Supporting Information

Serotonin-modified Hyaluronic Acid Hydrogel for Multifunctional Hemostatic Adhesive Inspired by a Platelet Coagulation Mediator

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Experimental Details

Synthesis of HA-serotonin Conjugate and HA-serotonin Hydrogel Formation: HA (molecular weight [MW] 200 kDa, Lifecore Biomedical, IL, USA) was dissolved in triple distilled water (TDW) at a concentration of 1 mg/ml. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, Thermo Fisher Scientific, Waltham, MA, USA) and NHS (Sigma-Aldrich, St. Louis, MO, USA) were added to the HA solution at an equal molar ratio to HA and stirred for 30 minutes at pH 5.5-6.0. Serotonin hydrochloride (Sigma-Aldrich) was added to the solution at a 1:1 molar ratio to HA and stirred overnight at room temperature at the same pH. Unreacted chemical and byproducts were removed by dialysis using a Cellu Sep T2 dialysis membrane with a MW cut-off of 6-8 kDa (Membrane Filtration Products Inc., Seguin, TX, USA) in 1× PBS (Sigma-Aldrich) in TDW at pH 5.0. The synthesized product was lyophilized and stored at 4°C until use. The successful conjugation of serotonin to HA was confirmed using ^{1}H-NMR at 300 MHz (Bruker, Billerica, MA, USA). Degree of substitution (DS) of HA-serotonin was measured using ^{1}H-NMR by calculating the integral area ratio of the peak of serotonin to that of the methyl groups of the HA backbone. The DS of HA-serotonin was also determined using ultraviolet-visible (UV-vis) light spectrophotometer (JASCO Corporation, Tokyo, Japan). The absorbance of HA-serotonin solution at 280 nm was measured with a quartz cuvette and the DS was calculated using a standard curve of 5-hydroxytryptamine solutions with serial dilutions. To induce hydrogel formation, HA-serotonin conjugate dissolved in PBS was mixed with several concentrations of diluted HRP (Sigma-Aldrich) solution and hydrogen peroxide (H_2O_2, Sigma-Aldrich) solution with an HA-serotonin:HRP:H_2O_2 volumetric ratio of 8:1:1.

Gelation Time of HA-serotonin Hydrogel: To measure the time for crosslinking, HA-serotonin hydrogels were formed in glass vials containing a stirring bar at a speed of 120 rpm. During the crosslinking reaction via the enzymatic oxidation by combinations of different

concentration of HRP and H_2O_2, we measured the times when the stirring bar speed was rapidly reduced by rheological changes (sol-gel transition) and when the rotation of the stirring bar stopped by the cohesive property of the formed hydrogel (gelation completion) (n = 3).

Rheological Analysis and Adhesive Force Measurement: All rheological analyses were performed using a model MCR 102 rheometer (Anton Paar, Ashland, VA, USA). The storage modulus (G′) and loss modulus (G″) of the hydrogel were measured in a frequency sweep mode at a frequency range of 0.1 to 10 Hz. The elastic modulus of the hydrogel was determined by calculating the average storage modulus of each hydrogel at 1 Hz (n = 3). Gelation kinetics of HA-serotonin hydrogel was measured in a time sweep mode at a 10% strain and 1 Hz frequency. Adhesive force of the hydrogel was measured in a tack-separation mode by recording the detachment force of the hydrogel between probe and base plate while pulling the probe at 10 μm/s (n = 3).

Swelling and Degradation Profiles of HA-serotonin Hydrogel: The swelling property of HA-serotonin hydrogel was assessed by measuring the weight of the remaining hydrogel at several time points. Briefly, the hydrogel was incubated in PBS at 37°C and the weight of the hydrogel at each time point was measured. The swelling ratio was calculated as $(W_t - W_i)/W_i \times 100$, where W_t represents the weight of hydrogel at each time point and W_i represents the weight of hydrogel at day 0 (n = 3). To investigate the degradation profile, HA-serotonin hydrogel that was fully swollen over 3 days was treated with hyaluronidase (2.5 U/ml, Sigma) in PBS at 37°C and the weight of remaining hydrogel was measured at each time point (n = 3; 1, 3, 5, 6, 7 hours after incubation).

