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Supporting Information

Mechanically active small intestinal submucosa hydrogel for accelerating chronic wound healing

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Experimental Sections

Materials

N-Isopropyl acrylamide (NIPAM) with the purity of 98.0% was purchased from Shanghai yuanye Bio-Technology Co., Ltd. Gelatin (type A, from porcine skin) was purchased from Sigma-Aldrich (USA). N,N'-methylene bisacrylamide (MBAA) of the ultra-pure grade was supplied from AMRESCO Inc. Ammonium persulfate (APS) with the purity of 98% was provided by Shanghai Titan Scientific in Shanghai. All other reagents and solvents were of analytical grade and supplied by Beijing Chemical Reagent Co., Ltd. (China), unless otherwise indicated.

Synthesis and characterizations of gels

The porcine jejunum was harvested from market pigs within 4 h of sacrifice. After fat was removed through mechanical removal of the tunica, serosa and tunica muscularis, small intestinal submucosa (SIS) was extracted from the porcine jejunum and then carefully washed with a saline solution. After washing, the SIS was freeze-dried at -80°C for 48 h using a freeze dryer. The dried SIS was pulverized using a freezer mill at -198°C to yield SIS powder with particles 10-20 μm in size. The obtained SIS powder was stirred for 48 h in an aqueous solution consisting of 3% acetic acid and 0.1% pepsin (25°C), and the final SIS digestion solution was freeze-dried again at -80°C to obtain a soluble SIS matrix. The obtained SIS matrix was sterilized using ethylene oxide gas.

To form a gel, the soluble SIS matrix was dissolved in phosphate-buffered saline (PBS) at concentrations of 10 mg/ml and neutralized to a pH of 7.4 with the addition of 2.5 M NaOH. Then, 0.1 g NIPAM were added and dispersed into SIS solution under constant stirring. Next, 10 mg MBAA were added and the mixture was stirred for 20 min to reach complete dissolution. Finally, 0.02 g of ammonium persulphate (APS) which was pre-dissolved in DI water was added dropwise into the solution under continuous stirring, and the radical polymerization was conducted at 4 °C. After 24 h, the obtained SIS-PNIPAm hydrogel was rinsed five times using phosphate buffer saline (PBS). G-PNIPAm or SIS-PAAm hydrogels were prepared in a similar

way, except 10 mg/ml gelatin solution instead of SIS solution, or acrylic amide (AAm) instead of NIPAm were used. The adhesive surface of hydrogel was achieved by adding carbodiimide reagents (e.g., EDC and NHS) according to a previously reported protocol.

The morphological of hydrogels at 25 ℃ and 37 ℃ were observed using a scanning electron microscope (SEM, JEOL JSM-7500F, Japan). The thermal response behaviour of hydrogel was evaluated by placing the hydrogel at 37 ℃ and measuring the volume change over time. The initial size and final size were denoted as L_0 and L, and the area strain was calculated by $1-(L_0/L)^2$. For degradation testing, various SIS-PAAm hydrogels were first weighed (W_0) and then continuously immersed in 0.5 U/mL collagenase solution at 37±1 °C for 12 h under gently shaking (60 rpm). At each time point, Zn FGHs were washed, lyophilized, and finally weighed (W_t). The weight loss was quantitatively determined as $(1 - W_t/W_0)\times100$ %. The rheological behavior of the hydrogel was measured using a rheometer (HR-1, TA Instrument, USA) with a 20 mm flat at room temperature.

Mechanical testing

The tensile tests were carried out on a STS10N tensometer (Xiamen East Instrument Co. Ltd.) equipped with a 10 N load cell, at the crosshead speed of 100 mm min^{-1}. The tested cylindrical samples were 15 mm long, and with a gauge length of 5 mm. For the cyclic tensile tests, a maximum strain of 5 was chosen. The adhesive tests were were carried out on a STS10N tensometer (Xiamen East Instrument Co. Ltd.). Two pieces of porcine skins were stick together by the hydrogel with the bonding area of 10×10 mm^2, by a pressure for 10 sec at ambient temperature and then the external pressure was removed immediately. The adhered substrates were submitted to a shear stress when applied to the tensometer, at a crosshead speed of 5 mm min^{-1} (lap-shear tests). The maximum stress during shear adhesive tests were recorded as the adhesive strength, calculated by the maximum force divided by the initial bonding area. For each mechanical test, at least 5 samples were tested for achieving the statistical data.

