Supporting Information

Injectable Cell-Laden Silk Acid Hydrogel

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1. Experimental Methods

1.1 Materials

Bombyx mori (*B. mori*) cocoons were obtained from a local store in Hangzhou, China. A regenerated silk fibroin solution was obtained following the degumming and dissolving procedure as a reported protocol.[1] The purified fresh solution was lyophilized for two days in a lyophilizer (FTFDS, Hangzhou, China) to obtain regenerated silk fibroin powder. Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and tris(hydroxymethyl)aminomethane (Tris), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Aladdin Co., Ltd. and used as received. Lithium chloride (LiCl), anhydrous dimethyl sulfoxide (DMSO), deuterated water (D_2O), deuterated dimethyl sulfoxide (DMSO-d_6), 8-anilino-1-naphthalenesulfonic acid (ANS), thioflavin T (ThT) and Nile red were purchased from J&K Scientific Ltd. and used as received. Calcein AM and propidium iodide (PI) were purchased from BEIJING LABLEAD Inc.

1.2 Preparation of silk acid

5.0 g of dry regenerated silk fibroin (RSF) powder was dissolved in 250 mL of 1.0 M anhydrous DMSO/LiCl solution. The solution was heated to 50 ° C before adding 40.0 g of succinic anhydride. The solution was stirred vigorously at 50 °C for 30 minutes. Following carboxylation, 600 mL of 1.0 M NaOH aqueous solution was added to neutralize the solution to a pH above 7 before dialyzing against DI water (dialysis tube, 3500 MWCO) for 72 hours (water was changed every 12 hours). The solution was centrifuged at 9000 rpm to obtain a purified SA solution. Finally, the purified SA solution was lyophilized for 2 days to obtain dry SA powder. The carboxylation degree of silk acid was determined by ^{1}H NMR result as in the previous report and calculated to be 9.5 %.

1.3 Preparation of silk acid hydrogel

Silk acid powder was dissolved in HEPES buffer solution (pH 7.4, 25 mM HEPES, 150 mM NaCl) with a concentration of 6 wt%. The obtained solution was incubated at 37 °C for 48 hours to allow the self-assembly of silk acid. For a comparison, regenerated silk fibroin solution was also diluted with HEPES buffer solution to obtain a final solution with a concentration of 6 wt% and incubated under the same conditions.

1.4 Detection of the absorbance of silk acid solution

Silk acid solution was added into a 96-well plate with each well of 150 μL and four duplicate wells for each condition. At predetermined time points (0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, and 60 hours), the absorbance of 550 nm of silk acid solution was measured on a Tecan Spark microplate reader (Männedorf, Switzerland). For investigating the influence of self-assembly conditions, silk acid was either incubated at different temperature (4, 25, 37, 60 °C), or dissolved with different concentrations (1, 2, 4, 6, 8 wt%), or

dissolved in different buffer solutions (PBS, HEPES, Tris-HCl, pH 7.4), or buffer solutions with different pH (PBS, pH 5, 6, 7, 8, 9), or buffer solutions with different NaCl concentrations (DI water, 15, 30, 75, 150, 300, 1000 mM).

1.5 Characterization of silk acid hydrogel

The Fourier transform infrared spectroscopy (FT-IR) was measured on a Bruker Invenio S FTIR spectrometer (Germany) equipped with an attenuated total reflectance (ATR) accessory (PIKE, USA). Silk acid was dissolved in HEPES buffer solution (6 wt%, pH 7.4, 25 mM HEPES, 150 mM NaCl) and allowed to self-assemble as described above. Samples were taken at different predetermined time points (0, 12, 24, 36, and 48 hours) for further freeze-drying before FT-IR measurements. For each measurement, the spectrum was recorded with 64 scans, a spectrum range of 400-4000 cm^{-1} and a resolution of 4.0 cm^{-1}. The peak devolution of the amide I region was performed using a home-developed Python package. The Gaussian peaks at 1620 cm^{-1} and 1698 cm^{-1} were assigned to the β-sheet structure, while the Gaussian peaks at 1645 cm^{-1} and 1685 cm^{-1} were assigned to random coil/helix and β-turn structures, respectively. The ratios of β-sheet structure were then estimated from the deconvolution.

