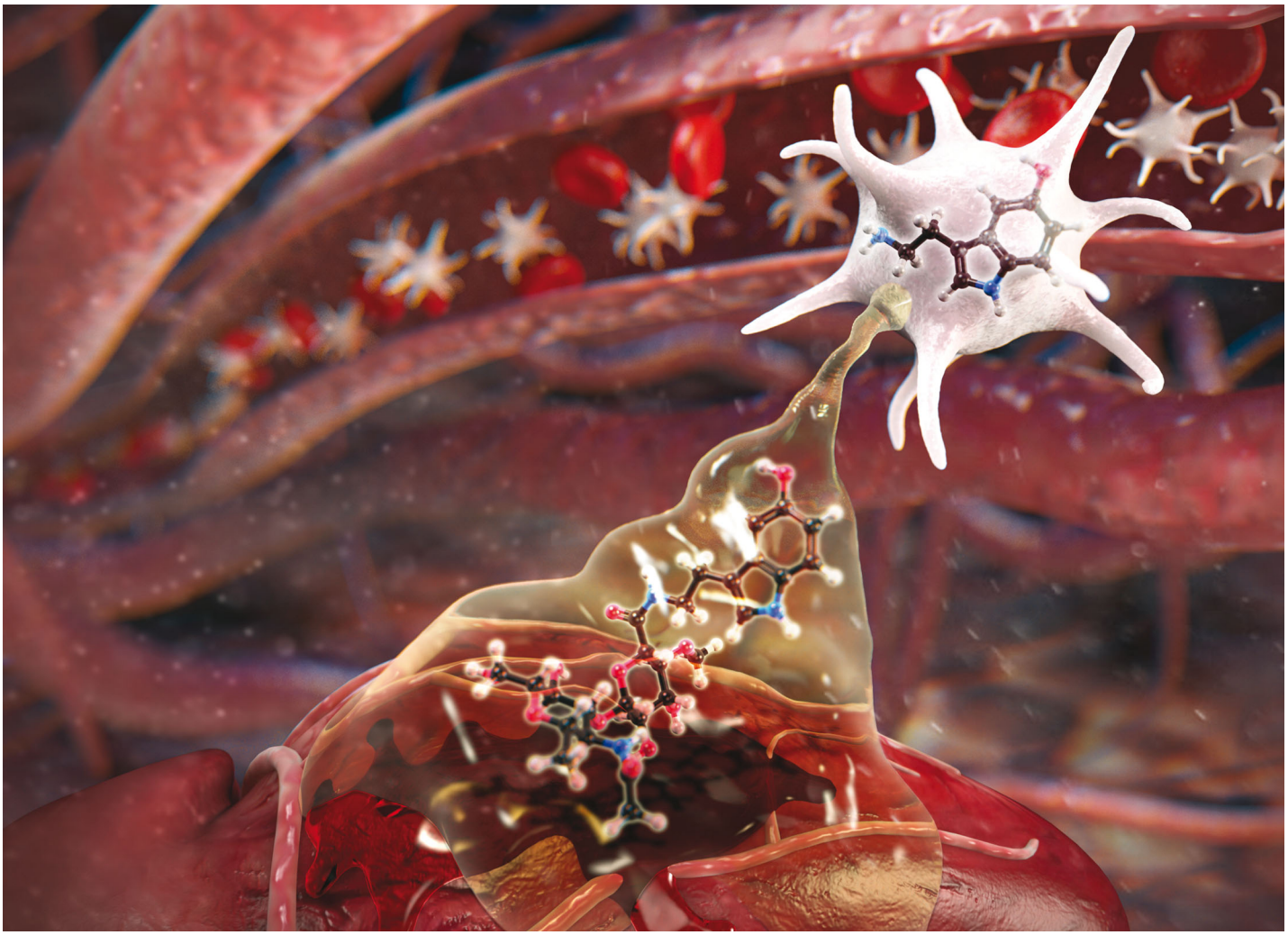

Highlighting research on a bio-inspired multifunctional hemostatic adhesive using a platelet-coagulating mediator serotonin by Prof. Seung-Woo Cho at Yonsei University, South Korea.

A serotonin-modified hyaluronic acid hydrogel for multifunctional hemostatic adhesives inspired by a platelet coagulation mediator

A serotonin-modified hyaluronic acid hydrogel not only exhibits a highly effective hemostatic performance resulting from the enhanced platelet activation mediated by conjugated serotonin, but also prevents abnormal tissue adhesion around the injured tissue. Our hydrogel would be clinically valuable as a multifunctional hemostatic adhesive in medical treatment for injury and surgical processes.

As featured in:

See Seung-Woo Cho *et al.*, *Mater. Horiz.*, 2019, **6**, 1169.

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A serotonin-modified hyaluronic acid hydrogel for multifunctional hemostatic adhesives inspired by a platelet coagulation mediator†

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Bleeding control is very important during operations and surgical treatments of wounds and traumatic injuries. This need has led to development and practical uses of various hemostatic agents. However, the currently available hemostatic agents have several limitations related to biocompatibility and hemostatic performance due to the presence of cytotoxic and immunogenic components and the individual differences in the blood coagulation system. In this study, a hydrogel system inspired by a blood clotting mediator in platelets was developed as a new class of hemostatic adhesive with improved performance and multi-functionality. The proposed hydrogel system was prepared using serotonin-conjugated hyaluronic acid, both of which are highly biocompatible as they are natural components of the body. Serotonin facilitates hemostasis by acting as a blood clotting mediator in platelets and acts as a crosslinker to form adhesive hydrogels. The serotonin-conjugated hyaluronic acid hydrogel exhibited significantly improved hemostatic capability *in vivo* with normal and hemophilic injuries compared with a commercially available fibrin-based hemostatic agent and prevented abnormal tissue adhesion after hemostasis. This hydrogel system, inspired by the platelet clotting mechanism, is a novel hemostatic adhesive that overcomes several limitations of existing hemostatic agents and could substantially improve bleeding control, thereby improving outcomes of surgical procedures.

New concepts

Currently available hemostatic agents need to be improved in terms of biocompatibility, tissue adhesiveness, and hemostatic capability. This work demonstrates a novel class of multifunctional hemostatic adhesive inspired by platelet coagulation process and its mediator to realize better biocompatibility and hemostatic performance. The hemostatic adhesive is developed by conjugating serotonin, one of the blood clotting mediators in platelets, to natural polymer hyaluronic acid. Oxidative cross-linking of serotonin molecules conjugated to hyaluronic acid backbone results in highly tissue-adhesive hydrogel with excellent biocompatibility due to nucleophilic reactivity of oxidative intermediates of serotonin. Importantly, serotonin-mediated platelet activation facilitates blood coagulation, thereby improving hemostatic performance of the hydrogel in wounded tissues, which is better than a commercially available fibrin-based hemostatic agent. Furthermore, our hemostatic hydrogel can effectively stop bleeding even in a hemophilia animal model deprived of innate blood coagulation pathways. Interestingly, the hydrogel layer deposited onto damaged tissues prevents abnormal tissue-adhesion through anti-biofouling effects of cohesive hyaluronic acid hydrogel. The multifunctionality of our bioinspired hydrogel provides the potential clinical benefits for effective hemostasis in wound treatment and surgical procedures.

