo* hemostasis properties of CSHAn

The hemostasis efficacy of CSHAn was further evaluated by the measurement of bleeding weight and hemostasis time both in the rat liver injury model and tail amputation model. In the rat liver injury model, a 5-mm penetrating wound was induced in the liver of the rats using a perforator. As shown in Fig 6A, the blank group exhibited the largest area of filter paper saturation within 2 min. The GS group had a marginally reduced wetted area compared to the blank group. By contrast, the CSHAn and CSHAn2 groups resulted in the smallest bleeding areas. The hemostasis outcomes are summarized in Fig. 6(B) and Fig. 6(C). The blood loss for the blank, GS, CSHAn0 and CSHAn2 were 603 ±37 mg, 486 ± 45 mg, 219 ± 32 mg and 141 ± 37 mg, respectively, with hemostasis time of 96.6 ± 8.9 s, 77.6 ± 7.4 s, 54.3 ± 7.9 s and 37.6 ± 6.2 s.

The assessment of hemostasis properties in the context of massive bleeding was conducted using a rat femoral artery injury model, which served as an intracorporeal hemostasis model. After cutting the artery with scissors, free bleeding was allowed for 3 s, followed by the application of the hemostatic material until bleeding stopped. As shown in Fig 6D, GS, CSHAn0 and CSHAn2 absorbed blood, and post-hemostasis CSHAn2 displayed fewer blood clots, indicating its exceptional ability to absorb blood and accelerate blood clotting. The hemostasis results are presented in Fig. 6(E) and Fig. 6(F). The blood loss for the blank, GS, CSHAn0 and CSHAn2 groups were 411 ±35 mg, 396 ± 15mg, 374 ± 22mg and 210 ± 19 mg, respectively, with hemostasis time of 120 ± 8.7 s, 101.0 ± 8.3 s, 73.0 ± 6.2 and 48.6 ± 6.5 s. These results further support the notion that CSHAn2 possesses outstanding rapid hemostasis capabilities, particularly scenarios involving hemostasis of wounds with massive bleeding. The results showed that CSHAn2 exhibited the superior hemostatic properties and held significant potential for the treatment of life-threatening bleeding.

The enhanced hemostatic properties of CSHAn2 can be attributed to three factors. First, the self-expanding nature of CSHAn2 upon compression enabled it to adequately fill the wound and apply perpendicular pressure to the injury site, thereby limiting further blood loss. Secondly, its interconnected 3D structure facilitates the rapid aggregation of blood cells and the activation of platelets, which enhance the hemostatic
response. Thirdly, the sustained and stable release of Ca²⁺ ions into the bloodstream aids in the conversion of prothrombin to thrombin, promoting the fibrin formation and resulting in a more stable blood clot.

Figure 6. In vivo hemostasis properties of CSHAn. (A) Photographs of CSHAn and GS post-

hemostasis in a rat liver penetrating defect model. (B) Blood loss in rat liver. (C) Hemostasis time in rat liver. (D) Photographs of CSHAn and GS post-hemostasis in a rat femoral artery injury model. (E) Blood loss in a rat femoral artery injury. (F) Hemostasis time in a rat femoral artery injury model. (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ****, p < 0.0001) using Student's t-test (two-sided), Error bars indicate SD. n = 3.

3.8 Interaction of blood pre-incubation CSHAn with BMSCs

Hematoma formation subsequent to hemostasis in bone defects represents the initial stage of bone healing.\cite{25, 43} Our objective was to elucidate the impact of material-blood clot interactions on cellular dynamics. This was achieved by co-culturing blood pre-incubated with CSHAn alongside BMSCs. The experimental design involved culturing BMSCs in the lower chamber of a well plate, with the material positioned in the upper chamber (Fig. 7A). The proliferation of BMSCs was detected by CCK-8 assays, which revealed that the cell viability of blood pre-incubation CSHAn0 and CSHAn2 was significantly higher than that of the Blank group (Fig. 7B). In addition, after 3 d of co-culture, BMSCs co-cultured with blood-preincubated CSHAn were observed to be more numerous in the same field of view compared to those without blood preincubation, and a notable change in cell morphology was observed (Fig. 7C), with the presence filamentous pseudopods on blood-preincubated CSHAn. The osteogenic differentiation of BMSCs was further evaluated by ALP staining and ARS staining of BMSCs co-cultured with CSHAn or blood-precultured CSHAn. ARS staining images and their semi-quantitative analysis revealed a higher abundance of mineralized nodules in CSHAn2 groups compared to other groups. The number of mineralized nodules further increased upon co-incubation of CSHAn with blood (Fig. 7D, F, G). Fig. 7H, I showed that the ALP activity of CSHAn0 was significantly higher than that of the blank group, and this enhancement was further augmented with the incorporation of HAn.

In essence, our findings highlight the potential of blood clot-infused materials, especially CSHAn2, as potent platforms for promoting bone defect repair and regeneration. These insights are paramount for advancing the realm of biomaterials, particularly those targeting bone regenerative applications.
Figure 7. Interaction between blood pre-incubated CSHAn and BMSCs. (A) Schematic representation of co-culture with BMSCs and blood pre-incubated CSHAn. (B) Proliferative effects of blood pre-incubated CSHAn co-cultured with BMSC over 1, 3, 5 days. (C) Morphological changes in BMSCs after 3 days of co-culture with blood pre-incubated CSHAn. (D) ARS staining for mineralization at 14 days post-osteogenic induction. (E) ALP staining at 7 days post-osteogenic induction. (F) Quantitative analysis of ARS by CSHAn. (G) Quantitative analysis of ARS by blood pre-incubated CSHAn. (H) Quantitative analysis of ALP activity by CSHAn. (I) Quantitative analysis of ALP activity by blood pre-incubated CSHAn. (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ****, p < 0.0001) using Student's t-test (two-sided), Error bars indicate SD. n = 3.
4 Conclusion

We have successfully engineered a series of composite cryogels CSHAn, crosslinked with a dual network of CS and HAn. CSHAn possessed an interconnected macroporous structure, ultrafast water absorption performance, and excellent shape memory properties, all of which contributed to their effectiveness in achieving hemostasis in non-compressible hemorrhage. The positively charged CS in CSHAn enhanced hemostatic effects by adhering to blood cells while the high aspect ratio nanowire structure of HAn promoted platelet adhesion and the release Ca$^{2+}$, triggering the coagulation cascade and accelerating the clot formation and hemostasis. In both a rat liver defect incompressible hemorrhage model and a rat femoral artery hemorrhage model, CSHAn2 outperformd Gelatin Sponges®. In addition, CSHAn2 enhanced the proliferation and differentiation of BMSCs. All these results demonstrate that the multifunctional CSHAn cryogels are excellent candidates as hemostatic materials for hemorrhagic bone defects and bone tissue regeneration.

Data availability statement

The raw data required to reproduce these findings are available within the paper. The processed data required to reproduce these findings are available within the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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