Electronic Supplementary Information

3D Printing of Artificial Skin Patch with Bioactive and Optically Active Polymer Materials for Anti-Infection and Augmenting Wound Repair

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Experimental

Materials

Sodium alginate (Alg, from brown algae), gelatin (Gel, from porcine skin) and verteporfin (Ver) were obtained from Sigma Aldrich. Hyaluronic acid (HA) was commercially acquired from Macklin Company. Laminin-derived peptide A5G81 (sequence: AGQWHRVSVRWGC) were customized from CHINESE PEPTIDE company. All the other chemical agents were purchased from Innochem or Alfa-Aesar and used as received without further purification. *Staphylococci aureus* (*S. aureus*) ATCC 6538 was acquired from China General Microbiological Culture Collection Center. Human umbilical cord mesenchymal stem cells (hMSCs) were obtained from State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences (Beijing, P. R. China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Xinjingke Biotechnology Co., Ltd (Beijing, China). Calcein-AM and propidium iodide (PI) were provided by Life Technologies (Beijing, China). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA).

Instruments

UV-vis absorption spectra were measured by a spectrophotometer (Thermo Scientific Evolution 201). Fluorescence spectra were recorded on a fluorometer (Hitachi F-4500). Size and zeta potential data were acquired on a Nano ZS90 (Malvern, UK) instrument. The MTT assay and diffusion data were recorded on a microplate reader (BioTek Synergy HT, USA). Phase contrast bright-field images were taken on fluorescence microscope (Olympus 1 × 71, Japan). Confocal laser scanning microscopy (CLSM) images were taken with a confocal laser scanning microscope (Olympus FV 1200-BX61, Japan). Calorimetric measurements were recorded on a TAM 2277-201 microcalorimetric system (Thermometric AB, Järfälla,

Sweden). Rheological tests were performed on a DHR-1 rheometer (TA Instruments) equipped with a temperature controller. Tensile tests were performed on M5 serries digital force gauge (MARK-10, USA). 3D printing experiments was performed on CPD1 printer (SUNP Biotech). Digital images were taken with a Canon EOS 550D camera.

Ink preparation

The ink containing Alg, Gel and HA was prepared in PBS under aseptic conditions. Briefly, PPV solution was diluted with PBS with the final concentrations of 0, 100, 200, 300 μg/mL, respectively. Then, Gel (4% w/v) was added to PBS (containing PPV) at 37 ℃ until Gel was completely dissolved. Alg (2% w/v) and HA (1% w/v) was added into the above system and the mixture was further shaken at 37 ℃ until forming a uniform hydrogel system, which was stored at 37 ℃. The procedure to prepare Gel/Alg/HA/Ver ink was same to above.

Size and Zeta potential measurements

50 μL ink containing PPV (0, 10, 20, 50, 1250, 2500, 5000 μg/mL, respectively) was diluted to be 1 mL with deionized water, and the above mixture was measured accordingly. The procedure to detect Gel/Alg/HA/Ver was same to the procedure above.

Cell culture

The formulation of hMSCs culture medium was 82% DMEM, 15% FBS, 1% Penicillin-Streptomycin, 1% NEAA, 1% L-Glutamine, and 1‰ FGF. hMSCs were cultured in the medium at 37 °C in a humidified incubator containing 5% CO_2.

Cytotoxicity of PPV or verteporfin by MTT assay

Briefly, hMSCs were seeded in 96-well plates at a density of 4×10^3 cells/well and cultured in

the medium at 37 ℃ for 12 h. Then, the medium containing PPV or Ver (0, 0.5, 1, 2, 4, 8, 16, 32, 64 μg/mL) was used to treat hMSCs at 37 ℃ for 24 h and 48 h, respectively. The medium was removed and MTT (0.5 mg/mL in culture medium, 100 μL/well) was added to the wells followed by incubation at 37 °C for another 4 h. The supernatant was removed again and DMSO (100 μL/well) was added to dissolve the produced formazan. After shaking the plates for 3 min, all the absorbance of the wells was taken on a microplate reader at 570 nm. The cell viability rate (VR) was calculated according to the following equation:

$$VR = A/A_0 \times 100\%$$

where A was the absorbance of the experimental group and A_0 was the absorbance of the control group.

Photodynamic therapy (PDT) properties of PPV and Ver molecule

PDT property of PPV and Ver molecule was characterized by 2,7-dichlorofluorescein (DCFH) assay for probing the production of reactive oxygen species (ROS). Briefly, PPV solution (1 μg/mL) containing DCFH (40 μM) were irradiated under white light (10 mW cm^{-2}) for 7 min, and the fluorescence intensity of the solution at 525 nm was recorded every minute with the excitation light located at 488 nm. This method was also used for PDT characterization of printed 3D structures (Gel/Alg/HA/PPV). PDT effect of Ver was also characterized according to the procedure above.