Introduction

Uncontrolled bleeding causes severe problems, including hypotension, organ dysfunction, and even death.[1–3] Over the years, several attempts have been made to manage excessive and uncontrolled bleeding by facilitating hemostasis in a variety of ways, including the application of hemostatic agents. Ancient remedies in Europe and America included the application of herbs and animal hides to wounds to staunch bleeding. In modern medicine, understanding the mode of action of hemostatic agents is important for successful treatment and improving the clinical outcomes. Several different types of topical hemostatic agents have been developed over the past decade.[4,5]

Although several established agents, including hemostatic sealants and tissue adhesives for hemostasis, have been used clinically, toxicity of the components and unsatisfactory hemostatic performance remain. For example, commercially available synthetic agents such as Bioglue (an albumin-based compound that is crosslinked using glutaraldehyde) may cause cytotoxic effects including restricted tissue growth, nerve dysfunction, and mutagenesis due to unreacted glutaraldehyde.[4] Concerns with thrombin-containing agents, such as Floseal and Tisseel,

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include inflammation and foreign body reaction against animal-derived proteins and the potential risk of transmission of viruses from the human-derived proteins.[4,6,7] In addition to the issues of biocompatibility, these fibrin-based agents require contact with blood as a source of fibrinogen in order to exert their hemostatic function. Thus, their hemostatic efficacy varies greatly depending on the blood coagulation system of each patient.[4] Another issue with fibrin-based glues is that the fibrin clot that remains after treatment may induce abnormal tissue adhesion or tissue fibrosis if the fibrinolytic system that removes the fibrin does not function properly.[8]

For these reasons, these hemostatic agents have recently been reexamined for their clinical safety and efficacy.[7,9–11] These investigations have determined that the ideal hemostatic agent, exhibiting good biocompatibility and effective hemostasis, should be composed of natural components of the body, with nonprotein-based main components and biomolecules that facilitate blood coagulation. To meet these criteria, approaches mimicking the natural blood coagulation pathways have been explored using natural compounds. Wang *et al.* recently reported the potential of a bio-inspired strategy using polyphosphate, a natural compound that can regulate the blood coagulation cascades, to improve the capability of chitosan-based hemostatic materials.[12] A nonprotein-based chitosan inspired by mussel adhesive chemistry has also been used to coat the surface of hemostatic hypodermic needles. This strategy completely prevented bleeding from tissue puncture following needle injection *via* intravenous and intramuscular routes.[13]

In this study, we developed a hemostatic adhesive hydrogel composed of hyaluronic acid (HA) conjugated with serotonin. In this hydrogel, serotonin functions as the active blood clotting component and the crosslinking moiety for gel formation. Serotonin is a blood coagulating mediator that can activate platelets and can also be released from the activated platelets for hemostasis.[14–16] Serotonin-mediated platelet activation induces the secretion of platelet granules containing various hemostatic factors, such as platelet factor 4, factor V, von Willebrand factor, and fibrinogen. These factors facilitate blood coagulation for hemostasis.[17] Therefore, serotonin could be an attractive and novel functional moiety for an effective hemostatic agent. We chose HA as the polymeric backbone of the hemostatic hydrogel. HA is one of the most commonly used natural polysaccharides in biomedical applications and medical products due to its excellent biocompatibility and bioactivity and the ease of functional group modification, which would also make it an ideal hydrogel backbone for hemostatic agents.[18–20]

Here, we developed a new biomacromolecule comprising HA conjugated with serotonin (HA–serotonin). HA was conjugated with serotonin to mimic the blood clotting mechanism of platelets. The HA–serotonin conjugate forms a hydrogel through oxidation-mediated crosslinking between serotonin molecules. The complex network of the hydrogel could play a role as a physical barrier to blood loss and locally concentrate serum elements,[21,22] thereby contributing to the synergistic improvement of the hemostatic performance of serotonin-mediated platelet activation. The HA–serotonin hydrogel strongly adheres to tissue because of its chemical reactivity with nucleophiles in the biomolecules, and it can form a thin hydrogel layer on the tissue surface that can effectively stop bleeding from the injured tissue. Furthermore, the HA–serotonin hydrogel barrier can simultaneously prevent abnormal adhesion on the bleeding site. This hydrogel is a new class of biocompatible and multifunctional hemostatic adhesive.

Results and discussion

HA–serotonin was synthesized by chemically conjugating serotonin to the HA backbone *via* a carbodiimide coupling reaction using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (Fig. 1a). The serotonin conjugation and the degree of substitution (DS) of carboxylic acid in the HA backbone were confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Fig. S1, ESI†). The presence of proton peaks in the serotonin moiety between 6.8 and 7.5 ppm, which indicate aromatic protons in the indole group, demonstrated successful HA–serotonin synthesis. The DS was determined to be approximately 20% by calculating the integral area ratio of the peak indicating aromatic protons in serotonin to that indicating protons in the methyl groups ($\sim$2.0 ppm) of the HA backbone. When we determined the DS by measuring the absorbance of HA–serotonin at 280 nm using ultraviolet-visible (UV-vis) spectroscopy (Fig. S2, ESI†), it was found that the serotonin molecules were conjugated to $\sim$9.1% of the carboxyl groups in the HA backbone. The DS determined from UV-vis-based quantification was approximately half of that from ^{1}H-NMR-based quantification. We assumed that the difference in the DS values between two methods is possibly due to the different operating principles of two spectroscopies and the spectrum change of serotonin within HA–serotonin polymer (absorbance peak = 280 nm) shifted from the peak of serotonin alone (absorbance peak = 275 nm).[23] HA–serotonin conjugates were crosslinked *via* enzymatic oxidation processes using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). This crosslinking strategy is considered safe and suitable for biomedical applications due to its substrate specificity and efficiency, mild reaction condition, and good cytocompatibility.[24–26] Enzymatic oxidation of HA–serotonin solution using HRP/H_2O_2 resulted in a slightly yellowish hydrogel construct within 20 to 30 seconds by the chemical bonding between oxidized products of 5-hydroxyindole in serotonin (Fig. 1b and Fig. S3a, ESI†). The stability of the resulting hydrogel was confirmed by a consistently higher storage modulus (G') than loss modulus (G'') in the measured frequency ranges from 0.1 to 10 Hz in rheometric analysis (Fig. S3b, ESI†).

