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Supporting Information

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Content:

Supplementary movie 1: A movie showing the performance of injectable HP-PEG/HA-SH hydrogel into wound site and the gelation occurs in 90 seconds.

Materials and Methods

Materials

Disulfiram, 2,2'-Azobis(2-methylpropionitrile) (AIBN), poly(ethylene glycol) diacrylate (PEGDA, Mn = 575 Da), and Irgacure 2959 were purchased from Sigma-Aldrich. The methyl ethyl ketone (butanone) was purchased from Fisher Scientific. Thiolated hyaluronic acid (HA-SH, HyStem™, Glycosan) was purchased from BioTime Inc., Alameda, CA. Streptozotocin (STZ) were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen. AlamarBlue was purchased from Sigma-Aldrich. All antibodies were purchased from Abcam Inc.

In situ RAFT polymerization of PEGDA

The reaction procedure is introduced briefly: disulfiram, AIBN, and PEGDA were added into a 100 mL two necks flask, following the designed feed ratio as showed in Table S1. All the chemicals were dissolved in the 40.81 mL butanone. The reaction will begin after the 30 min bubbling by Argon for deoxygenation. The *in situ* RAFT polymerization was stirred at 500 rpm and kept at different temperatures (Table S1) in oil bath. The samples were collected at scheduled time points, and were monitored by gel permeation chromatography (GPC). The final products were purified by precipitation and dialysis, respectively. After the freeze drying, the pure polymer products were obtained as faint yellow liquid.

GPC characterization

Number average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI) were measured by GPC (Agilent Technologies, PL-GPC 50) equipped with a refractive index detector (RI), a viscometer detector (VS) and a dual angle light scattering detector (LS 15° and LS 90°). The columns (30 cm PLgel Mixed-C, two in series) were eluted by dimethylformamide (DMF) with 0.1% LiBr at a flow rate of 1 mL min^{-1} at 60 °C and calibrated with poly(methyl methacrylate) standards (PMMA). All samples were dissolved in DMF at a concentration of 5 mg mL^{-1} and passed through a 0.2 µm filter before analysis.

Proton and carbon nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) analysis

^{1}H-NMR and ^{13}C-NMR were performed on a 400 MHz Varian NMR system spectrometer and was analyzed using MestReNova processing software. The chemical shifts were referenced to the solvent peak of chloroform-d (7.26 ppm for ^{1}H-NMR and 77.16 for ^{13}C-NMR). The structure of the HP-PEG polymer is easily recognizable with the simple integration of the peaks e, b, a and c all correlating to the structure outlined; the vinyl peaks are located between chemical shifts of 6.5 to 7.5 ppm.

The polymer composition was calculated from **Equation. S(1)-(3)**:

$$n = \frac{(f)+(g)}{2} \quad (1)$$

$$2n + 2p = \frac{(b)}{2} \quad (2)$$

$$18n + 18p = \frac{(c)}{2} \quad (3)$$

The acrylate content and the branching degree of the HP-PEG were calculated from the following **Equation S(4) and (5)**:

$$\text{Acrylate Content (mol\%)} = \frac{n}{n+p} \times 100 \quad (4)$$

$$\text{Branching Content (mol\%)} = \frac{p}{n+p} \times 100 \quad (5)$$

Rheological assessments

The hydrogels were prepared with HP-PEG (in water) and HA-SH (1% w/v), which were characterized by TA Discovery Hybrid Rheometer. 200 μL mixed solution was added to spread on a parallel plate (diameter: 20 mm) with a frequency of 1 Hz and a strain of 0.5%, and the storage modulus G' and loss modulus G" were monitored with time. Frequency sweep tests were conducted with a frequency range of 0.1 to 100 rad s^{-1} at a constant strain of 0.5%.

Injectable HP-PEG/HA-SH hydrogels swelling property and degradation profile

Swelling ratio was used to introduce hydrogel water retention capacity:

$$\text{Swelling ratio} = W_t / W_0 \times 100\%$$

Weight of sample at scheduled time was record as W_t, and the initial weight of sample as W_0. 5% w/v of different HP-PEG/HA-SH hydrogels were incubated in Transwell® permeable inserts (BD. Bio Coat, PET track-etched membrane, 3.0 μm). PBS was added into Transwell® permeable inserts and the whole system was incubated in a shaker at 37 ºC at 150 revolutions per minute. Weights of swollen gels were obtained at scheduled time points until gels had totally disappeared. Experiments were performed five times for each type of hydrogel at the same condition.

Scanning Electron Microscopy (SEM) of the hydrogels

HP-PEG_1/HA-SH hydrogel samples were prepared for SEM test (AMETEK® Quanta 3D FEG). Briefly, hydrogels with final polymer concentration of 5% w/v were prepared using the same method. Liquid nitrogen was used to freeze the hydrogels. The samples were freeze-dried overnight and coated with gold before SEM test.

Protein adsorption assay

To evaluate the anti-fouling property of the HP-PEG/HA-SH hydrogels, the amount of FBS adsorbed on the surface were determined by a BCA kit. The protein adsorption of 10 wt% PEGDA, HP-PEG, HP-PEG/HA-SH, and Chitosan/Collagen hydrogel was assessed by an *in vitro* single adsorption experiment in PBS. PEGDA and HP-PEG hydrogels were formed by exposure to UV light in the presence of a photoinitiator (0.5 wt%, Irgacure 2959). Chitosan/Collagen hydrogel was formed by chitosan and collagen.

Samples of 25 mg were incubated in PBS for 12 h to reach swelling balance, and then incubated in 10% FBS. The samples were then rinsed three times with PBS for 10 min each at scheduled time points. After drying at room temperature, the samples were treated with 1% sodium dodecyl sulfate (SDS) (0.5 mL) to remove the surface adsorbed protein for 1 h. The

amounts of adsorbed protein were quantified using a Micro BCA protein assay kit. Absorbance at 562 nm was recorded on a plate reader (SpectraMax® M3).

In vitro cell culture and cytotoxicity analysis

Rat adipose-derived stem cells (ADSCs) were extracted from rat adipose tissue following the method reported before.[1] ADSCs were cultured in DMEM (Sigma) with 10% FBS and 1% penicillin as supplements. For *in vitro* cytotoxicity study, cells (10×10^3 per well) were first cultured for 24 h in 96-well flat-bottomed microtitre plates to permit cell attachment, then exposed to different concentrations of HP-PEG polymers (from 50 μg to 1,000 μg) for 24 h. Cell viability was evaluated by AlamarBlue assay, which was considered an index to the metabolic activity of viable cells. The cytotoxicity of mouse embryonic fibroblast cells (3T3) cell line was carried out as the same procedure.