Drug Release Profiles of HA-serotonin Hydrogel: To test the potential of HA-serotonin hydrogel as a drug delivery carrier for controlled and sustained drug release, VEGF (Peprotech, Rocky Hill, NJ, USA) was encapsulated in HA-serotonin hydrogel. VEGF-containing HA-serotonin hydrogels were incubated in PBS or hyaluronidase solution (0.5 U/ml in PBS) for 96 hours. Solution was collected from each sample at several time points (3, 6, 9, 12, 24, 36, 48, 72, and 96 hours). The cumulative amount of released VEGF in the retrieved solution was quantified using a human VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA).

Chemical Analysis of Crosslinking Mechanism of HA-serotonin Hydrogel: XPS spectra were obtained using a K-alpha X-ray Photoelectron Spectrometer (Thermo Fisher Scientific). The samples used for the XPS analysis were prepared by lyophilizing HA-serotonin solution with HRP (before oxidation group) and HA-serotonin hydrogel crosslinked by using HRP and H_2O_2 (after oxidation group) on a titanium (Ti) substrate purchased from National Nanofab Center (Daejeon, South Korea). The FTIR spectrum was acquired using a Vertex 70 FTIR spectrometer (Bruker) in the attenuated total reflectance mode. The transmission spectra of the samples from before and after oxidation were obtained at wavelengths of 900 to 1700 cm^{-1}. The absorbance spectra of HA-serotonin solution containing HRP before and after triggering oxidation by adding H_2O_2 were measured using a model V-650 UV-vis spectrometer (JASCO Corporation) every 5 minutes for 1 hour.

In Vitro Biocompatibility of HA-serotonin Hydrogel: To examine the biocompatibility of the HA-serotonin hydrogel *in vitro*, HepG2 cells and hADSCs (1.0×10^6 cells per 100 μl of hydrogel) were encapsulated in the hydrogel crosslinked via enzymatic oxidation using 12 U/ml HRP and 1 mM H_2O_2. The viability of cells cultured in the hydrogel was evaluated using the Live/Dead viability/cytotoxicity kit (Invitrogen, Carlsbad, CA, USA) at days 0 and

7 in the culture following the manufacturer's protocol. The stained cells were observed using a model IX73 fluorescence microscope (Olympus, Tokyo, Japan), and the ratio of viable cells (green) to dead cells (red) was quantified by manual counting from the acquired images (n = 3). HepG2 cells were cultured with high glucose Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). hADSCs were cultured with MesenPRO-RS^{TM} medium (Invitrogen).

Assessment of Prevention of Adhesion by HA-serotonin Hydrogel in Mouse Model of Abdominal Adhesion: All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Yonsei University (approval number: IACUC-A-2201705-577-04 and IACUC-A-201803-706-01). To investigate the ability of HA-serotonin hydrogel to prevent adhesion we used a modified method to induce abdominal adhesion in a mouse model.[1, 2] ICR, female, 4-week-old mice (Orient Bio, Seongnam, Gyonggi-do, Korea) were anesthetized with a mixture of ketamine (100 mg kg^{-1}, Yuhan, Seoul, South Korea) and xylazine (20 mg kg^{-1}, Bayer Korea, Ansan, South Korea) by intramuscular injection. Abdominal hair was shaved off and the shaved region was sterilized. One anterior round incision was made and a 1.5 cm incision was made in the peritoneal cavity. A 0.5 × 0.5 cm area of partial peritoneal damage with bleeding was created using sandpaper. The damaged tissue was covered immediately with HA-serotonin hydrogel solution. After gelation, the wound area was closed with 6-0 prolene suture (Ethicon, Somerville, NJ, USA). Mice subjected to the same surgical procedure with no treatment served as a control group. After 7 days, the mice were sacrificed, and the degree of peritoneal cavity adhesion was visually assessed.

Ex Vivo Coagulation Test for Hemostatic Capability of HA-serotonin Hydrogel: To evaluate the hemostatic capability of HA-serotonin hydrogel *ex vivo*, ICR, female, 4-week-old mice (Orient Bio) were anesthetized and the whole blood was collected in a syringe containing sodium citrate (Sigma-Aldrich). PBS, serotonin, HA, or HA-serotonin was added to the blood sample and incubated at 37°C for 1 hour. The blood clot remaining after PBS washing was weighed. PRP solution from whole blood sample was isolated and immediately reacted with PBS, serotonin, HA, or HA-serotonin. After a 1-hour incubation, the levels of platelet factor 4 and Factor V in the PRP solution were measured by ELISA (Mouse CXCL4/PF4 Quantikine ELISA kit, R&D Systems and Mouse Coagulation Factor V ELISA kit, Abbexa Ltd., Cambridge, UK).