For the analysis of the gelation kinetics of the HA–serotonin hydrogel, the gel–sol transition time and gelation completion time at different concentrations of HRP and H_2O_2 were measured during the gelation of HA–serotonin solution crosslinked by HRP/H_2O_2 in glass vials containing a stir bar. The gelation time increased considerably with the concentration of HRP, but increase in H_2O_2 concentration did not show a similar

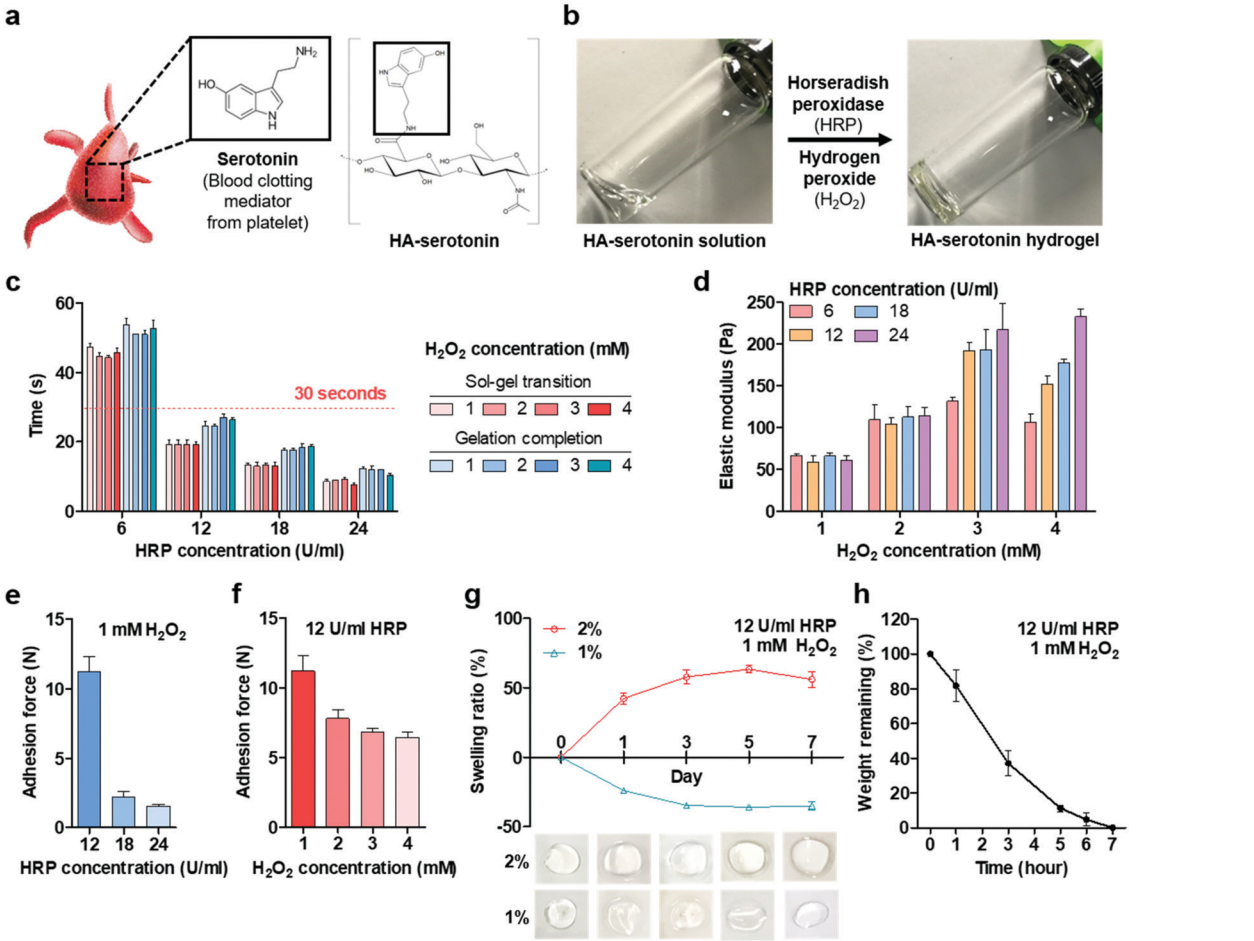

Fig. 1 Characterization of HA–serotonin hydrogel. (a) Schematic representation and chemical structure of a bio-inspired HA–serotonin hemostatic hydrogel. (b) Formation of HA–serotonin hydrogel *via* enzymatic oxidation of HA–serotonin solution using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). (c) Sol–gel transition time and gelation completion time of HA–serotonin hydrogels crosslinked using different concentrations of HRP and H_2O_2 (n = 3). (d) Average elastic modulus (G') of HA–serotonin hydrogels at different concentrations of HRP and H_2O_2 (n = 3). (e) Adhesive force of HA–serotonin hydrogels formed using different concentrations of HRP at a fixed concentration of H_2O_2 (1 mM) (n = 3). (f) Adhesive force of HA–serotonin hydrogels formed using different concentrations of H_2O_2 at a fixed concentration of HRP (12 U ml^{-1}). The adhesive force was measured using a rheometer in the tack test mode (n = 3). (g) Measurement of swelling property of HA–serotonin hydrogels formed with different concentrations of pre-gel solution upon incubation in PBS at 37 °C (n = 3). (h) Enzymatic degradation profile of HA–serotonin hydrogel formed with 12 U ml^{-1} HRP and 1 mM H_2O_2 by hyaluronidase treatment (2.5 U ml^{-1}) (n = 3).

influence (Fig. 1c); this result was consistent with the findings from other studies involving crosslinking by HRP/H_2O_2.[24] In particular, the gelation times using 12, 18, and 24 U ml^{-1} HRP were less than 30 seconds, but the gelation times using 6 U ml^{-1} HRP were approximately 50 seconds, which was somewhat longer than for other HRP groups (Fig. 1c). Likewise, HA–serotonin hydrogels crosslinked under different oxidative conditions exhibited different physical and rheological properties. In general, the average storage modulus of HA–serotonin hydrogels (at 1 Hz) formed by HRP/H_2O_2 increased proportionally to the increase in the concentration of H_2O_2 and HRP (Fig. 1d). It was also previously reported that the mechanical properties of hydrogels can be controlled by changing the concentration of HRP/H_2O_2, with the influence of the concentration of H_2O_2 being especially influential.[24]