To evaluate cytotoxicity of the HP-PEG/HA-SH hydrogels, single cell suspensions of ADSCs or 3T3 were mixed with HA-SH (1% w/v) and HP-PEG solutions (10% w/v) (both were dissolved in DMEM supplemented with 10% FBS and 1% P/S). Final cell density was 1.0×10^6 cell mL^{-1}. 35 μL of the mixture was pipetted onto a Teflon surface and left at room temperature for 15 min for complete crosslinking. Then, the hydrogels were transferred into a 24-well plate and incubated at 37 °C in 5% CO2 with medium change every 3 days (n = 4 per group). An AlamarBlue assay was used to assess cell viability of both cell lines for 24, 48, 72 and 96 h.

Differentiation test for ADSCs encapsulated in the HP-PEG$_1$/HA-SH hydrogel

Cell suspension of ADSCs were mixed with HA-SH (1% w/v) and HP-PEG$_1$ solutions (10% w/v) as described above. 35 μL of the mixture was pipetted onto a Teflon surface and covered with another Teflon tape. The thickness of the hydrogels was altered by the thickness between two Teflon tapes, which is approximately 1.6 mm. Then hydrogels were left at room temperature for 15 min for complete crosslinking. The final concentration of HA-SH is 0.5% w/v; HP-PEG$_1$ is 5% w/v. Final cell density was 1.0×10^6 cell mL^{-1}. The cell-seeded hydrogels were transferred into a 24-well plate. The same of cells as in the hydrogel was seeded in the 24-well plate (1 mL fresh cell media was added into each well; *n=4*). The whole system was then incubated at 37 °C in 5% CO_2.

Immunofluorescence for Oct4, Sox2, and Nanog for ADSCs was carried out to detect the undifferentiated status. HP-PEG/HA-SH/ADSCs was first fixed with 4% paraformaldehyde, and then reacted with antibodies against Oct4, Sox2, and Nanog. After washed with PBS/Triton X-100, the hydrogel reacted with corresponding secondary antibodies, and counterstained with DAPI. Immunofluorescence was observed with a fluorescence microscope.

For *in vitro* differentiation assessment, ADSCs were induced by relative induction media and media without growth factors, separately.

Adipogenic induction medium: Preadipocyte basal medium-2 supplemented with 16.6% FBS, 50 μg mL^{-1} ascorbate-2 phosphate, 10^{-7} M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-1-methyl-xanthine and 10 μg mL^{-1} insulin.

Chondrogenic induction medium: add ITS + (Gibco-BRL) and 10 ng mL^{-1} TGF-1 (Preprotech, Rocky Hill, NJ) into MSC differentiation medium - chondrogenic (PT3925).

Osteogenic induction medium: MSC differentiation medium-osteogenic (PT3924) supplemented with 16.6% FBS, 50 μg mL^{-1} ascorbate-2 phosphate), 10^{-8} M dexamethasone and 10 mM -glycerophosphate.

After separate induction, ADSCs were stained by oil red O, Alizarin red, and safranin-O to detect the morphology.

Growth factors secretion of ADSCs encapsulated in the HP-PEG$_1$/HA-SH hydrogel

Conditioned media was collected from ADSCs seeded in the hydrogels or plated in wells after 1, 4, and 7 days incubation. Angiogenic protein levels of PIGF, VEGF, and TGF-β were quantified using multi-plex ELISA system (Sigma).

Animals

6-8-week old male Sprague Dawley (SD) rats with body weights range between 180-240 g were used for adipose-derived mesenchymal stem cells (ADSCs) harvest, subcutaneous hydrogel implantation, and inducing diabetic rat model. Rats were fed *ad libitum* water and rodent diet, and housed in the Animal Experimentation of the Chinese Academy of Medical Sciences and Peking Union Medical College Institute of Biomedical Engineering-approved animal care guidelines. All procedures were approved by the Chinese Academy of Medical Sciences and Peking Union Medical College Institute of Biomedical Engineering.

***In vivo* biosafety and degradation profile of HP-PEGs/HA-SH hydrogels in a subcutaneous implantation model**

A subcutaneous implantation was used to test the *in vivo* biosafety and biodegradation of the injectable HP-PEG$_1$/HA-SH (5% w/v) and HP-PEG$_2$/HA-SH hydrogel (5% w/v). After anesthesia with an intraperitoneal injection of 8% chloral hydrate, four incisions with 1 cm full-thickness transverse each were made on both sides of the shaved dorsum of SD rats. Prepared hydrogels (100 μL) were implanted subcutaneously and incisions were closed with 5-0 nylon suture (Ethicon, Somerville, NJ) and covered with a sterile occlusive dressing (Tegaderm; 3M, St. Paul, MN). Wounds were examined every other day and hydrogels were harvested at 3, 7, 11, 14, and 21 days post-wounding. Residual hydrogel was taken and weighted (4 samples for each condition).

Diabetic animal model

In order to generate diabetic animals, STZ was used to chemically induce healthy SD rats to develop type 1 diabetes *via* cauda vein injection (45 mg kg^{-1} of STZ dissolved in a 0.1 M sodium citrate buffer). Diabetes was verified by serum glucose levels after the injection. Serum glucose level > 16.7 mmol L^{-1} for at least 4 weeks was defined as successfully developed diabetic rat model. The serum glucose levels were tested pre- and post-injection, and every 7 days during the experiments with a blood glucose meter (OneTouch Ultra® Blood Glucose Monitoring System).

Humanized diabetic wound model and treatment groups

To better mimic the human wound healing process, a splinted excisional wound model was utilized to prevent wound contraction and allow wound healing through re-epithelialization and granulation tissue formation. Briefly, after full thickness excisional skin wound of 1.1 cm diameter circle was created on the dorsum, wound was circumscribed by silicone rings sutured onto the skin surface surrounding the excision area with 5-0 nylon. Four wounds were generated on each animal. Animals were randomized into the following five treatment groups: directly local injection of ADSCs, S&N (INTRASITE◊GEL, a commercially available product from Smith & Nephew), injectable HP-PEG/HA-SH hydrogel, injectable HP-PEG/HA-SH/ADSCs hydrogel, and blank (no treatment) group (n = 16 wounds per treatment group). For the direct ADSCs local injection group, 200 μL (2.5×10^6 cell mL^{-1}) cell suspension was injected subcutaneously around the wound edge. For the injectable hydrogel with (concentration of ADSCs in the hydrogel: 2.5×10^6 cell mL^{-1}) or without ADSCs groups, 200 μL solution was injected directly onto the wound surface.

Digital images were taken at scheduled time points (days 0, 11, 21 post-procedure). The wound area was measured by two blinded evaluators *via* Image J software (n = 8 wounds for each condition). Wound closure rate (%) was defined as: (origin wound area - residual wound area at day 'X') / origin wound area × 100%.