Isothermal titration microcalorimetry (ITC) characterization

Briefly, the stainless-steel sample cell was loaded with 0.6 mL of water or Gel/Alg/HA solution. The PPV solution was inserted to the above solution dropwise with a Hamilton syringe. The system was stirred at constant rate of 60 rpm by a gold propeller. All the measurements were performed at 25.00 ± 0.01 ℃. The procedure to measure Gel/Alg/HA/Ver

system was same to the procedure above.

Rheological properties

Rheological measurements were taken with a DHR-1 rheometer (TA Instruments) with a 20 mm parallel-plate geometry. Temperature-scan tests were conducted at 1% strain and 10 rad/s. The hydrogel was kept at 4 ºC for 5 min, and then the temperature was elevated at a speed of 0.5 ºC/min from 4 ºC to 40 ºC. Shear-thinning test were measured by increasing the shear rate from 1 to 1500 s^{-1} at 25 ºC. Strain-scan tests were performed at 10 rad/s and 25 ºC accompany by varying the strain from 0.01%-1000%.

3D printing

3D printing for fabricating artificial skin patch was conducted with a commercial 3D printer (CPD1, SUNP Biotech). Briefly, the print platform was cooled to 4 ºC before printing, and 5 mL ink was loaded into a BD syringe with a 220 μm nozzle followed by equipping on the printing arm with the temperature of 26 ºC. The 3D structures were printed onto a 60 mm Petri dish, which were then immediately crosslinked with 3 mL sterilized 2% w/v $CaCl_2$ solution for 10 min.

PPV Diffusion Studies.

The PPV diffusion out of ink was determined by measuring the fluorescence intensity of PPV in water. The formulation was PPV (300 μg/mL), Gel (4% w/v), Alg (2% w/v) and HA (1% w/v). Briefly, the printed structures after crosslinking were washed with water and immersed into water at 37 ºC for 24 h. PPV diffusion was determined by measuring the fluorescence intensity of PPV (data at 573 nm was collected under excitation wavelength of 455 nm) at every time point.

Tensile tests of the printed Gel/Alg/HA/PPV artificial skin patch.

The formulation in the tensile testes was PPV (300 μg/mL), Gel (4% w/v), Alg (2% w/v) and HA (1% w/v). Briefly, the printed patches (30 mm × 15 mm) with 30, 60° and 90° pore geometry were printed followed by performing the tensile tests with a speed of 0.9 mm/min.

Preparation of *S. aureus*

10 μL single colony of *S. aureus* solution was transferred into 10 mL NB liquid culture medium and incubated overnight at 37 ℃ under constant shaking of 180 rpm. Then, the *S. aureus* solution was centrifuged (7100 rpm for 2 min) to remove the NB medium and washed with PBS thrice. After discarding PBS, the remaining *S. aureus* were suspended in PBS with OD_{600} = 1.0.

***In vitro* antibacterial activity**

The photodynamic antibacterial activity of 3D printed artificial skin patch (Gel/Alg/HA/PPV ink) towards *S. aureus* was evaluated by the reduction in colony-forming units (CFUs) through spread plate method. Briefly, 10 μL of the *S. aureus* suspensions (OD_{600} = 1.0) was added on the surface of 3D printed artificial skin patches (1 cm ×1 cm). After 5 min incubation in the dark, the above system was exposed to white light (60 mW cm^{-2}) for 15 min. Then, 40 μL PBS was added and the collected solution was further diluted for 1×10^4 folds with PBS. 100 μL of the obtained *S. aureus* suspensions was spread on the solid NB agar plate. Then, CFUs were counted and the CFU reduction was calculated according to the equation:

$$\text{CFU reduction (\%)} = (N_0\text{-}N)/N_0 \times 100\%$$

where N_0 is the mean number of the *S. aureus* colonies in the dark control group while N is

the mean number of the *S. aureus* colonies in every experimental groups.