HA–serotonin hydrogels adhered strongly to tissue because the serotonin derivatives, which include serotonin radical and tryptamine dione generated during serotonin oxidative processes, can bind to biomolecules such as proteins containing amines, thiol, and phenol.[27] Here, the adhesive force of HA–serotonin hydrogels was measured by a tack-separation test using a rheometer. When the concentration of H_2O_2 was fixed (1 mM), the adhesive forces decreased sharply as the concentration of HRP increased (Fig. 1e); however, the decrease was gradual when the HRP concentration was fixed (12 U ml^{-1}) and the H_2O_2 concentration increased (Fig. 1f). We assumed that the faster oxidation rate obtained by increasing the concentration of the enzyme (HRP) and substrate (H_2O_2) could enhance the cohesion of the HA–serotonin backbone by accelerating internal crosslinking between conjugated serotonin molecules. This may lead to fewer interactions between oxidized serotonin and other substrates, thus decreasing adhesiveness. Based on these results, HA–serotonin hydrogels that were crosslinked using 12 U ml^{-1} HRP and 1 mM H_2O_2 appeared to be most suitable for further

applications in terms of hydrogel handling related to gelation time and adhesive property. Thus, these concentrations were chosen for the subsequent experiments. The swelling properties of HA–serotonin hydrogel crosslinked using the optimized concentrations of HRP and H_2O_2 differed drastically depending on the concentration of the HA–serotonin solution. The hydrogel prepared from 2% (w/v) HA–serotonin solution swelled over 50% from the initial state over 3 days and then remained stable. In contrast, the hydrogel prepared with 1% (w/v) HA–serotonin solution shrank to about 35% of its initial state over 3 days and was maintained in the shrunken state (Fig. 1g). In seeking to explain the reverse swelling profile between the 1% and 2% HA–serotonin hydrogels, we assumed that the optimal oxidative conditions of 12 U ml^{-1} HRP and 1 mM H_2O_2 were exhausted for serotonin oxidation in 2% (w/v) HA–serotonin, but were not fully exhausted in 1% (w/v) HA–serotonin with a lower serotonin content. The excess oxidative in the latter condition might induce additional side reactions, such as oxidative degradation of the HA backbone, which could weaken the structural integrity and mechanical property of HA hydrogel.[28–30] The biodegradability of HA–serotonin hydrogel was confirmed by enzymatic degradation with 2.5 U ml^{-1} hyaluronidase using hydrogel formed in the 2% (w/v) HA–serotonin solution, 12 U ml^{-1} of HRP, and 1 mM of H_2O_2. The HA–serotonin hydrogel was completely degraded by hyaluronidase (Fig. 1h), indicating that the HA–serotonin hydrogel can be naturally removed *in vivo* after achieving hemostasis.

Owing to its biodegradability and high binding affinity to diverse nucleophiles, HA–serotonin hydrogel can also serve as a delivery platform for controlled and sustained drug release. As a demonstration, HA–serotonin hydrogel loaded with vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, was exposed to a physiological condition using phosphate-buffered saline (PBS) supplemented with 0.5 U ml^{-1} hyaluronidase at 37 °C. VEGF was released in a sustained manner from the hydrogel for up to 96 hours of incubation (Fig. S4, ESI†). In contrast, VEGF was barely released from the HA–serotonin hydrogel under the same conditions, except for the absence of hyaluronidase, probably due to covalent tethering of the VEGF to oxidized serotonin derivatives in the hydrogel. The observations provide evidence supporting the value of the HA–serotonin hydrogel for the controlled and sustained *in vivo* delivery of therapeutic drugs, including growth factors, cytokines, and antibodies, indicating the potential of the HA–serotonin hydrogel-based system to simultaneously facilitate hemostasis and tissue repair.

Next, we elucidated the underlying crosslinking mechanisms of HA–serotonin hydrogel based on the oxidative products of serotonin (Fig. 2a).[31,32] X-ray photoelectron spectroscopy (XPS) was used to confirm the proposed chemical reactions for crosslinking of HA–serotonin hydrogel. Each sample for XPS analysis was prepared by lyophilizing either a drop of the HA–serotonin solution with HRP alone (before oxidation group) or the HA–serotonin hydrogel formed by oxidative crosslinking using HRP and H_2O_2 (after oxidation group) on a titanium substrate. As shown in Fig. 2b, the C1s spectrum of both groups contains five common bands associated with C–C, C–O–C, C═O, O–C═O, and C═C bonds. During the oxidative crosslinking process, 5-hydroxyindole residues in the conjugated serotonin form various oxidative products, such as hydroxyindole radical and indole-4,5-dione, which can further react with each other and form several kinds of dimers (Fig. 2a). In the XPS spectrum of the oxidation group, the peak intensity associated with the C–C and C–O–C bonds formed between the conjugated serotonin increased and the peak intensity associated with the C═O bonds indicating indole-4,5-dione also increased, when compared with the intensities of the corresponding peaks in the spectrum of the control (before oxidation) group (Fig. 2b). Interestingly, the bands associated with the π–π* interaction and C–Ti interaction appeared in the spectrum of the oxidation group, additionally demonstrating the oxidative crosslinking mechanisms and the surface adhesive mechanism of the HA–serotonin hydrogel. The proposed crosslinking mechanism was also confirmed using Fourier transform infrared (FTIR) spectroscopy (Fig. 2c). The peak related to the C–O–C stretching vibration of phenyl ether at 1255 cm^{-1} was detected in the FTIR spectrum for HA–serotonin hydrogel crosslinked using HRP and H_2O_2 (after oxidation group), but not in that for HA–serotonin solution with only HRP (before oxidation group), which indicated the formation of diphenyl ether structures between the conjugated serotonin molecules within HA–serotonin hydrogel.[33] In addition, the peaks related to the C–C stretching vibration of the aromatic ring in the spectrum for the control group (before oxidation group) at 1462, 1541, and 1593 cm^{-1} slightly shifted to the neighborhoods at 1466, 1518, and 1585 cm^{-1}, respectively in the oxidation group, which demonstrated the deformation of the aromatic ring and indicated the formation of biphenolic products.[34–36] The chemical reactions during crosslinking were further examined by analyzing the absorbance changes using UV-vis spectroscopy (Fig. 2d). After beginning the enzymatic oxidation by HRP and H_2O_2, both the spectral peaks at ~400 nm, which likely corresponded to the quinone group, and the spectral peaks at ~287 nm, demonstrating various oxidized components like biphenols and biphenyl ethers derived from crosslinked serotonin molecules, gradually increased with the time of incubation. The observations could be evidence of oxidative covalent coupling between the molecules with aromatic hydroxyl groups like phenol or catechol.[37–40] Through insights from previous literatures, we could hypothesize that physical crosslinking between the indole groups in serotonin molecules may also contribute to crosslinking to form HA–serotonin hydrogel since there could be a variety of possible inter-molecular interactions between indole groups and its dimers, such as hydrogen bonding, ionic, cation–π, and π–π interactions.[41,42] Thus, in addition to oxidative chemical crosslinking *via* enzymatic reaction, those non-covalent interactions could contribute to crosslinking of HA–serotonin hydrogel by inducing self-assembly and aromatic ring stacking.[41,42]

Interestingly, HA–serotonin can serve as a multifunctional hydrogel with both tissue adhesive and anti-fouling properties. As mentioned, oxidized intermediates of serotonin can bind to other molecules like proteins by forming covalent bonds with functional groups, such as amine and thiol groups, by nucleophilic addition.[27] The proposed tissue adhesive mechanisms of HA–serotonin hydrogel using these chemical properties of