Histological analysis

At scheduled time points, wound tissues were excised and immediately fixed in 4% formaldehyde/PBS solution overnight. Samples were then dehydrated with a graded series of ethanol and embedded in paraffin. Tissues were then sectioned into slices with a thickness of 5 μm and stained by hematoxylin and eosin (H&E, Sigma-Aldrich), and Masson's trichrome (Sigma-Aldrich) and visualized by an optical microscope.

Masson's trichrome stained sections were utilized to determine the collagen deposition and remodeling, which stains the collagen blue, cytoplasm red and nuclei black. We measured the normal dermis thickness around the wounds and the newly formed dermis thickness, using the Image J software (n = 8 wounds for each condition) with a minimum of 6 measurements per sample. Percentage of neo-dermis thickness (%) was calculated as (neo-dermis thickness / the surrounded normal dermis thickness) × 100%.

Immunohistochemistry and immunofluorescence

For the immunohistochemical staining, 5 μm thickness paraffin sections were deparaffinized, washed three times in PBS for 5 min. Then the sections were blocked with 5% serum for 30 min. Next, the slides were incubated in primary antibodies, anti-Keratin 10 (1:300), CD31 (1:200), α-SMA (1:25), VEGF (1:50), CD11b (1:100), TNF-α (1:200), and IL-1β (1:200). All tissues were imaged at 400 ×, five high power microscopic fields for each separate wound sample by three blinded evaluators. The inflammation response was identified by the expression of inflammatory cytokines which was quantified by the positively stained cells. Vascularization was evaluated by counting CD31-positive and α-SMA positive staining cells. Re-epithelialization rate was defined as: (the length of newly formed epidermis of both sides /original wound length) × 100%.

Immunofluorescence was also performed after sections' fixation in aceton at -20 ºC. TNF-α, CD31, and α-SMA antibodies were used to perform the immunofluorescent staining. Nuclei

were stained with DAPI (purchased from Abcam Inc.). Immunofluorescence images were acquired with an AxioCam HRm camera mounted on a Zeiss Imager M2 microscope.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Statistical differences between two groups were determined using the student's unpaired *t* test. A *p* value < 0.05 was considered statistically significant.

Reference:

[1] Y. Dong, W.U. Hassan, R. Kennedy, U. Greiser, A. Pandit, Y. Garcia, W. Wang, Performance of an in situ formed bioactive hydrogel dressing from a PEG-based hyperbranched multifunctional copolymer, Acta Biomater. 10 (2014) 2076–2085. doi:10.1016/j.actbio.2013.12.045.

Supplementary Table 1. *In situ* polymerization conditions and results.

	Feed molar ratio Disulfiram: AIBN: PEGDA	Reaction temperature (°C)	Results
Entry 1	1:0:25	70	No polymer after 24h
Entry 2	0:1:25	70	Gelation within 15min
Entry 3	1:2:25	70	Figure 2A
Entry 4	1:1.4:25	70	Figure 2B
Entry 5	1:1.4:25	60	Figure 2C
Entry 6	1:1.4:25	80	Figure 2D

Supplementary Table 2. Blood glucose level of type 1 diabetic rats at different time periods. To make sure a diabetic animal model is successfully established, blood glucose was tested before STZ injection, day 0, 7, 14, 21 and 28 days post-wounding.

	Different time after STZ injection (day)					
	-14	0	7	14	21	28
Average blood glucose level (mmol L^{-1})	5.4±0.7	24.1±0.7	23.5±1.9	24.0±3.1	24.1±3.0	24.5±2.5

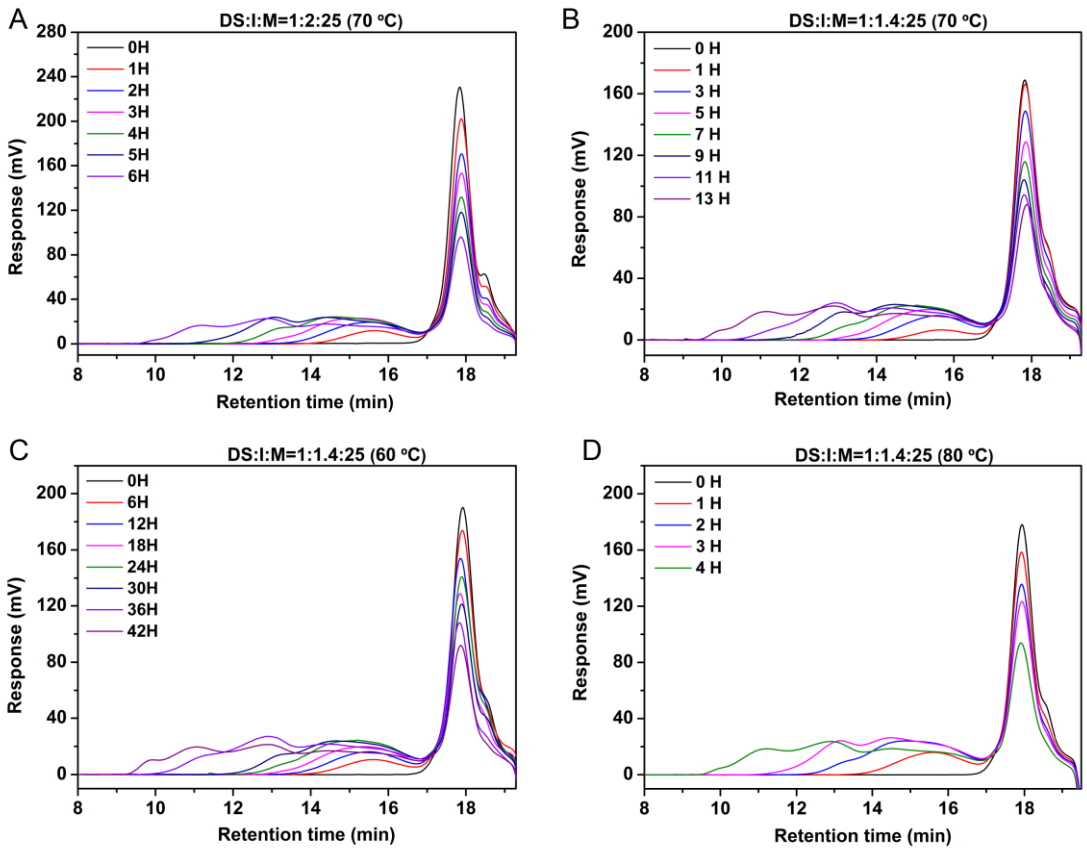

Figure S1. GPC traces of *in situ* RAFT polymerizations (Entry 3-6).