The circular dichroism (CD) spectroscopy was collected on Applied Photophysics Ltd. Chirascan V100 spectrometer (United Kingdom). The self-assembled silk acid was diluted to 0.6 mg/mL and added into the quartz cell with an optical path of 0.2 mm before CD measurements. Each measurement was recorded with a spectrum range of 180-260 nm, a step size of 0.5 nm, and 3 scans for average.

1.6 Fluorescence detection of fluorescence probe in silk acid hydrogel

Silk acid solution (6 wt%, pH 7.4, 25 mM HEPES, 150 mM NaCl) was prepared as previously described. Three fluorescence probes, ANS, ThT, and Nile red, were added to the silk acid solution with a concentration of 0.01 mg/mL, respectively. 100 μL of solution was added into a 96-well black plate and incubated at 37 °C. At predetermined time points (0, 12, 24, 36, 48 hours), the fluorescence spectra of the probes were measured on a BioTek Synergy H1 microplate reader (United States), with the fluorescence spectra of silk acid solution as the background. The excitation wavelength of ANS was 360 nm, with a spectrum range of 400-500 nm and a step size of 2 nm. The excitation wavelength of ThT was 440 nm, with a spectrum range of 470-570 nm and a step size of 2 nm. The excitation wavelength of Nile red was 590 nm, with a spectrum range of 615-710 nm and a step size of 2 nm.

1.7 Confocal images of fluorescence probe in silk acid hydrogel

Silk acid solution (6 wt%, pH 7.4, 25 mM HEPES, 150 mM NaCl) was prepared as previously described. Three fluorescence probes, ANS, ThT, and Nile red, were added to the silk acid solution with a concentration of 0.02 mg/mL, respectively. 20 μL of solution was added onto the glass slide, covered by a coverslip, and incubated at 37 °C. At predetermined time points (0, 12, 24, 36, and 48 hours), the confocal images were collected on a Zeiss LSM 980 confocal microscope equipped with an Airyscan module (Germany).

1.8 Rheological study on silk acid hydrogel

Rheological studies were conducted on TA instruments ARES-G2 rheometer (Waters, United States). Silk acid hydrogel was prepared as previously described (6 wt%, pH 7.4, 25 mM HEPES, 150 mM NaCl) and was loaded on the plate of the rheometer, fitted with a parallel plate of 25 mm diameter and a gap distance of 0.5 mm at predetermined time points (0, 12, 24, 36, 48 hours). The loading plate was set at 37 ℃. Oscillation strain sweeps were performed from 0.01 to 100% at an angular frequency of 6.28 rad/s. Angular frequency sweeps were performed from 0.628 to 628 rad/s at 2.5% strain. Flow curve measurements were performed with a shear rate sweep from 0.01 to 10 s^{-1}. Time sweep measurements were performed with alternating high (100%) and low (2%) strain for five cycles in 300 seconds.

1.9 Cell culture

Mice fibroblast cell line L929 was purchased from the BeNa Culture Collection. Human umbilical vein endothelial cells (HUVEC) were provided by Hunan Fenghui Biotechnology Co., Ltd. Rat bone marrow-derived mesenchymal stem cells (rBMSCs) were isolated from the femurs and tibias of six-week-old female Sprague Dawley rats.[2] All animal experiments were conducted in compliance with guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Westlake University (AP#21-055-GCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in an incubator with a 5 % CO_2 atmosphere at 37 °C.

1.10 Cell encapsulation and cell viability analysis

Silk acid was dissolved in DMEM with a concentration of 6 wt% and incubated at 37 °C. After 30 hours, L929, HUVEC, and rBMSCs were encapsulated into silk acid hydrogel at a seeding density of 10^6 cells/mL. Silk acid hydrogel was incubated at 37 °C for another two hours for complete gelation before being loaded onto culture dishes with glass bottoms. 200 μL DMEM with 10% FBS was added to immerse silk acid hydrogel for further incubation.

For the injection of cell-laden hydrogel, silk acid hydrogel encapsulated with three cell lines was loaded into 1.0 mL syringes, respectively. After complete gelation, silk acid hydrogel was extruded through a 21-gauge needle into culture dishes with a glass bottom. 200 μL DMEM with 10% FBS was added to immerse silk acid hydrogel for further incubation.

A live/dead assay was utilized to assess cell viability. After incubation for 24 or 72 hours, cells within silk acid hydrogel were stained with 4 μM calcein AM and 4 μM PI for one hour before being washed with PBS three times. The fluorescent images of stained cells were obtained using Leica THUNDER DMI8 fluorescent microscope (Germany), and the cell viability was quantified with ImageJ according to the following formula: cell viability% = (alive cells) / (total cells) × 100%.