Cytocompatibility testing

Cytocompatibility in vitro was conducted by culturing L929 fibroblast cells with complete RPMI medium 1640 (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution (Gibco) in a 5% CO_2 incubator at 37 ℃, The tested hydrogels immersed into complete medium to make extracts and sterilized the extracts by 0.2 μm filter. The cells were treated with trypsin-EDTA (Gibco) and resuspended cells with the extracts. The cells with the density of 1×10^3 were seeded into each well (96-well plate) and allowed to grow for 1, 3, 5, and 7 days. The cytocompatibility of the hydrogels were analysed by cell counting kit-8 (CCK-8) assay (Bimake) and Live/Dead assay. CCK-8 assay was conducted as follows: After a specific period of incubation in 96-well plate, CCK-8 solution was diluted by 10 times with the extracts.[1] After the removal of the original medium, 100 μL CCK-8 reagent were added into each well, which were co-cultured with the cells in a 5% CO_2 incubator at 37 ºC for 2 hr before measurement of the absorbance with a microplate reader (BIO RAD) at a wavelength of 450 nm. After CCK-8 assay, Live-Dead assay was carried on. 2 μM calcein AM (in DPBS) and 4 μM EthD-1 (Invitrogen) working solution were added into wells. The 96-well plate was then incubated in a 5% CO_2 incubator at 37 ℃ for 20 min. A laser scanning confocal microscope (Nikon, Japan) was used to observe the morphologies of the cells. For each group, 4 parallel experiments were conducted for obtaining the convincing results.

Evaluation of *in vitro* angiogenesis

To evaluate cell migration, wound healing assay was conducted. For wound healing assay, HUVECs were seeded in 24-well plates at a density of 5000 cells/well. Twenty-four hours after cells being seeded, a series of 1.5 mm-linear wound were scratched by blue tip and washed with PBS for three times. Various extracts were added to continue the incubation for another 24 h. Wound closer was observed by an optical microscope and analyzed using ImageJ software. HUVECs (0.6×10^4 cells/300 μL) were suspended in the extracts of various hydrogels, and then seeded in 24 well plate precoated with Matrigel (300 μL/well). Images were taken after 12 h culture and analyzed by Image J. The expression of angiogenesis chemokines were analyzed by RT-PCR. Briefly, HUVECs were cultured in various extracts for 3 days in 12-well plates (5

× 10^4 cells/well), Angiopoietin-1 (Ang-1), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGF-R2) genes expression were determined by RT-PCR.

Evaluation of *in vitro* macrophage polarization

The RAW264.7 cells were seeded on 24-well plates with a density of 1×10^4 cells/well and stimulated with 0.1 μg/mL lipopolysaccharide (LPS, Sigma) for 12 h. Then, the medium was replaced by various extracts, and the normal medium was used as the control group. After they were cultured for 12h, immunofluorescence staining was performed to evaluate the polarization of macrophage phenotypes. Briefly, the cells were fixed by 4% paraformaldehyde for 30 min and blocked with 10 wt% BSA solution. Next, the cells were incubated with antibody of CD206 (141709, Biolegend, USA), or iNOS (696803, Biolegend, USA) at 37 ℃ for 2 h. Finally, the images of cells were captured with a confocal laser scanning microscope (CLSM, TCS SP8, Leica) after counterstaining the nuclei with Hoechst 33528. The images were quantitatively analyzed by the ImageJ software. The quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to quantify the expression of IL-6, TNF-α, IL-10 and CD206.

Wound healing

All the animal experiments were complied with the guidelines of the Tianjin Medical Experimental Animal Care, and animal protocols were approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Co., Ltd (protocol number YSY-DWLL-2022078). To investigate the regeneration of chronic wound healing with SIS-PNIPAm *in vivo*, 16 diabetic SD rats were used to establish a full-thickness wound model. Diabetic SD rats were induced by intraperitoneal injection of STZ (50 mg/kg) once a day for 5 consecutive days. After anesthetizing, shaving, and sterilization, two full-thickness circular wounds (d = 1 cm) were made on either side of the spine, respectively. The wounds were randomly divided into four groups, i.e., Blank (PBS-treated) group, G-PNIPAm group, SIS-PAAm group, and SIS-PNIPAm group. The images of wounds were captured by a digital camera and analyzed with ImageJ at day 0, 3, 7, and 11.

The animals were sacrificed on day 3 and 11, and the skin samples were fixed with 4% paraformaldehyde for at least 24 h. Sections in 5 μm thickness were prepared for histological analysis. On day 3, eight rats (4 wounds for each group) were sacrificed, the regenerated skin tissues were homogenized in PBS, and the quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to quantify the expression of VEGF, TGF-β, IL-1β, IL-6, IL-4, and IL-10. The skin wound sections were also stained with the corresponding antibodies (including F4/80 and CD206) according to the standard protocol for immunofluorescence staining, while the nuclei were stained with DAPI. On day 11, eight rats (4 wounds for each group) were sacrificed for histological analysis. Hematoxylin-eosin (H&E) staining were performed on the consecutive tissue sections.

Statistical analysis

All quantitative data were shown in the form of mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Turkey's test was used for statistical analysis. Differences between groups of $p < 0.05$ was regarded as statistically significant. $p < 0.01$ and $p < 0.001$ were realised as highly significant.