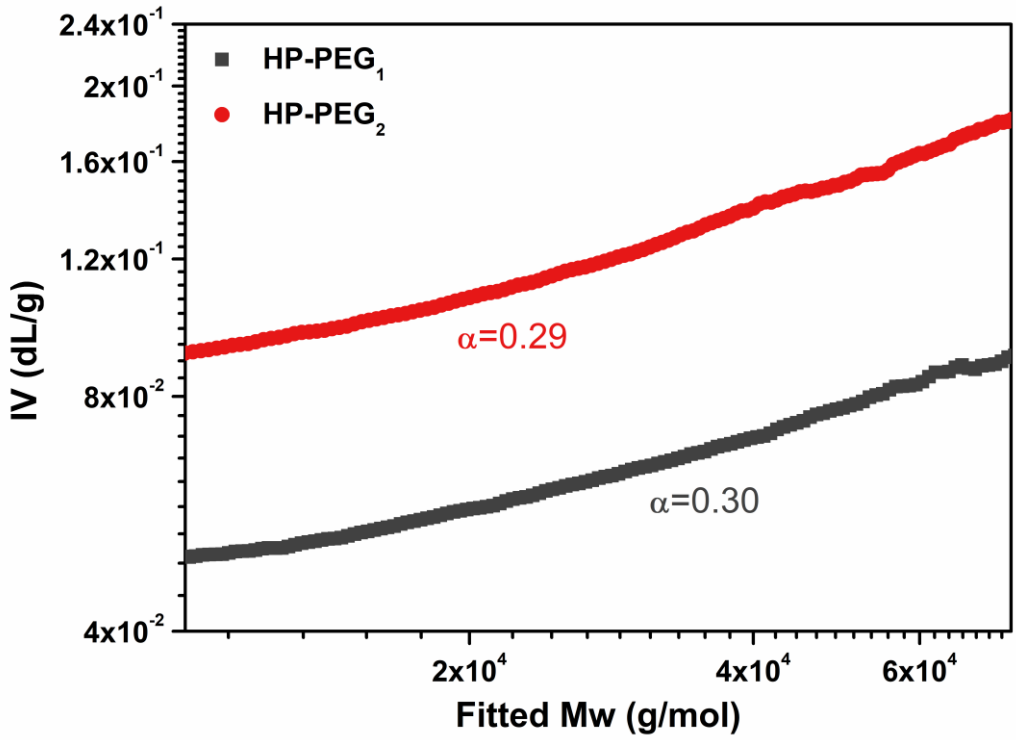

Figure S2. Mark-Houwink plots alpha (α) value of HP-PEG polymers from GPC tests. α values were obtained by analyzing purified HP-PEG polymers suggesting a hyperbranched structure of the resultant polymers. The α value is much lower than that of a linear structure (the parameter is between 0.5-1.0), indicating typical highly branched structure.

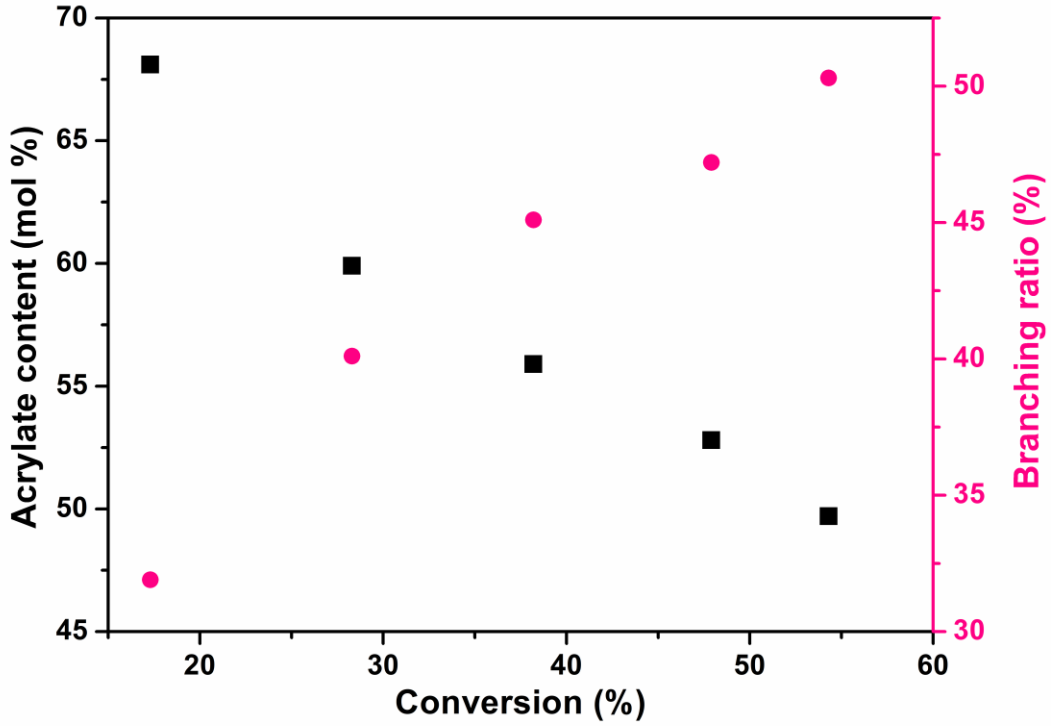

Figure S3. Dependence of the acrylate content and branching ratio on the monomer conversion were calculated by NMR spectrums of entry 4. The polymer composition, acrylate content, and the branch ratio were calculated from the Equation**. S (1)-(5)**.