Measurement of Blood Clotting Kinetics: To measure the time for blood clotting, 100 μl of mouse blood was placed in glass vials containing a stirring bar at a speed of 120 rpm, and then hemostatic agents (HA-serotonin hydrogel and fibrin glue) were treated to blood samples. We measured the time when the stirring bar speed was rapidly reduced by rheological changes due to blood clotting ($n = 4$). Fibrinogen and thrombin for fibrin glue were purchased from Sigma-Aldrich.

In Vivo Hemostatic Capability of HA-serotonin Hydrogel in Mouse Model of Liver Incision: The *in vivo* hemostatic capability of hydrogel was evaluated in a mouse model with liver hemorrhage as previously reported.[3, 4] Briefly, ICR, female, 4-week-old mice (Orient Bio) were anesthetized and an incision was made on the abdominal area. Sterilized filter papers were placed beneath the liver. Bleeding was induced using an 18 G needle (Korea Vaccine Co., Gyeonggi-do, Korea) and the damaged area was immediately covered with HA-serotonin hydrogel or fibrin glue (TISSEEL, Baxter, Vienna, Austria). The filter papers were exchanged every 30 seconds until the end of the observation. The collected filter papers were weighed to

measure blood mass. After completing this assessment of bleeding, the peritoneum and incised area were closed with 6-0 prolene suture. Untreated mice served as a control group. After 7 days of treatment, the mice were sacrificed and physiological status was examined. The *in vivo* hemostatic capability was also evaluated with the same procedure using the endogenous Factor VIII-deficient knock-out mice with hemophilia (B6;129S-F8^{tm1Kaz}/J, 6 weeks old; Jackson Laboratory, Bar Harbor, ME, USA).

Histological Analyses of Liver Tissue: After 3 days of treatment, the mice were sacrificed and liver tissues were harvested for histological analysis. The tissues were fixed with 10% (v/v) neutral buffered formalin (Sigma-Aldrich) and processed with a tissue processor (Leica Biosystems, Wetzlar, Germany). The prepared tissue-embedded paraffin blocks were sectioned at 5-μm thickness and stained with hematoxylin (Sigma-Aldrich) and eosin Y (Samchun Chemicals, Seoul, Korea) (H&E) or Toluidine Blue O (Sigma-Aldrich).

Statistical Analysis: All quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed to determine statistical significance by calculating p values < 0.05 or 0.01 using Students' t-test or ANOVA (GraphPad Software, CA, La Jolla, USA).

References

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2 C. D. Marshall, M. S. Hu, T. Leavitt, L. A. Barnes, A. T. Cheung, S. Malhotra, H. P. Lorenz and M. T. Longaker, *J. Vis. Exp.*, 2016.
3 K. Kim, H. Lee and S. Hong, *J. Vis. Exp.*, 2016.
4 K. Kim, M. Shin, M. Y. Koh, J. H. Ryu, M. S. Lee, S. Hong and H. Lee, *Adv. Funct. Mater.*, 2015, **25**, 2402-2410.