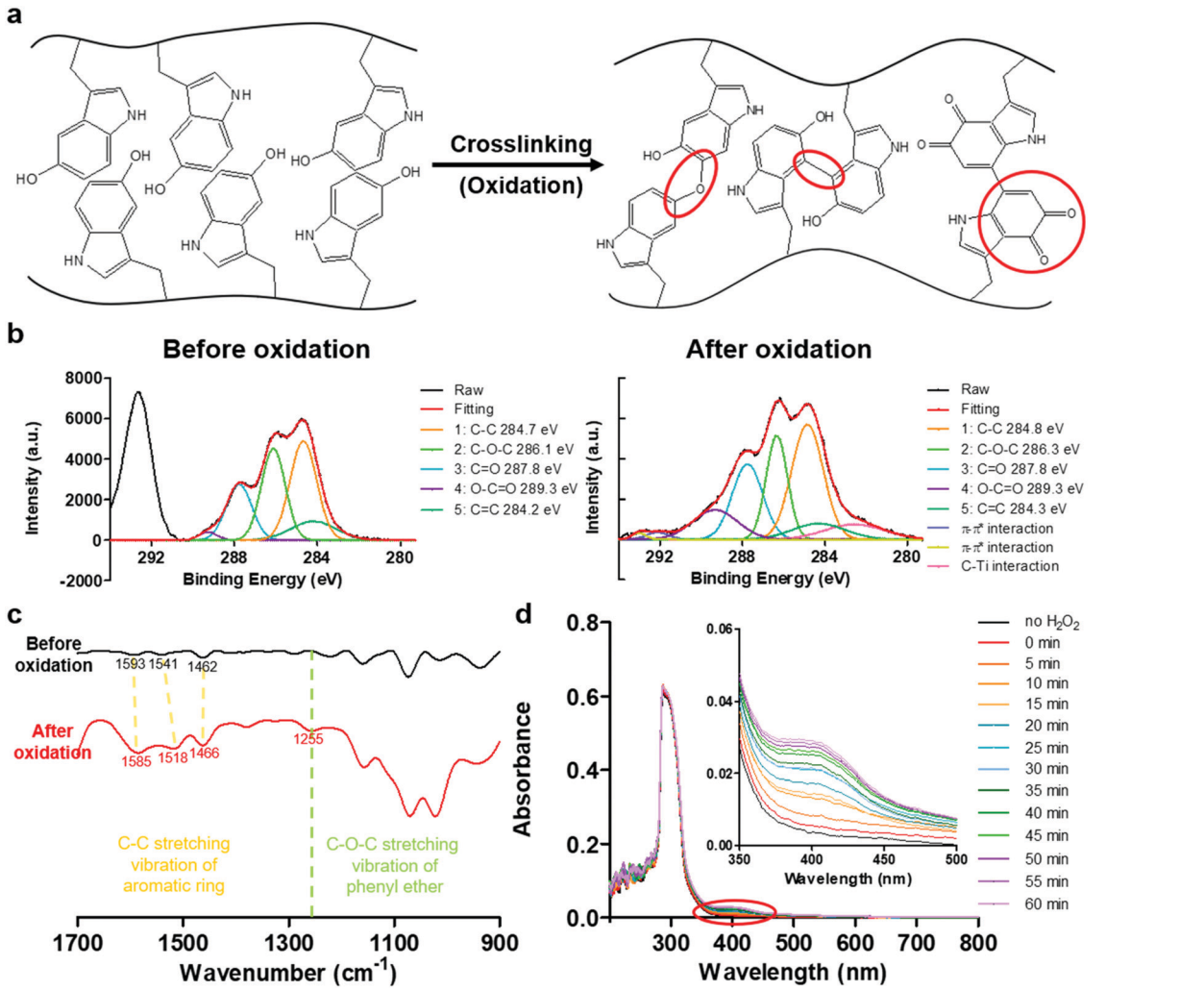

Fig. 2 Chemical analysis of crosslinking mechanisms of HA–serotonin hydrogel. (a) Proposed crosslinking chemical mechanisms *via* oxidative of conjugated serotonin molecules within HA–serotonin polymers induced by HRP and H_2O_2. (b) XPS and (c) FTIR spectra of uncrosslinked HA–serotonin solution (before oxidation) and HA–serotonin hydrogel crosslinked *via* enzymatic oxidation using HRP and H_2O_2 (after oxidation). (d) UV-vis absorbance profiles of HA–serotonin hydrogel during gelation triggered by addition of H_2O_2.

serotonin are illustrated in Fig. 3a. We assume that the formation of imine by Schiff base reaction between dione from oxidized intermediates of serotonin and amine groups in proteins might also contribute to the tissue adhesiveness of HA–serotonin hydrogels.[43,44] Due to the adhesive properties gained during oxidative crosslinking, the HA–serotonin hydrogel can tightly adhere to the tissue surfaces to create a thin and stable hydrogel layer (Fig. 3b and Fig. S5a, ESI†). Upon completion of gelation after 10 min, the HA–serotonin hydrogel no longer displayed adhesiveness to substrates (Fig. S5b, ESI†). This may be because almost all the oxidative intermediates of serotonin were consumed during the cohesive crosslinking involved in hydrogel formation and the binding to nucleophiles that occurred during tissue adhesion. Thus, it is likely that no adhesive moieties are available for tissue adhesion after gelation completion. In addition, because HA has a strong overall negative charge and strongly interacts with water, the HA–serotonin hydrogel can provide further resistance to protein adsorption and cell adhesion, leading to the anti-biofouling effect of HA–serotonin hydrogel layer formed on the tissue surface.[45,46] These hydrogel features likely contribute to the prevention of abnormal tissue adhesion at bleeding and damaged sites after hemostatic application of HA–serotonin hydrogel (Fig. 3c). To verify this anti-adhesion effect, a mouse abdominal wall abrasion model was established.[47,48] Seven days post-operatively, untreated abraded abdominal walls tightly adhered to surrounding organs like the intestine, indicating abnormal tissue adhesion. In contrast, abraded walls treated with the HA–serotonin hydrogel did not adhere to any surrounding organs and tissues, indicating the prevention of abnormal adhesion by the HA–serotonin hydrogel layer on the tissue surfaces (Fig. 3d and e).

Serotonin modification of HA hydrogel could facilitate blood coagulation and improve the hemostatic capability of the HA adhesive. Since serotonin mediates blood clotting in platelets,[14–16] we expected that HA–serotonin hydrogel would facilitate hemostasis *via* serotonin-mediated platelet activation by inducing the