Supplementary Figures

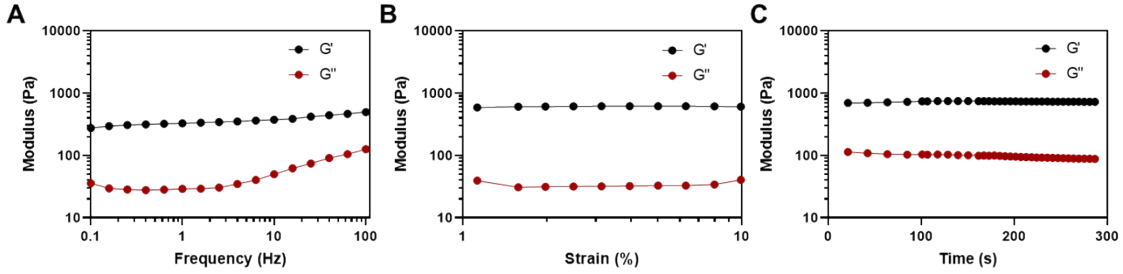

Fig. S1. Rheological behavior of SIS-PNIPAm hydrogel. G': storage modulus; G'': loss modulus. (A) Frequency spectra. (B) Strain spectra. (C) Time spectra.

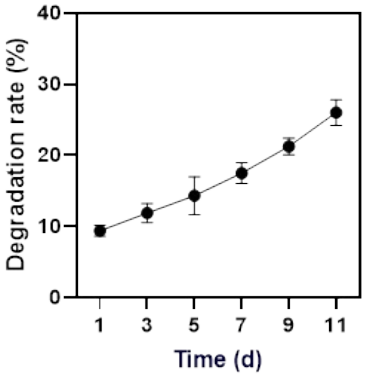

Fig. S2. Degradation profile of SIS-PNIPAm in 0.5 U/mL collagenase solution detected at predetermined time points.

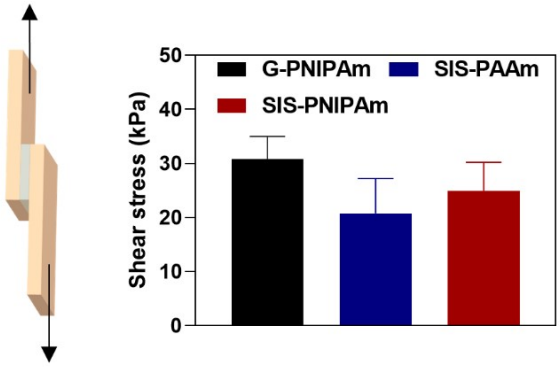

Fig. S3. Lap shear adhesive strength of various hydrogels.

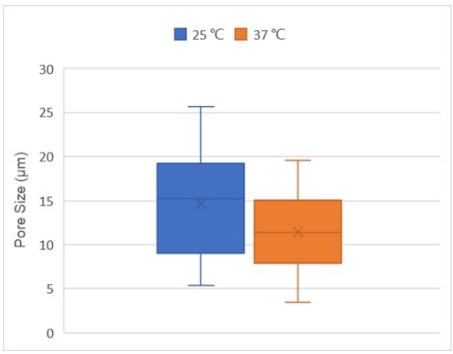

Fig. S4. Pore size of SIS-PNIPAm hydrogel at 25 ℃ and 37 ℃.

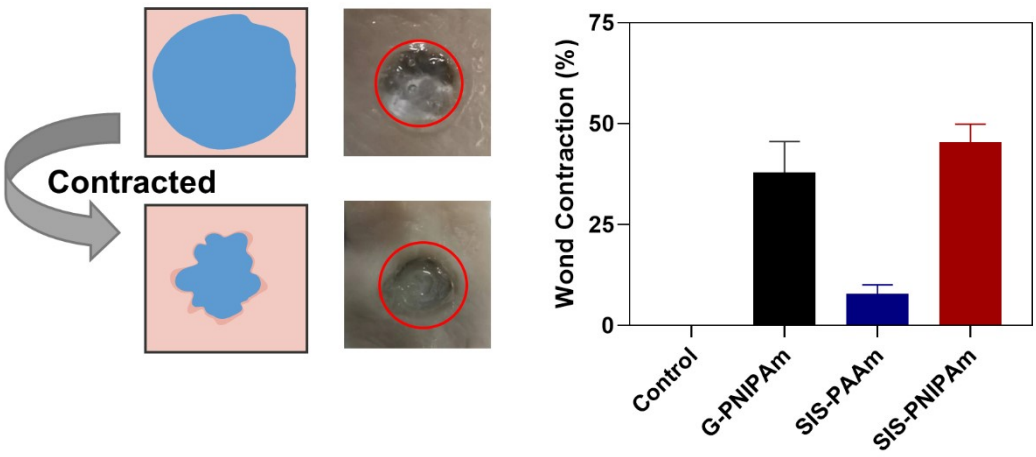

Fig. S5. *Ex vivo* wound contraction of fresh porcine skin using various hydrogel matrices. The representative figures show a wound treated with SIS-PNIPAm hydrogel.