Supplementary Figures

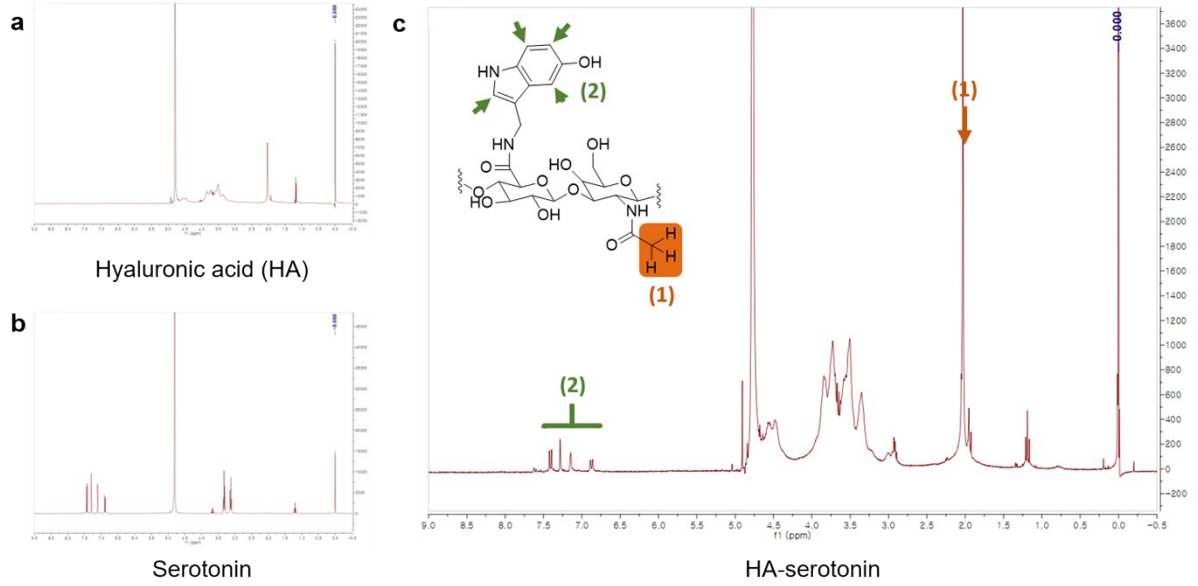

Figure S1. Characterization of the synthesis and chemical structure of HA-serotonin conjugate by proton nuclear magnetic resonance (^{1}H-NMR) analysis. NMR spectra of a) hyaluronic acid (HA), b) serotonin, and c) HA-serotonin conjugate. The peak at (1) indicates the protons of methyl groups in HA backbone and the peaks at (2) indicate the aromatic protons in serotonin. Deuterium oxide was used as a solvent for NMR analysis.

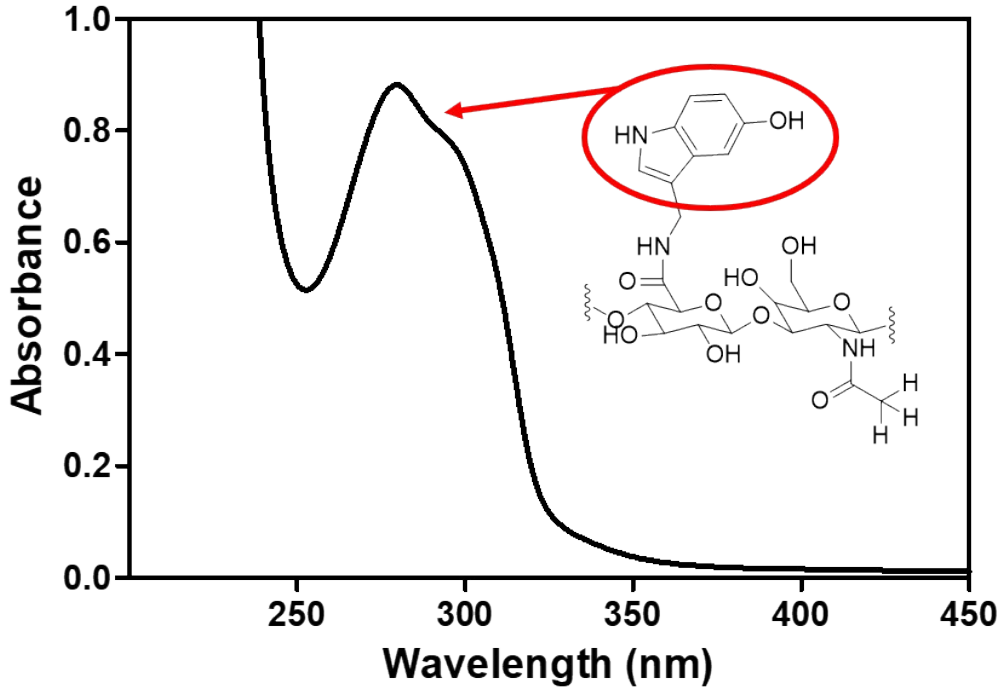

Figure S2. Characterization of HA-serotonin conjugate using UV-vis spectroscopy (with a quartz cuvette).