The relative metabolic activity of the cells in SA hydrogel was determined by AlamarBlue assay (Invitrogen™) according to the manufacturer's directions. HUVEC were encapsulated into the SA hydrogel at a seeding density of 10^6 cells/mL. After incubation for 1, 3, 5, and 7 days, hydrogels were rinsed with

PBS and incubated in DMEM medium with 10% AlamarBlue reagent for 4 h at 37 °C with 5 % CO_2. Following incubation with the reagent, aliquots (100 μL) were placed into black 96 well plates, and the fluorescence was quantified using a plate reader with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Acellular hydrogels were used to adjust for background fluorescence.

For the in situ imaging of cell growth, the cell-laden hydrogel was placed into the KEYENCE microscopic system (BZ-X800) with total environmental control, including gas, temperature, and humidity, to record live cell time-lapse sequences. HUVEC was used as a model cell and encapsulated in the SA hydrogel with a seeding density of 10^5 cells/mL. Images were captured every 20 minutes. Images presented within the manuscript were cropped and adjusted for contrast and brightness to illustrate cell growth better.

1.11 Enzymatic degradation test of silk acid hydrogel

To conduct the *in vitro* enzymatic degradation test, SA hydrogels (200 μL) were incubated in 1 mL of 3.5 U/mL protease XIV, 120.0 U/mL collagenase I or 40 U/mL α-chymotrypsin enzyme solutions at 37 °C under steady shaking. PBS buffer was used as the negative control. After one day of incubation, solid residuals were collected by centrifugation at 5000 rpm for 5 minutes and washed with DI water twice. After drying at 60 °C to constant weight, the residuals were weighed. SA hydrogel without immersing enzyme solutions was also dried weight and taken as the blank. The percentages of weight remaining were calculated by comparing the weight of the residuals and blank.

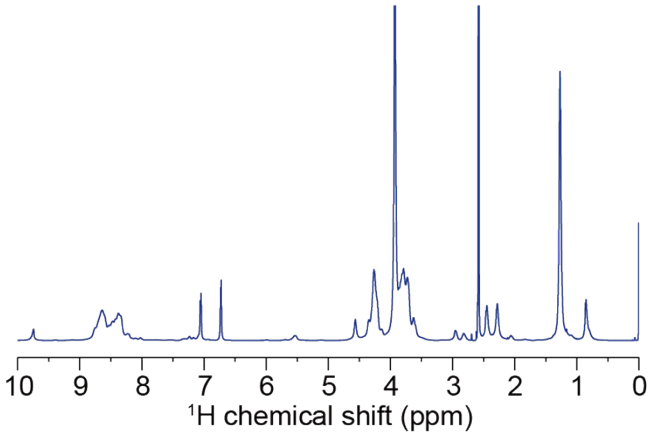

Fig. S1 ^{1}H NMR spectrum of SA in DMSO-d_6/LiCl.