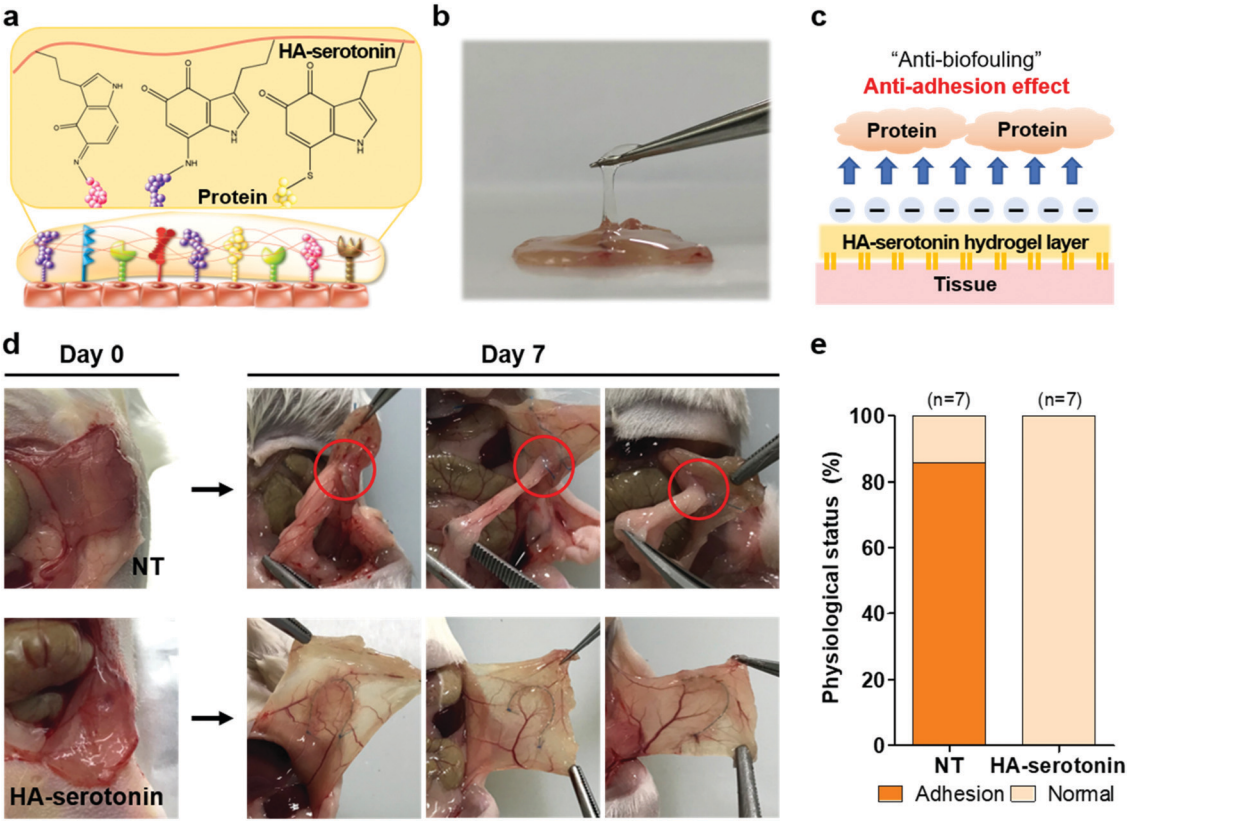

Fig. 3 Prevention of post-surgical abdominal adhesion by HA–serotonin hydrogel layer. (a) Schematic illustration of the chemistry for tissue-adhesiveness of HA–serotonin hydrogel through oxidative processes of conjugated serotonin and its intermediates. (b) Gross view of HA–serotonin hydrogel applied to the tissue surface of mouse abdominal wall. (c) Proposed mechanism of anti-adhesion effect by thin HA–serotonin hydrogel layer adhered onto the tissue surface. (d) Tissue morphology of abraded regions in the mouse abdominal wall abrasion model with no treatment (NT group) and HA–serotonin hydrogel treatment (HA–serotonin group), immediately after (day 0) and 7 days after the operation. (e) Frequency of occurrence of abnormal tissue adhesion on the abraded abdominal wall of the mice at day 7 after operation (n = 7).

release of various coagulating factors in platelet granules for hemostasis (Fig. 4a). To validate this suggestion, we compared the quantity of coagulation factors released from the platelet granules when the platelet-rich plasma (PRP) was incubated with each sample (PBS, serotonin, HA, and HA–serotonin). Enzyme-linked immunosorbent assay (ELISA) for coagulation factors demonstrated that when the serotonin solution and HA–serotonin solution were individually mixed with PRP, there was a statistically significant increase in the level of factors released from platelet alpha-granules in the activated platelets (Platelet factor 4 and factor V) as compared with the baseline (PBS group), but there was no increase in the level of those factors when only the HA solution was mixed with PRP (Fig. 4b and c). We also tested whether serotonin-mediated platelet activation by HA–serotonin can facilitate blood coagulation. When the whole blood was mixed and incubated with each sample (PBS, serotonin, HA, and HA–serotonin) on the glass substrate, the formation of blood clots was prominent in both the serotonin and HA–serotonin groups, while only traces of remaining blood were observed in the PBS and HA groups (Fig. 4d). Especially, the blood clot mass in the HA–serotonin group was larger than even that in the serotonin group. We assumed that the improved blood coagulation in the HA–serotonin group resulted from the synergistic effect by the biologically enhanced hemostasis *via* serotonin-mediated platelet activation and enhanced physicochemical interaction by the HA polymeric network in HA–serotonin hydrogel. We have also compared quantitatively the blood clotting kinetics of HA–serotonin and fibrinogen/thrombin-based system (fibrin), a representative of the existing hemostatic agent, by measuring the blood clotting time of each system per unit amount of material (Fig. S6, ESI†). The blood clotting time of both hemostatic agents decreased as the amount of materials increased. Interestingly, HA–serotonin exhibited the blood clotting kinetics more dependent on material dose than fibrin agent did, indicating that control of hemostatic performance to cope with diverse clinical situations may be much easier and more efficient with HA–serotonin than with the existing hemostatic agents like fibrin glue. Such flexible control in blood clotting kinetics by simply changing material doses could suggest another competitive advantage of HA–serotonin hydrogel over conventional hemostatic agents for realistic applications.

Finally, we evaluated the hemostatic performance of HA–serotonin hydrogel in a mouse liver hemorrhage model. Before the *in vivo* experiments, we checked the cytotoxicity of the developed HA–serotonin hydrogel. Live/Dead staining indicated that most encapsulated cells (HepG2 cells and human adipose-derived stem cells; hADSCs) were highly viable in the