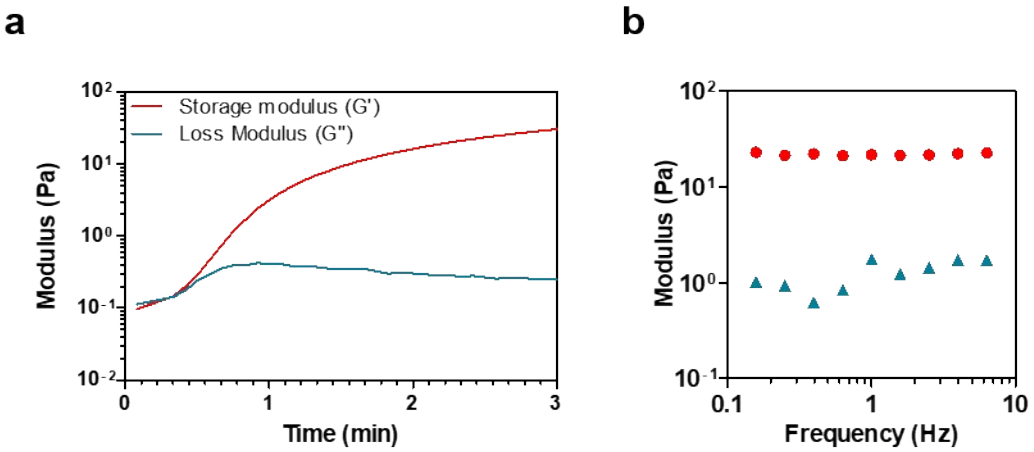

Figure S3. Rheological analysis of HA-serotonin hydrogel. a) Gelation kinetics of HA-serotonin hydrogel crosslinked using 12 U/ml HRP and 1 mM H_2O_2 was analyzed by a time-dependent rheological measurement. b) Storage modulus (G′) and loss modulus (G″) of HA-serotonin hydrogel crosslinked using 12 U/ml HRP and 1 mM H_2O_2 were measured at frequencies ranging from 0.1 to 10 Hz in frequency sweep mode using a rheometer.

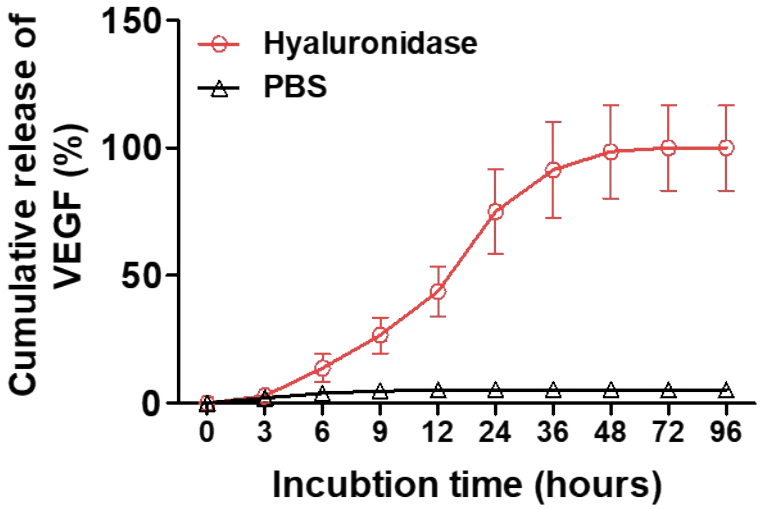

Figure S4. *In vitro* release profiles of VEGF from HA-serotonin hydrogels at each time point (3, 6, 9, 12, 24, 36, 48, 72, and 96 hours) during incubation in PBS and hyaluronidase solution (0.5 U/ml) at 37°C.