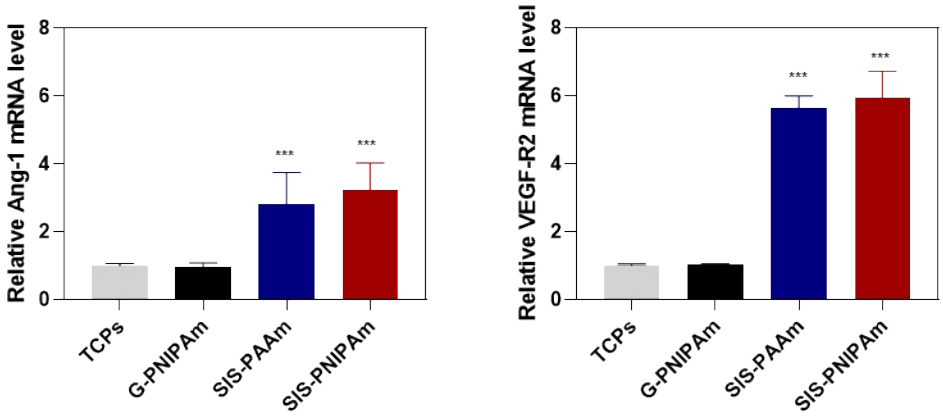

Fig. S6. RT-qPCR analysis for Ang-1 and VEGF-R2 genes expression in HUVECs.

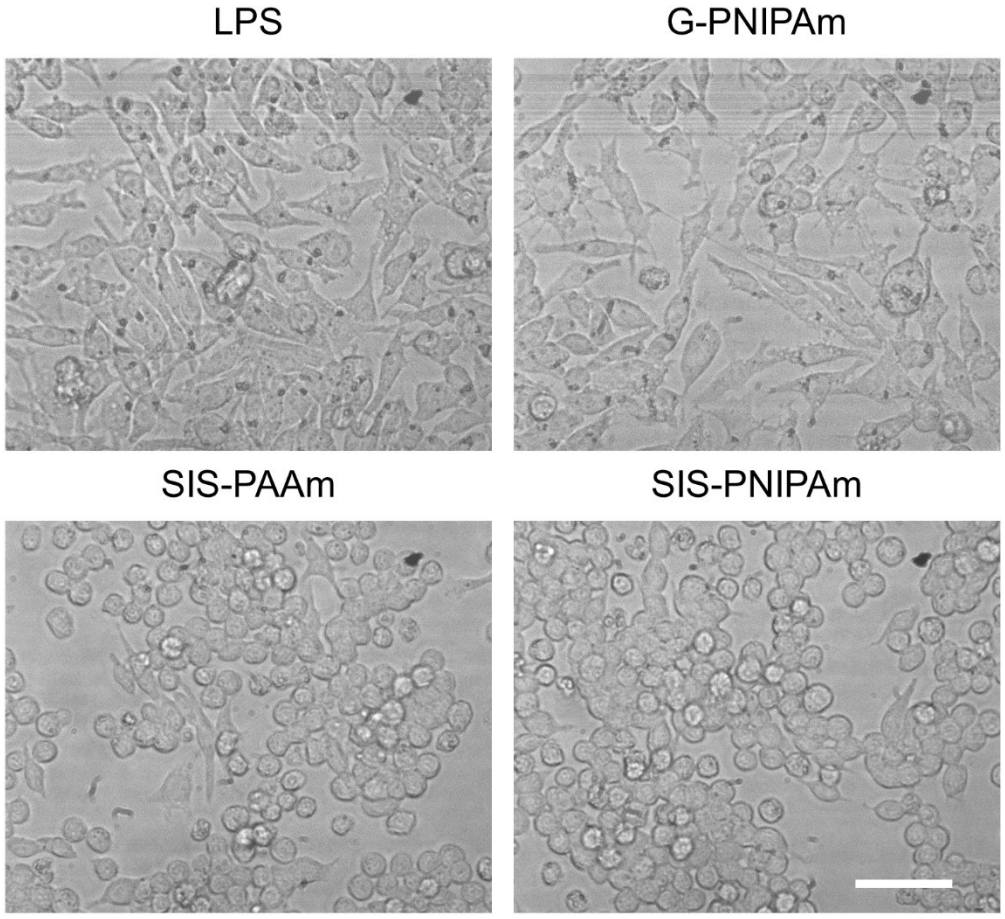

Fig. S7. Morphological changes of RAW246.7 cells.