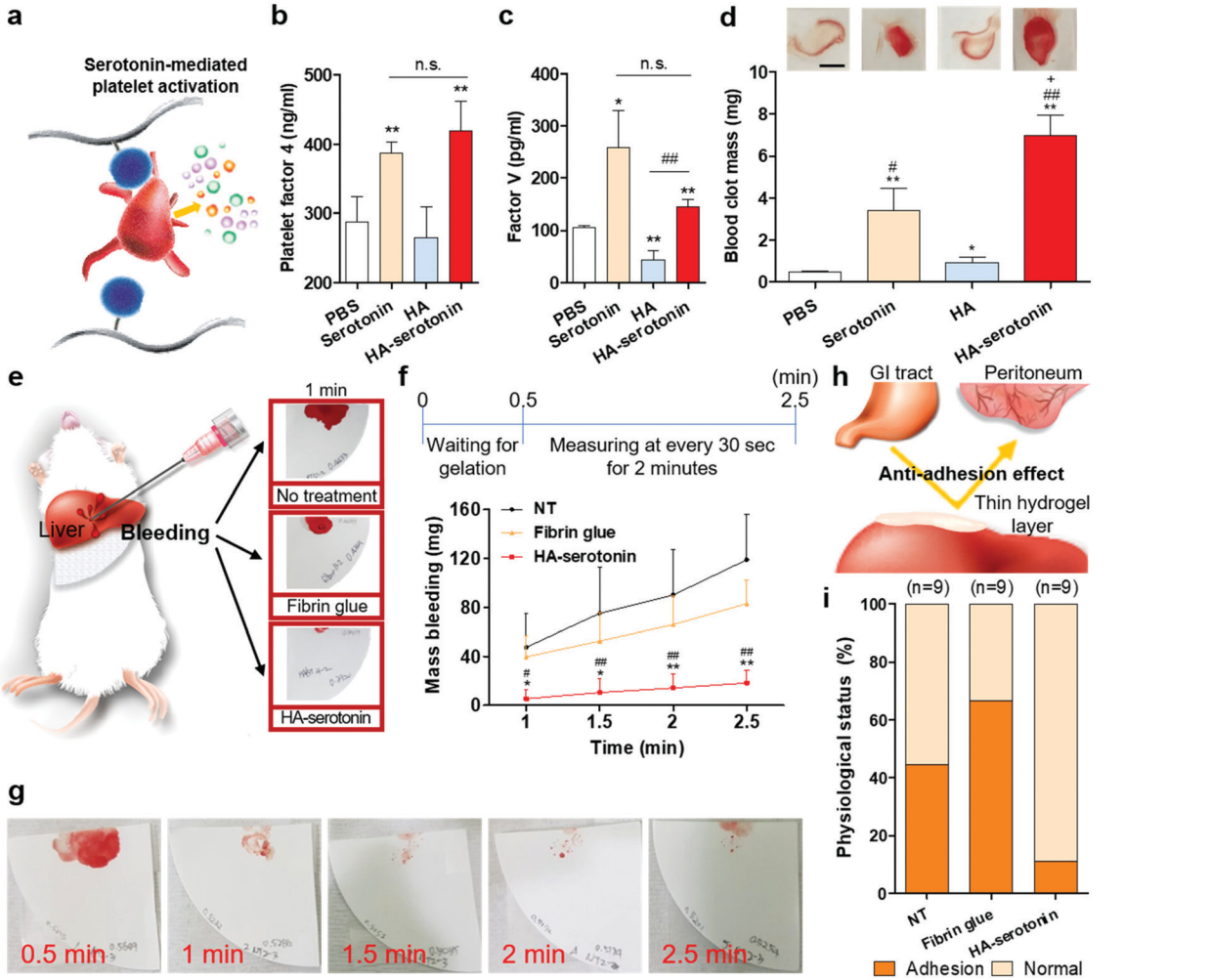

Fig. 4 Enhanced blood coagulation and *in vivo* hemostatic capability of HA–serotonin hydrogel with an anti-adhesion effect. (a) Schematic representation of the platelet activation mediated by conjugated serotonin molecules in HA–serotonin hydrogel. ELISA analysis to quantify the concentration of (b) platelet factor 4 and (c) factor V in the PRP solution mixed and incubated with PBS, serotonin, HA, or HA–serotonin solution for 60 minutes ($n = 4$ for platelet factor 4 and $n = 3$ for Factor V) (*$p < 0.05$ and **$p < 0.01$ *versus* PBS group, ##$p < 0.01$ *versus* HA group). (d) Blood clot mass from the mixture of the whole blood with PBS, serotonin, HA, and HA–serotonin solution on glass after incubation for 60 min and washing in PBS ($n = 3$, *$p < 0.05$ and **$p < 0.01$ *versus* PBS group, #$p < 0.05$ and ##$p < 0.01$ *versus* HA group, +$p < 0.05$ *versus* serotonin group). (e) Schematic illustration of the mouse liver hemorrhage model and representative photographs of the papers to check the bleeding level from the damaged liver treated by HA–serotonin or fibrin glue (positive control) and without treatment (negative control) at 1 minute after the injury. (f) The accumulated amount of bleeding mass in non-treated (black), fibrin glue-treated (yellow), and HA–serotonin-treated (red) mice every 30 seconds for 2 minutes beginning 30 seconds after treatment of hemostatic agents ($n = 5$). Two-way analysis of variance (ANOVA) was used to determine the statistical significance (*$p < 0.05$, and **$p < 0.01$ *versus* NT group, #$p < 0.05$ and ##$p < 0.01$ *versus* fibrin glue group). (g) Photographs of the papers that absorbed blood from HA–serotonin-treated liver every 30 seconds up to 2.5 minutes after treatment. (h) Schematic description of *in vivo* anti-adhesion effect of HA–serotonin hydrogel accompanied by hemostatic function. (i) The frequency of abnormal tissue adhesion near the surgical sites in mice after hemostasis test ($n = 9$). The tissue adhesion was checked at day 1, 3, and 7 in the hemostasis test by sacrificing three mice per each group (NT, fibrin glue, and HA–serotonin) at each time point.

HA–serotonin hydrogel during the oxidative crosslinking process for gelation and three-dimensional culture for 7 days (Fig. S7a and b, ESI†). The hemostatic capability of the HA–serotonin hydrogel was evaluated by comparison with a commercially available hemostatic agent (fibrin glue) as a positive control group. The HA–serotonin hydrogel and fibrin glue were applied to mouse liver with bleeding induced by an 18-gauge needle puncture, and the amount of bleeding from liver of each group was measured by using filter papers (Fig. 4e). Although the amount of bleeding in both groups treated with the same amount of hemostatic agents (Fibrin glue and HA–serotonin hydrogel) decreased compared to that in no treatment (NT) group, bleeding mass was significantly reduced by HA–serotonin hydrogel treatment in comparison to fibrin glue treatment ($p < 0.01$, two-way ANOVA test; Fig. 4f), indicating that the HA–serotonin hydrogel could exhibit better hemostatic performance than commercially available fibrin-based hemostatic agent. Photographs of the filter papers used for the hemostasis test using the HA–serotonin hydrogel captured every 30 seconds after bleeding initiation confirmed that the HA–serotonin hydrogel could stop bleeding within 30 seconds and substantially reduce mass bleeding as a result of highly efficient hemostasis (Fig. 4g). No apparent