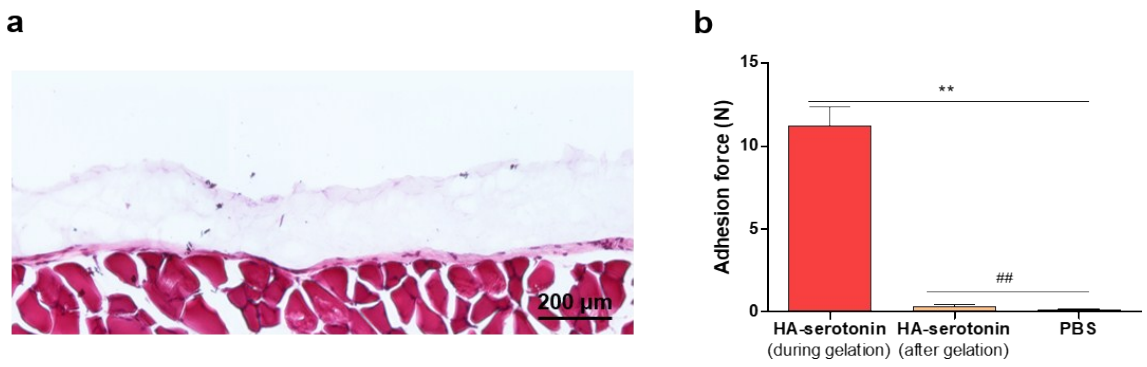

Figure S5. Tissue adhesiveness and enzymatic oxidation-dependent adhesiveness of HA-serotonin hydrogel. a) Histological analysis using H&E staining of HA-serotonin hydrogel adhered onto the surface of abdominal wall of a mouse. Scale bar = 200 μm. b) Adhesive force of HA-serotonin hydrogel during gelation, HA-serotonin hydrogel after complete gelation, and PBS as a negative control was measured by a tack-separation test using a rheometer ($n = 3$, $^{**}p < 0.01$ versus HA-serotonin after gelation group and PBS group, $^{\#\#}p < 0.01$ versus PBS group).

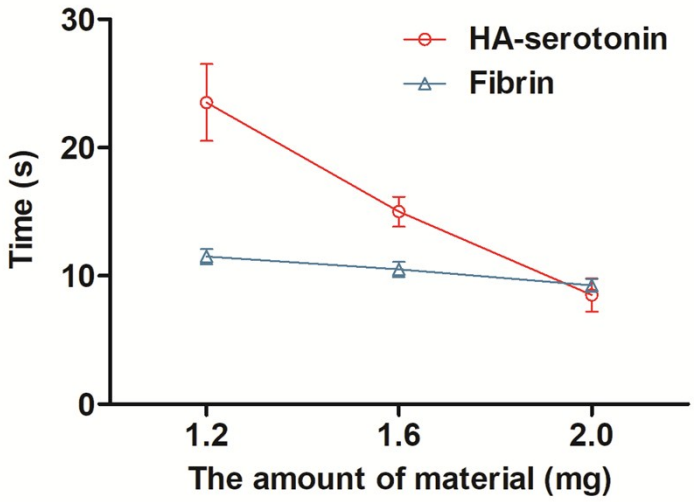

Figure S6. Comparison of the blood clotting kinetics of HA-serotonin and fibrin per the amount of material.