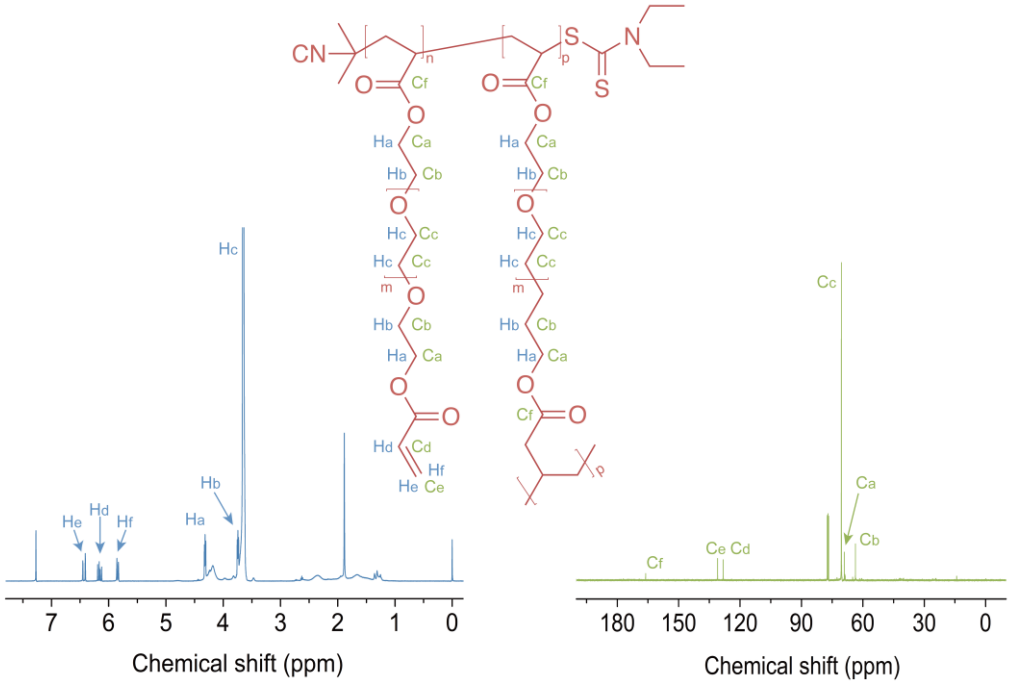

Figure S4. ^{1}H-NMR and ^{13}C-NMR spectrums of a typical HP-PEG polymer (Mw = 10 kDa) characterizing the chemical structure. The ^{1}H-NMR spectra clearly shows the moiety of disulfiram (4.0 ppm) and AIBN (1.9 ppm) at the end of the polymer chains. The acrylate group peaks can be clearly seen between 5.8 ppm and 6.6 ppm.

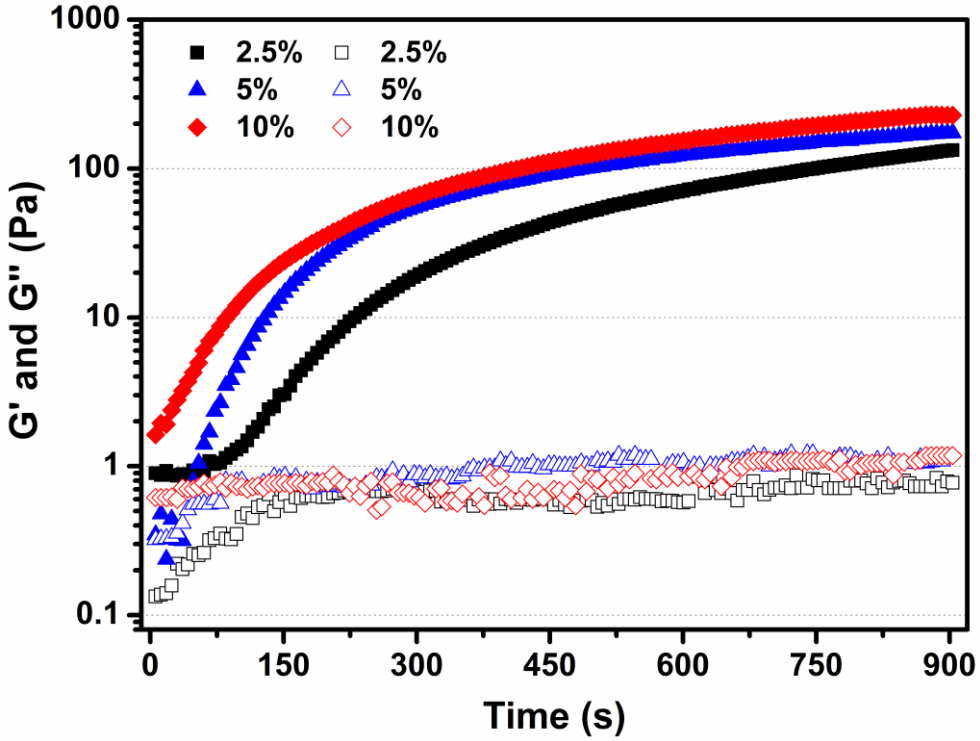

Figure S5. Gelation process of different concentrations of injectable HP-PEG$_2$/HA-SH hydrogels and G' (storage modulus) and G" (loss modulus) characterized by rheometer.

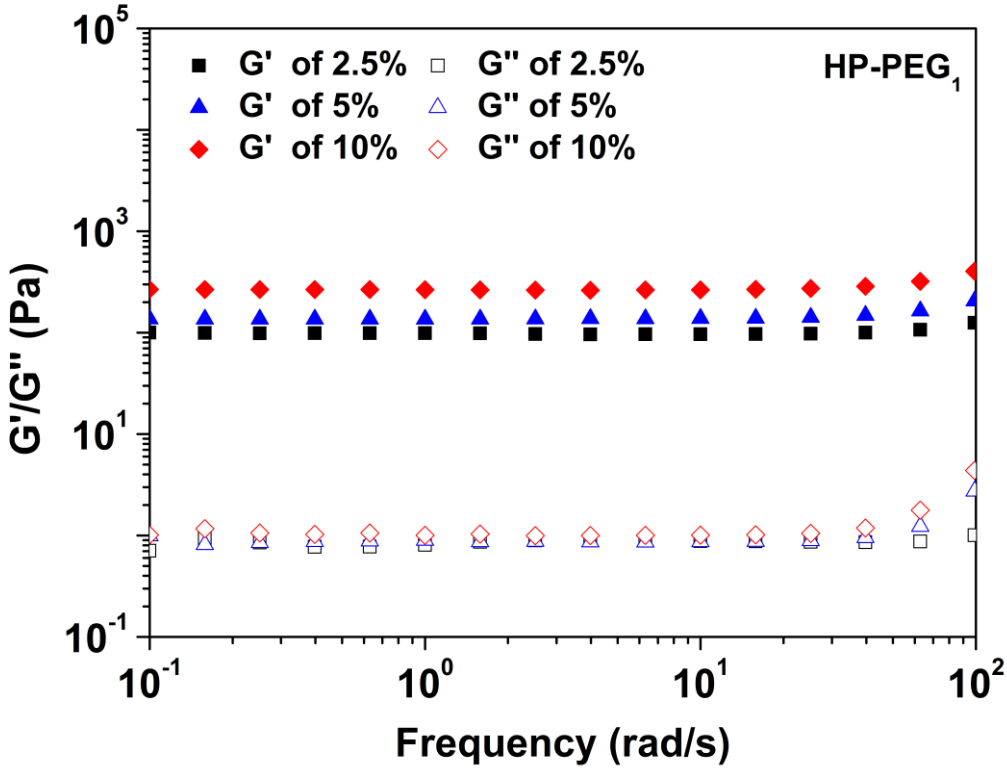

Figure S6. Frequency sweep tests of HP-PEG_1 hydrogels at a constant strain of 1%.