In vivo antibacterial activity

In vivo antibacterial activity of 3D printed skin patches was evaluated on a murine model. The ink of PPV (300 μg/mL), Gel (4% w/v), Alg (2% w/v) and HA (1% w/v) was used for 3D printing of antibacterial skin patches. Sprague Dawley (SD) rats (6-8 weeks, Beijing) were divided in to 3 groups (bacterial-infected group, bacterial-infected + PPV skin patch + dark group, and bacterial-infected + PPV skin patch + light group), and then anaesthetized with pentobarbital (2 wt.%, 2 mL/kg). Next, full-thickness circular wounds (12 mm in diameter) were created followed by injection of *S. aureus* suspensions (1.0×10^8 CFU mL^{-1}, 100 μL). After the *in vivo* incubation for 15 min, the rats of bacterial-infected group were bandaged after another 30 min incubation; the rats of bacterial-infected + PPV skin patch + dark group were implanted with PPV skin patch and bandaged after 30 min incubation in dark; the rats of bacterial-infected + PPV skin patch + light group were irradiated with white light (60 mW cm^{-2}) for 30 min followed by bandage. The wounds were imaged on 1- and 4-day post-infection accompanying with sacrificing rats and wound tissue harvest. The tissues were separated and homogenized in 150 μL sterilized PBS buffer and then diluted 10 folds with PBS. 100 μL of the obtained suspensions was spread on the solid NB agar plate for overnight incubation at 37 °C. The wound tissue sections were also utilized for H&E staining analysis.

Cytotoxicity of A5G81 peptide by MTT assay

Briefly, hMSCs were seeded in 96-well plates at a density of 4×10^3 cells/well and cultured in the medium at 37 ℃ for 12 h. Then, the medium containing A5G81 peptide (0, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200 μg/mL) was used to treat hMSCs at 37 ℃ for 24 h, 48 h and 72 h, respectively. The culture medium was removed and MTT (0.5 mg/mL in culture medium, 100

μL/well) was added to the wells followed by incubation at 37 °C for 4 h. The supernatant was removed again and DMSO (100 μL/well) was added to dissolve the produced formazan. After shaking the plates for 3 min, all the absorbance of the wells was taken on a microplate reader at 570 nm. The cell viability rate (VR) was calculated according to the following equation:

$$VR = A/A_0 \times 100\%$$

where A was the absorbance of the experimental group and A_0 was the absorbance of the control group.

Promoted cell migration by A5G81

Promoted cell migration by A5G81 was investigated by cell scratch test. Briefly, hMSCs were seeded in 6-well plates at a density of 1×10^6 cells/well and cultured in the medium at 37 ℃ until the bottom of the cell plates was fully covered. The medium was discarded, and three parallel cell scratch lines were created. The cells were incubated with A5G81 (0 and 25 μg/mL) for 1 h. Then the cells were imaged after another 0 h, 12 h, 18 h and 24 h incubation.

Promoted cell attachment by A5G81

Briefly, 6-well plates were soaked with A5G81 (0 and 25 μg/mL) for 24 h at 37 ℃. Then, hMSCs were seeded in 6-well plates at a density of 1×10^6 cells/well. The cells were imaged after another 0 h, 2 h, 4 h and 18 h incubation.

Synthesis of HA-A5G81 and fabrication of artificial skin patch with high cell affinity

HA-A5G81 was synthesized according to the literature,[1] and the modification ratios were measured by a standard TNBS assay. The content of A5G81 on HA-A5G81 was measured to be 71.8 μg/mg, the concentration of HA-A5G81 was made to be 0, 12.5, and 25 μg/mg by mixing with unmodified HA. The ink of PPV (300 μg/mL), Gel (4% w/v), Alg (2% w/v) and

HA (1% w/v, containing HA-A5G81) was applied for 3D printing artificial skin patches with high cell affinity.

Promoted cell attachment on Gel/Alg/HA-A5G81/PPV artificial skin patch

Artificial skin patch with high cell affinity were fabricated with the ink of Gel/Alg/HA-A5G81/PPV (the concentration of HA-A5G81 was made to be 0, 12.5, and 25 μg/mg by mixing with unmodified HA). Then, hMSCs were seeded on the skin patches in 6-well plates at a density of 1×10^6 cells/well. The cells were imaged after another 0 h, 2 h, and 4 h incubation.

Live/dead cell imaging of hMSCs on Gel/Alg/HA-A5G81/PPV artificial skin patch

Artificial skin patch with high cell affinity were fabricated with the ink of Gel/Alg/HA-A5G81/PPV (the concentration of HA-A5G81 was made to be 25 μg/mg by mixing with unmodified HA). Then, hMSCs were seeded on the skin patches in CLSM dishes at a density of 1×10^6 cells/well. After 24 h-incubation, the cells were incubated with fresh medium containing calcein AM/PI for 30 min at 37℃. CLSM images were acquired after washing with PBS for three times. The fluorescence of AM and PI were collected at the range of 500-540 nm (λ_{ex}: 488 nm) and 570-650 nm (λ_{ex}: 559 nm), respectively.