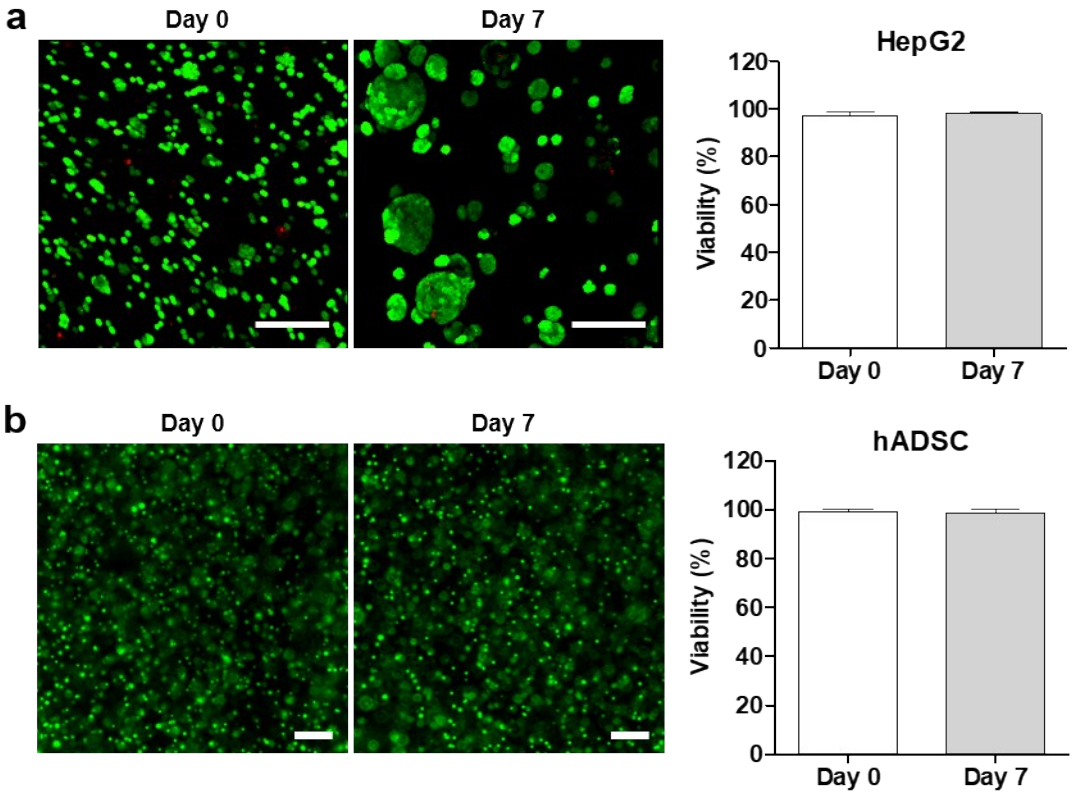

Figure S7. *In vitro* cytotoxicity test of HA-serotonin hydrogel. Live/Dead staining (left panels) and the viability (right panels) of a) HepG2 cells and b) human adipose-derived stem cells (hADSCs) encapsulated in HA-serotonin hydrogel immediately after gelation (day 0) and at day 7 after cell culture. Scale bars = 200 μm.

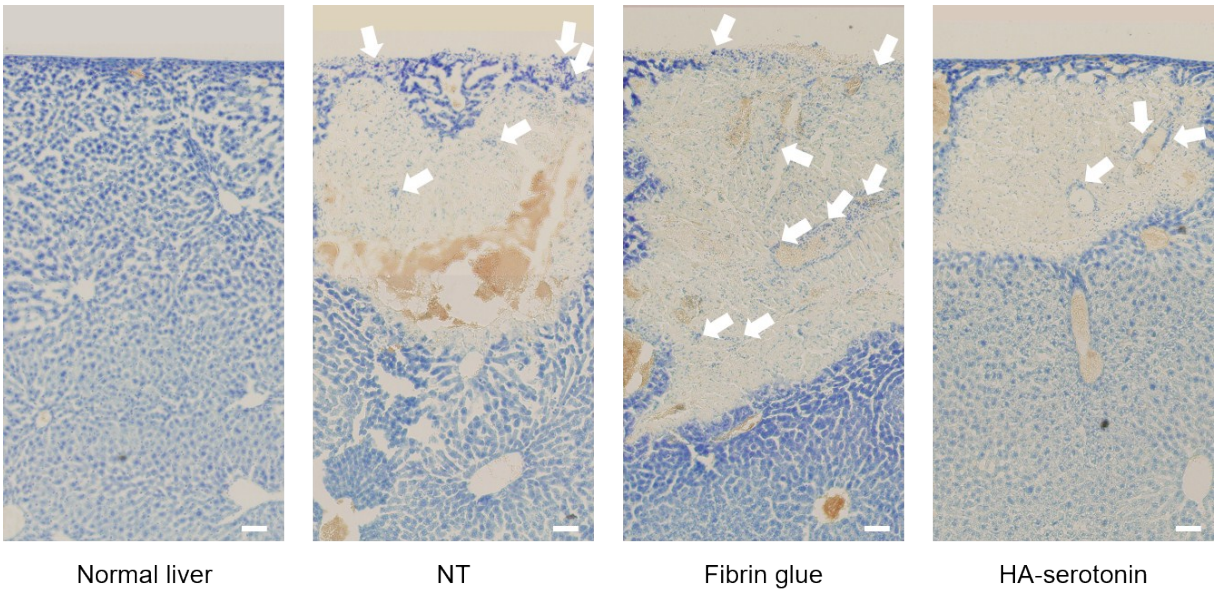

Figure S8. *In vivo* biocompatibility of HA-serotonin hydrogel. Toluidine blue staining of the tissues of normal liver and wound sites in damaged livers harvested from the mice used for the hemostasis test (NT: non-treated group, Fibrin glue: fibrin glue-treated group, and HA-serotonin: HA-serotonin-treated group) 3 days after operation. Scale bars = 200 μm.

Supplementary Movie

Movie S1. Hemostatic capability of the HA-serotonin hydrogel in a hemophilia mouse model. Hemostasis tests in a liver hemorrhage model of a Factor VIII-deficient hemophilia mouse without any treatment (No treatment group, left) and with the treatment of HA-serotonin hemostatic adhesive (HA-serotonin group, right).