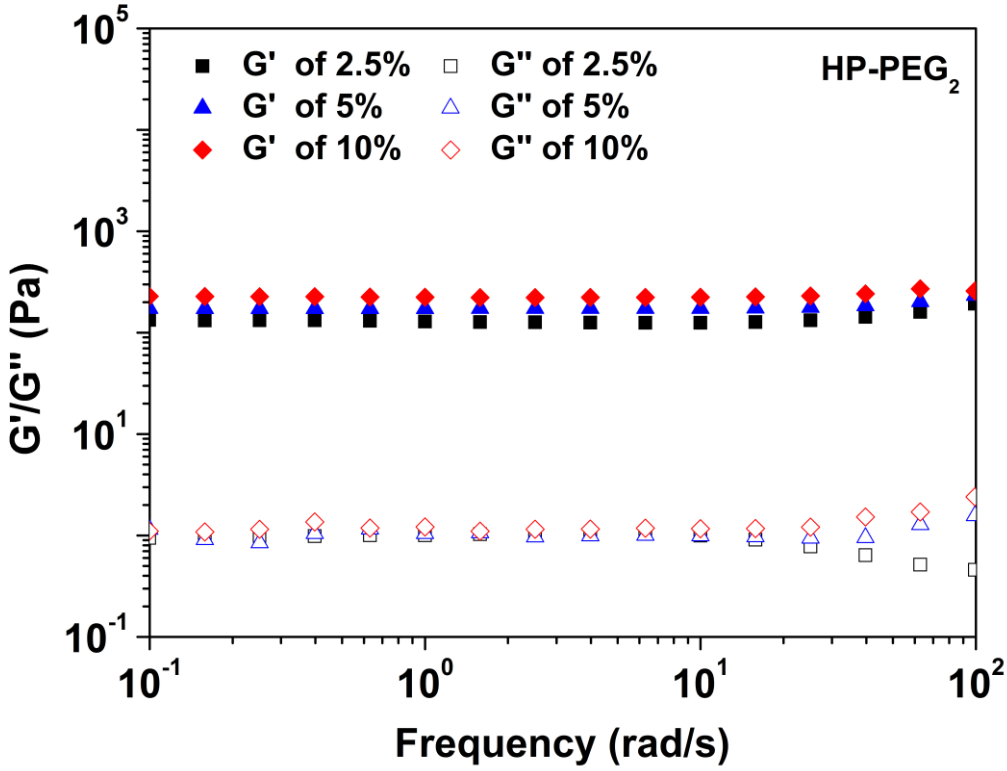

Figure S7. Frequency sweep tests of HP-PEG_2 hydrogels at a constant strain of 1%.

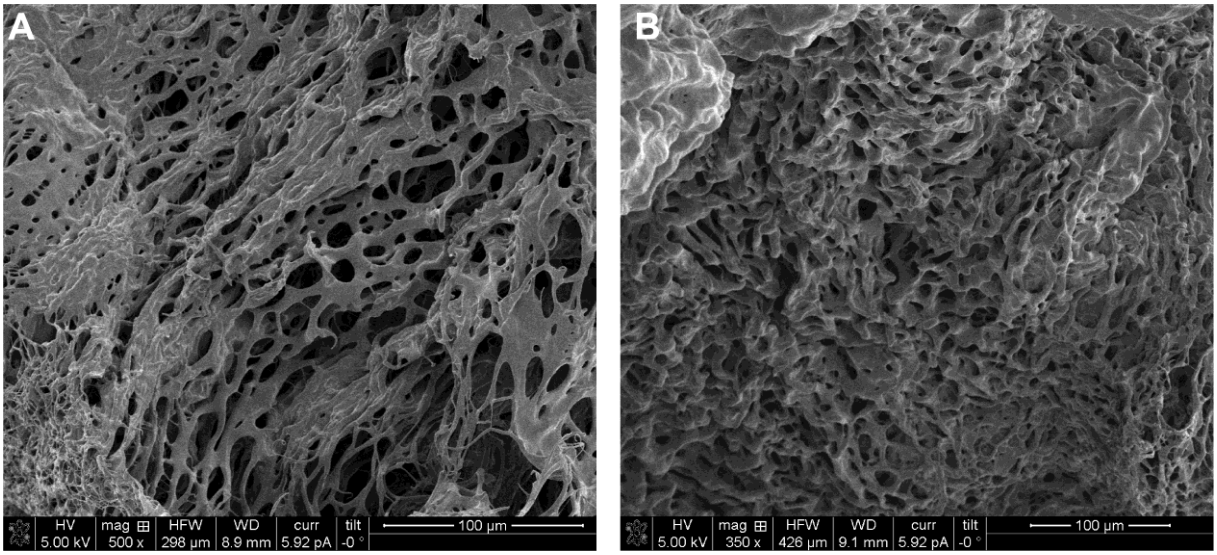

Figure S8. SEM images of HP-PEG$_1$/HA-SH. A, 5% w/v; B, 10% w/v. Scale bars: 100 µm.

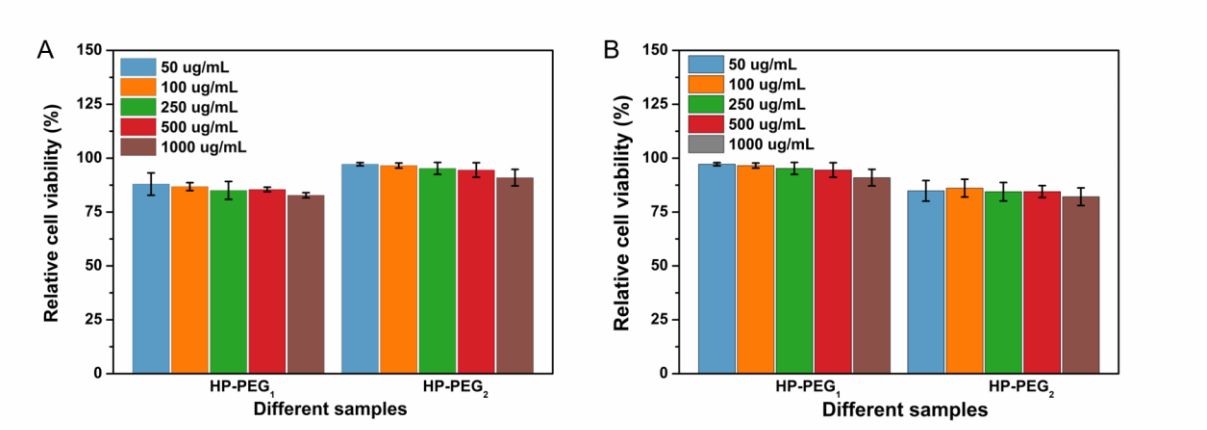

Figure S9. Cytotoxicity of HP-PEGs by alamarBlue assay. A, Cytotoxicity of 3T3 cells for two types of HP-PEGs at various polymer concentrations. B, Cytotoxicity of rADSCs for two types of HP-PEGs at various polymer concentrations. Both polymers show negligible toxic to rADSCs and 3T3 fibroblasts after 24 h incubation with various concentrations. And the HP-PEG_1 demonstrate higher viability for rADSCs.