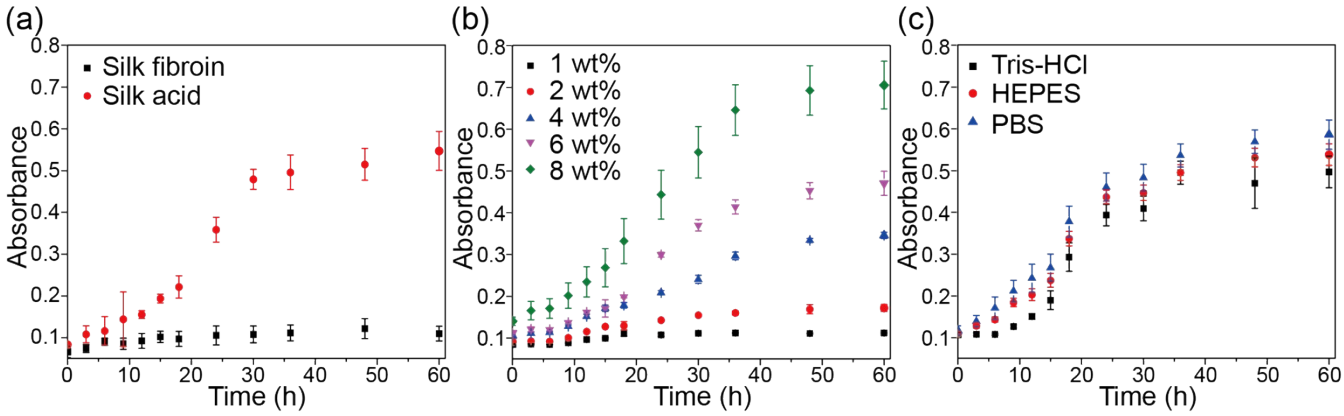

Fig. S2 (a) the absorbance changes of silk acid and silk fibroin solutions with time. (b) The absorbance changes of silk acid solutions with different concentrations. (c) The absorbance changes of silk acid solutions with varying buffer solutions. Data were presented as mean ± s.d., n=4.

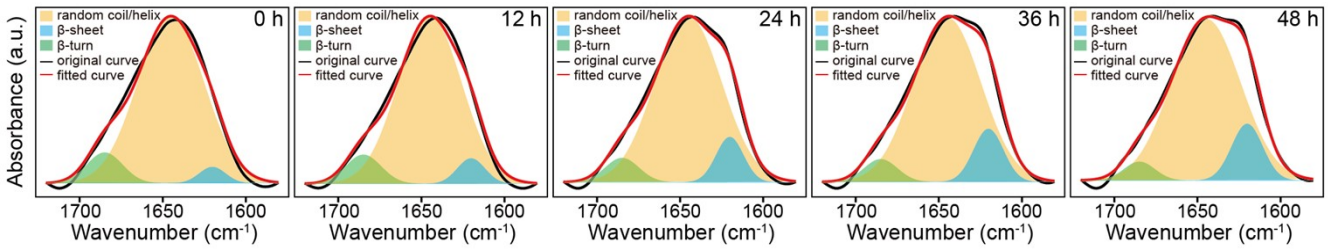

Fig. S3 The deconvolution of the amide I region for FT-IR spectrum of silk acid with different gelation times.

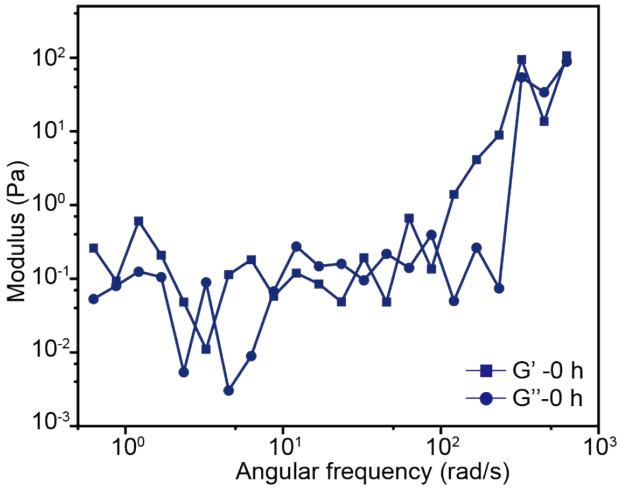

Fig. S4 Angular frequency sweeps on the silk acid solution.