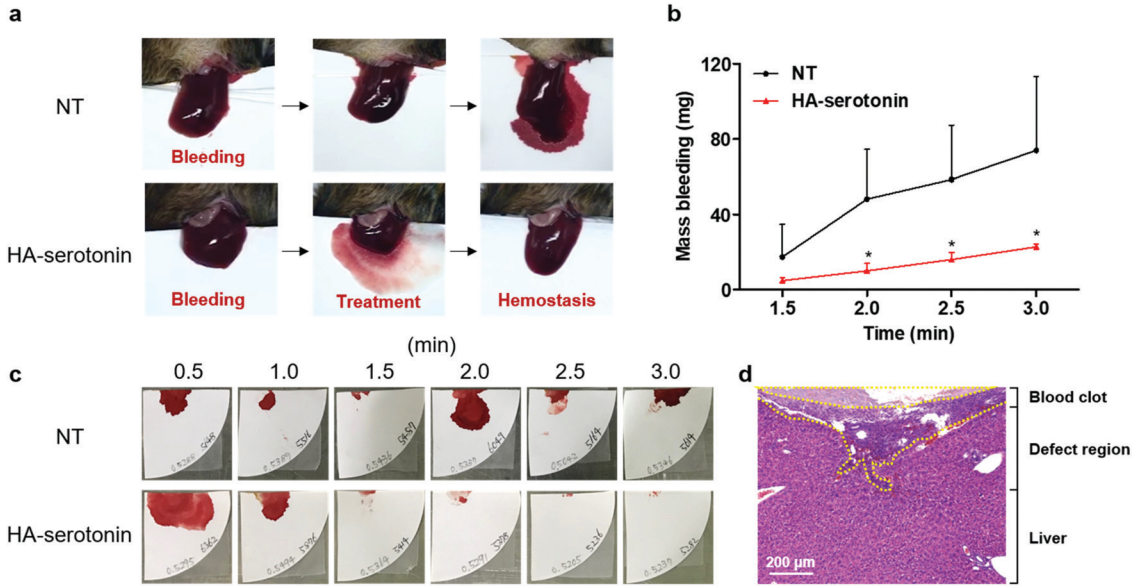

Fig. 5 Hemostasis induced by HA–serotonin hydrogel in a mouse model of hemophilia. (a) HA–serotonin-mediated hemostasis in a liver hemorrhage model of Factor VIII-deficient hemophilia mice without treatment (NT, top) and with treatment using HA–serotonin hemostatic adhesive (HA–serotonin, bottom). (b) The accumulated blood loss from hemophilia mice over time after bleeding without any treatment (NT, black) and with the treatment of HA–serotonin hemostatic adhesive (HA–serotonin, red) (n = 3). One-tailed t-test was used to determine statistical significance (*$p < 0.05$ *versus* NT group). (c) Photographs of the blood-absorbed papers from non-treated and HA–serotonin-treated hemophilia mice every 30 seconds up to 3 minutes after treatment. (d) Histological analysis (H&E staining) of the damaged liver harvested from HA–serotonin-treated hemophilia mice 3 days after the hemostasis test. Scale bar = 200 μm.

physiological abnormality was observed in the mice treated with HA–serotonin hemostatic hydrogel for at least 7 days after the experiment. After the bleeding test, histological analysis was conducted with the liver tissues retrieved from each group to check whether treatment with HA–serotonin hydrogel has adverse effects on the tissues. Toluidine blue staining to detect immune cells did not reveal any unusual immune response in the regions treated with HA–serotonin hydrogel compared with the tissues from normal liver or the untreated group, confirming the safety and biocompatibility of HA–serotonin hydrogel for medical use (Fig. S8, ESI†). Additionally, the examined HA–serotonin hydrogels displayed anti-adhesion behavior to repel tissues surrounding the damage tissue, including the gastrointestinal (GI) tract and peritoneum, by forming a thin layer on the sites of bleeding (Fig. 4h). Abnormal tissue adhesion was frequently found in the untreated (44%) and fibrin glue (66%) groups (n = 9), while only a single case among nine experimental cases was found in the HA–serotonin group (11%) (Fig. 4i). Thus, HA–serotonin hydrogel has a significant potential as a multifunctional medical adhesive that is simultaneously a very effective hemostatic material with anti-adhesion behavior.

Hemostatic materials enabling bleeding control in coagulopathic situation provide a great clinical significance. The development of hemostatic swabs made of chitosan–catechol conjugate was previously demonstrated for bleeding control in mouse and rat models with diabetic coagulopathy.[23] To further evaluate the clinical benefits of our HA–serotonin in more clinically relevant situations, the HA–serotonin hydrogel was tested on a mouse strain with hemophilia (B6;129S-F8^{tm1Kaz}/J). When liver hemorrhage was induced in hemophilic mice with an 18 G needle, bleeding was continuous at the injured sites in the untreated group and was ultimately fatal (Fig. 5a). In contrast, bleeding could be successfully stopped using HA–serotonin adhesive hydrogel (Fig. 5a). The amount of bleeding mass in the mice treated with HA–serotonin hydrogel was significantly decreased compared to that in the untreated group ($p < 0.05$, Student's t-test at each time point; Fig. 5b). Photographs and movies of the filter paper from each group revealed only a trace of absorbed blood in the paper 1 min after HA–serotonin treatment, while bleeding was continuously observed in untreated mice (Fig. 5c and Movie S1, ESI†). Remarkably, histological analysis (hematoxylin and eosin staining) revealed a blood clot-like structure in the bleeding region of the liver retrieved from the mice that had a complete hemostasis using HA–serotonin (Fig. 5d), even though innate blood coagulation is almost impossible in mice with hemophilia. We assumed that the blood coagulation could be effectively induced by HA–serotonin hydrogel even in a hemophilic condition due to the synergistic effect of serotonin-mediated platelet activation and the physical barrier imposed by the adhesive polymeric network within HA–serotonin hydrogel. HA–serotonin-mediated hemostasis is thought to bypass the factor VIII-mediated coagulation pathway, which is deficient in hemophilia.[49,50] Our results demonstrated the potential clinical benefit of HA–serotonin hydrogel for effective hemostasis in the clinically relevant situations and diseases.

Conclusions

In conclusion, we have developed and described a new bio-inspired hemostatic material with a novel crosslinker. The HA–serotonin hydrogel is multifunctional with hemostasis and

anti-adhesion evident *in vivo*. The HA–serotonin hydrogel system as a hemostatic adhesive possesses several distinct advantages. First, the biocompatibility of our HA derivative is best compared with the existing hemostatic agents, reflecting the use of compounds and polysaccharide naturally resident in the body as the main components. The thin hydrogel layer tightly adheres to the tissue surface at sites of bleeding. The tissue adhesiveness of the HA–serotonin hydrogel system is superior to current hemostatic agents. The hydrogel layer can also restrict adhesion. The dual functions of hemostasis and anti-adhesion of the single biomaterial could have practical and economic benefits. Serotonin-mediated platelet activation conferred by the conjugated serotonin and the physical interaction of the hydrogel network can synergistically improve the hemostatic performance of the HA–serotonin hydrogel, creating the possibility for effective hemostasis even when innate blood coagulation is impaired, as in patients with hemophilia. Considering the biocompatibility and nucleophilic affinity of the HA–serotonin hydrogel, its utility could be further expanded to tissue engineering scaffolds and drug delivery carriers. The unique bio-inspired hydrogel system with the multifunctional performance advantages summarized above could overcome the limitations of existing hemostatic agents, and could prove valuable in improving medical treatment and consequent clinical outcomes.

Experimental section

Experimental details are available in the ESI.†

Conflicts of interest

The authors declare no conflicts of interest.

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