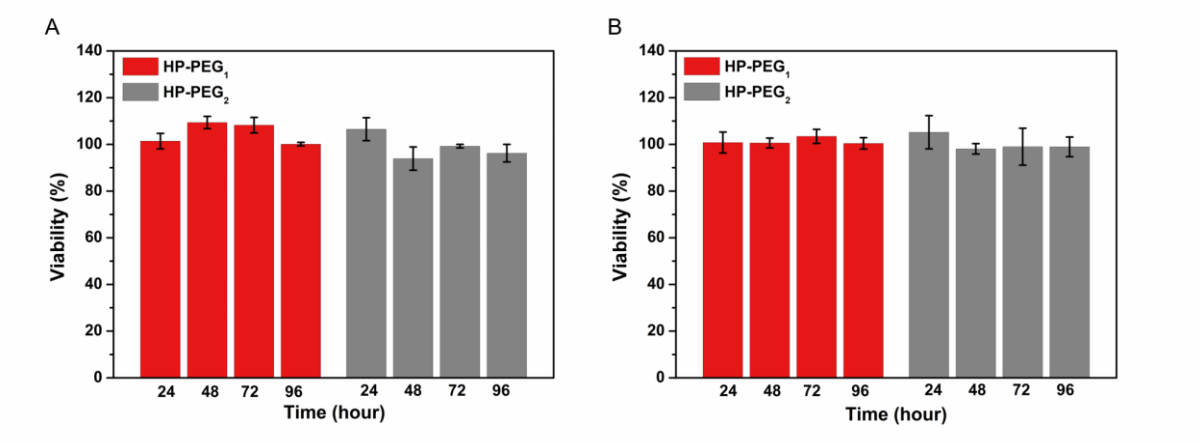

Figure S10. Cytotoxicity of injectable HP-PEG/HA-SH hydrogels (5 wt%) using AlamarBlue assay in a Transwell system. A, Both hydrogels exhibit high viability at 24 h incubation for 3T3 cells; B, Both hydrogels exhibit approximately 100% cell viability after 24, 48, 72, and 96 h incubation for rADSCs.

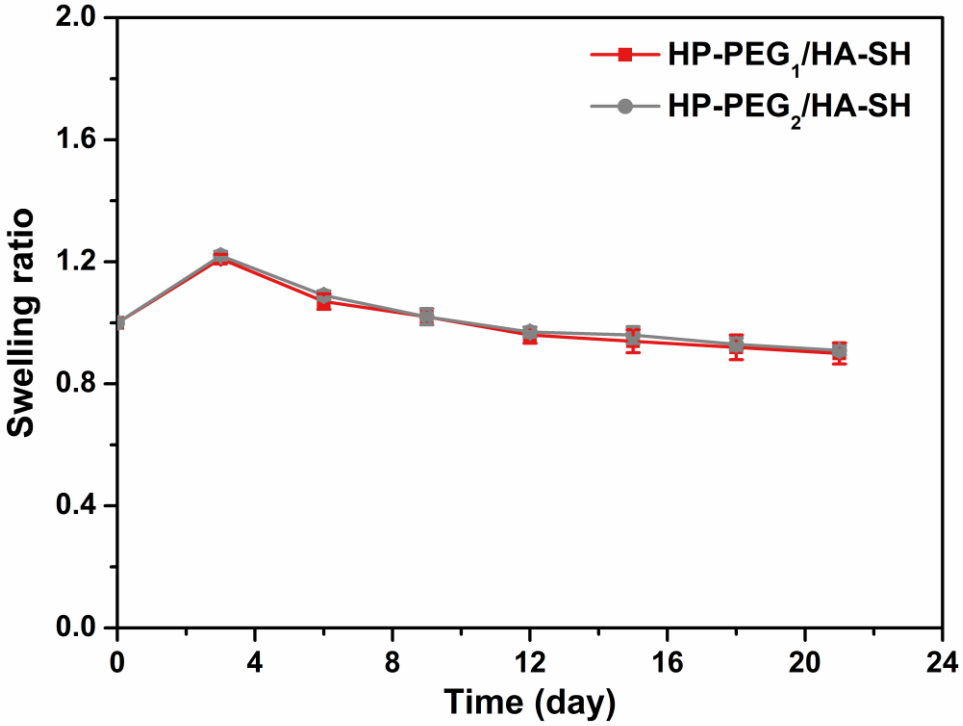

Figure S11. Subcutaneous implantation was performed to examine the *in vivo* degradation profile of HP-PEG$_1$/HA-SH and HP-PEG$_2$/HA-SH hydrogel. The hydrogels exhibit swollen from the first 3 days post-implantation reaching approximately 1.2 the original weights and then start to losing weight. There are 0.9 original weights remaining at 21 days post-implantation. No evidence of rejection or infection was observed during the whole experiment period highlighting the excellent biocompatibility of the HP-PEG/HA-SH hydrogels.