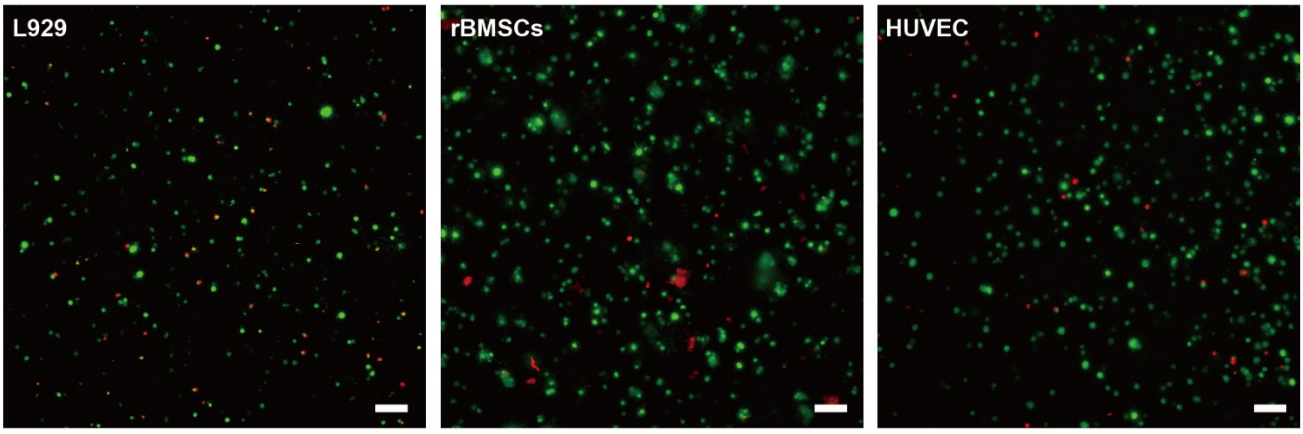

Fig. S5 The L929, rBMSCs, and HUVEC were seeded and cultured inside the SA hydrogel for 24 hours. Cells were stained with calcein AM and PI for live/dead staining. The scale bar is 100 μm.

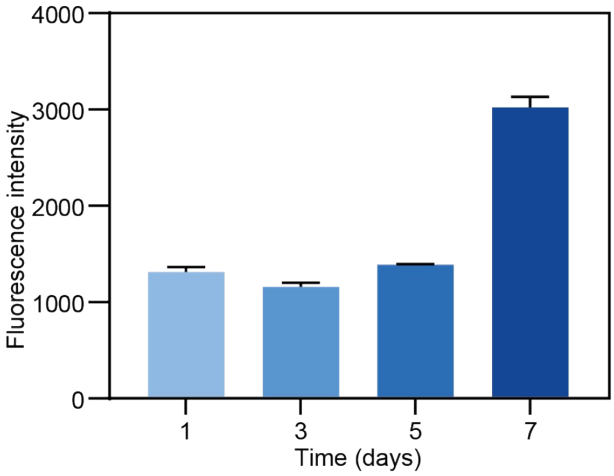

Fig. S6 The Alamarblue assay results of the HUVEC cultured in the SA hydrogel on day 1, day 3, day 5, and day 7.

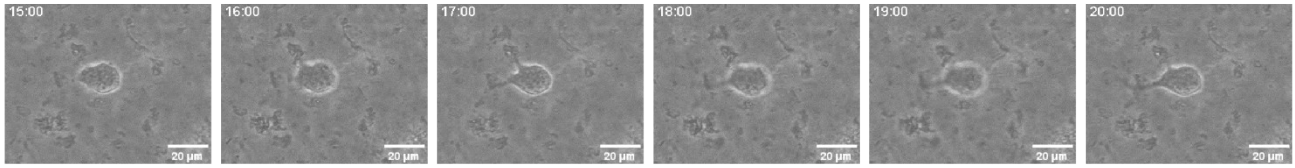

Fig. S7 *In situ* live cell imaging. The HUVEC were encapsulated in the SA hydrogel and placed into the microscopic system with total environmental control. The cell growth and volume expansion were shown in the incubation time from 15 to 20 hours. The scale bar is 20 µm.

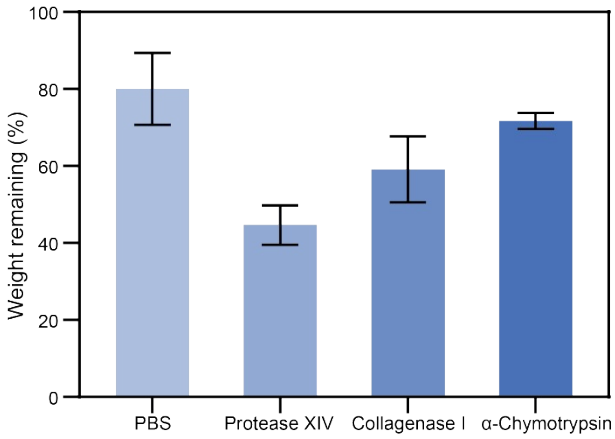

Fig. S8 Weight remaining of SA hydrogels after one day of *in vitro* degradation. SA hydrogels were immersed in 3.5 U/mL protease XIV, 120 U/mL collagenase I or 40 U/mL α-chymotrypsin at 37 °C under gentle shaking. PBS was used as a control.

References:

1. D. N. Rockwood, R. C. Preda, T. Yucel, X. Wang, M. L. Lovett and D. L. Kaplan, *Nat Protoc*, 2011, **6**, 1612-1631.
2. H. Zhu, Z.-K. Guo, X.-X. Jiang, H. Li, X.-Y. Wang, H.-Y. Yao, Y. Zhang and N. Mao, *Nature Protocols*, 2010, **5**, 550-560.