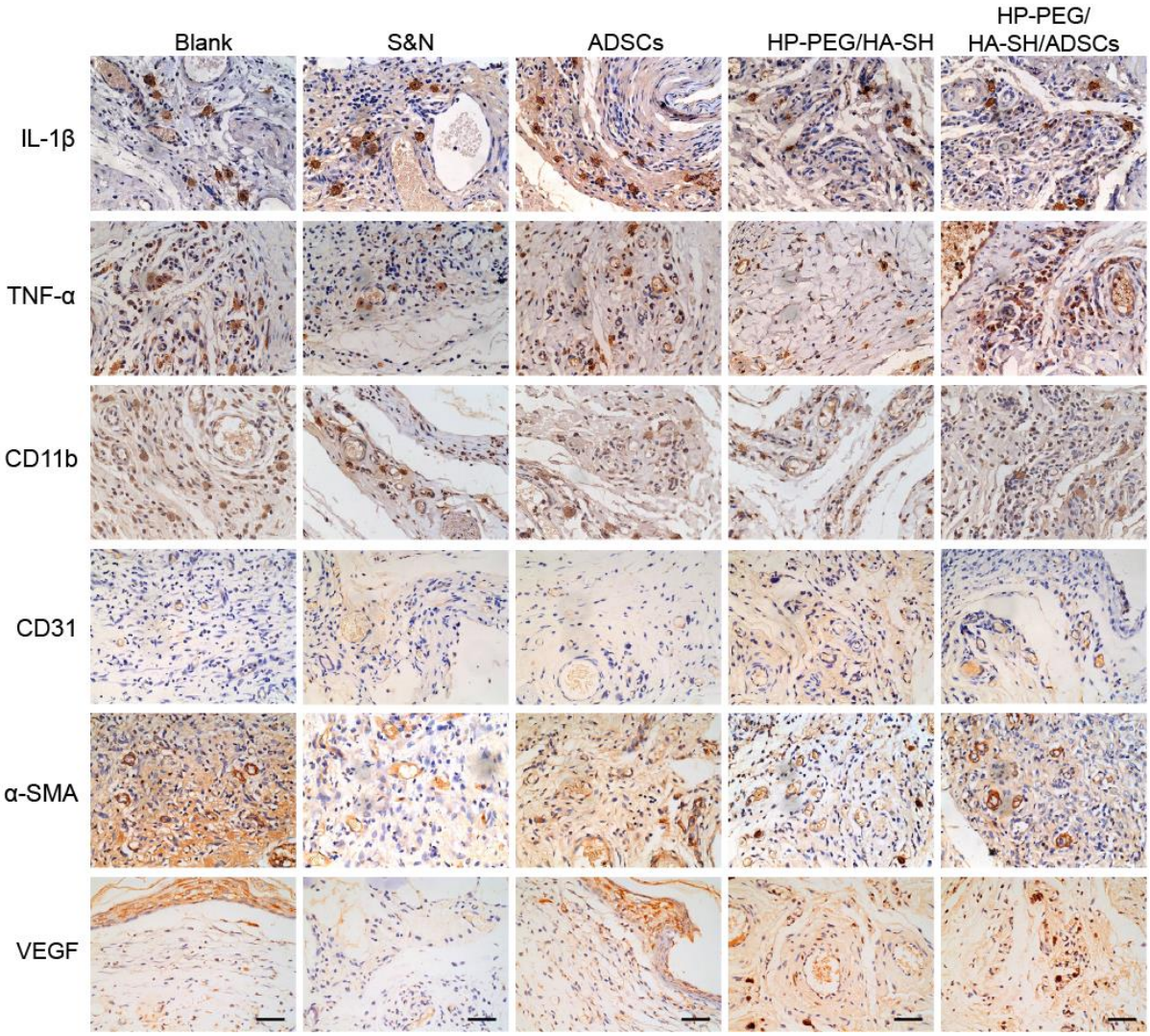

Figure S12. Immunohistochemical staining for diabetic wound sections. Representative images of tissue sections at 11 days post-wounding (IL-1β, TNF-α, and CD11b) and 21 days post-wounding (CD31, α-SMA, and VEGF) for each treatment group. Scale bar: 100μm.

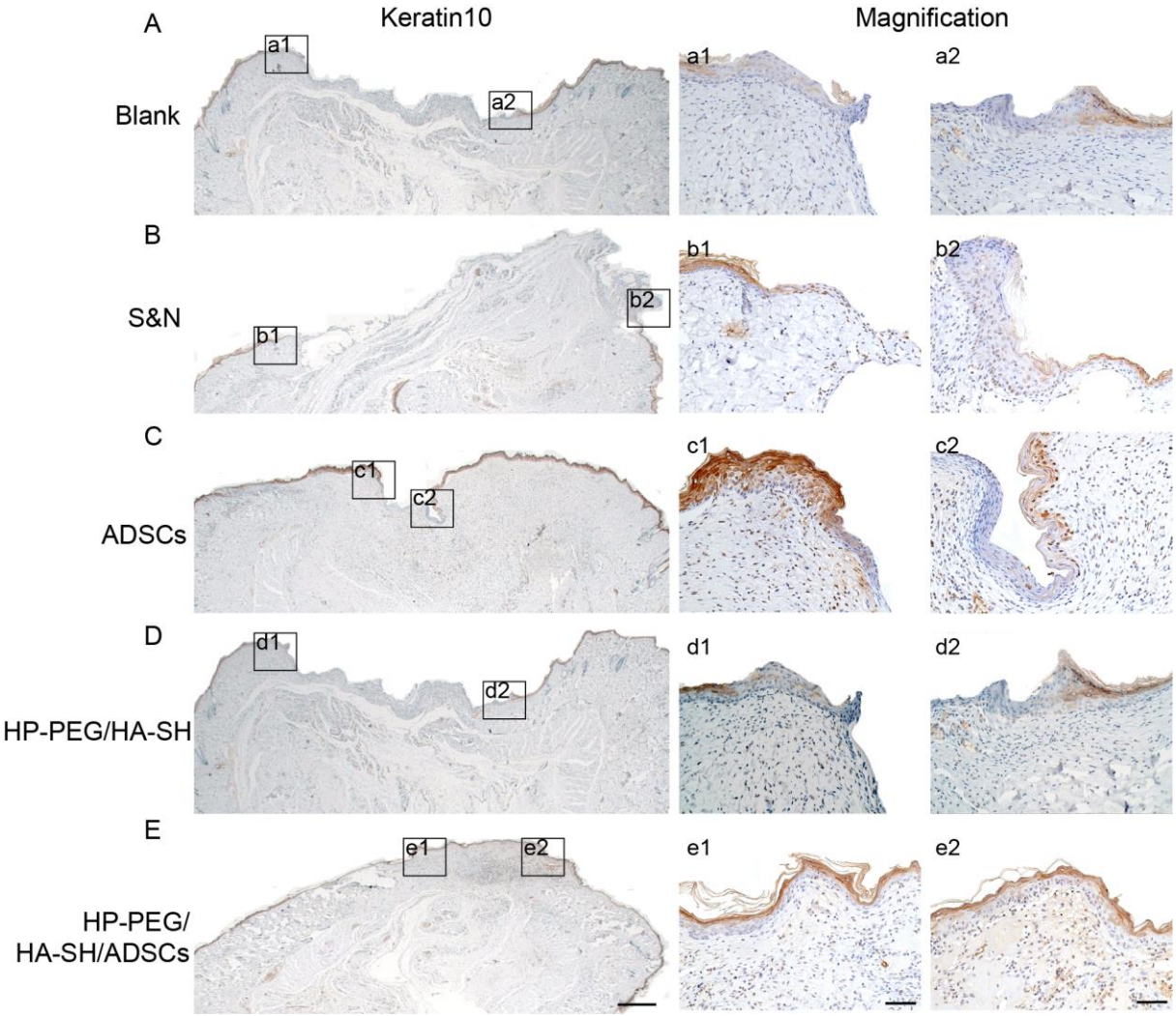

Figure S13. Immunohistochemical staining sections of Keratin-10 at 21 days post-wounding for diabetic wounds. A-E, Different treatment groups show different level of re-epithelialization. (a1, a2)-(e1, e2), Magnifications of the corresponding boxed areas shown on the left. Scale bar: (A-E), 1mm; (a1, a2)-(e1, e2), 100 μm.