***In vivo* wound healing studies**

3D printed artificial skin patches with Gel/Alg/HA-A5G81 (25 μg/mg)/PPV or Gel/Alg/HA/PPV ink were used for *in vivo* wound healing studies. Sprague Dawley (SD) rats (6-8 weeks, Beijing) were anaesthetized with pentobarbital (2 wt.%, 2 mL/kg) and the dorsal area of rats was depilated. 2 full-thickness circular wounds (12 mm in diameter) were created on the upper back of each rat, then Gel/Alg/HA-A5G81/PPV and Gel/Alg/HA/PPV skin

patches were placed directly on the wound area, which were further covered with a circular polydimethylsiloxane (PDMS) grid (14 mm in diameter) to protect the patches from dropping. PDMS grid and skin were sewed up together using 4-0 monofilament medical silk braided sutures (Shanghai Pudong Jinhuan Medical Products Co., Ltd.) followed by covering with TegaDerm TM dressing (3M, St. Paul, MN, USA) for protection and ventilation. The wounds were imaged on 0, 5, 9 and 19 d post wounding. The rats were sacrificed and the wound tissues were harvested accordingly for histological analysis.

In vivo biodegradability

3D printed artificial skin patches from Gel/Alg/HA-A5G81/PPV ink was implanted to the subcutaneous layers of SD rats through skin incision without creating full-thickness excisional wounds. The wound sites with the surrounding skin were collected at 1, 3, 7 and 14-day post transplantation for H&E staining characterizations.

Animal welfare statement

The animal experiments were approved and performed in compliance with the policy of Research Ethic Committee in the Institute of Zoology, Chinese Academy of Sciences in Beijing, China on animal use and ethics (Approval number: IOZ-IACUC-2020-087).

Statistical analysis

Statistical analysis was performed in OriginPro 2016 software. Histopathological studies were analyzed by ImageJ and CaseViewer software.

References

[1] Y. Zhu, Z. Cankovaa, M. Iwanaszkob, S. Lichtorc, M. Mrksicha, G. A. Ameera, *Proc. Natl. Acad. Sci*., 2018, **115**, 6816-6821.

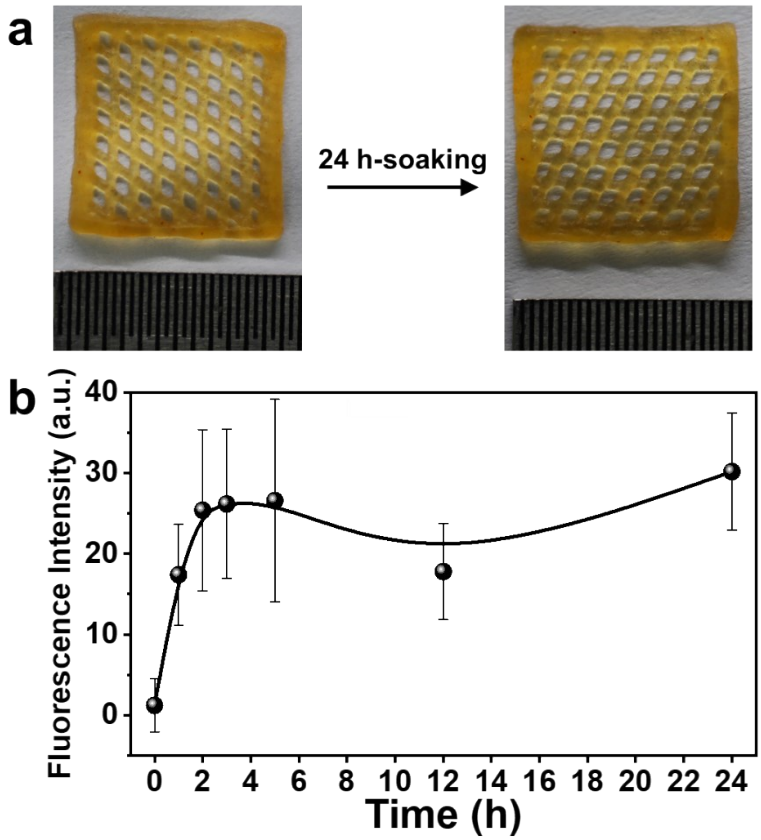

Figure S1. (a) Gel/Alg/HA/PPV patch images before and after 24 h-soaking. (b) Diffusion curve of PPV from Gel/Alg/HA/PPV patch within 24 h. PPV diffusion was determined by measuring the fluorescence intensity of PPV (data at 573 nm were collected under excitation wavelength of 455 nm) at every time point.

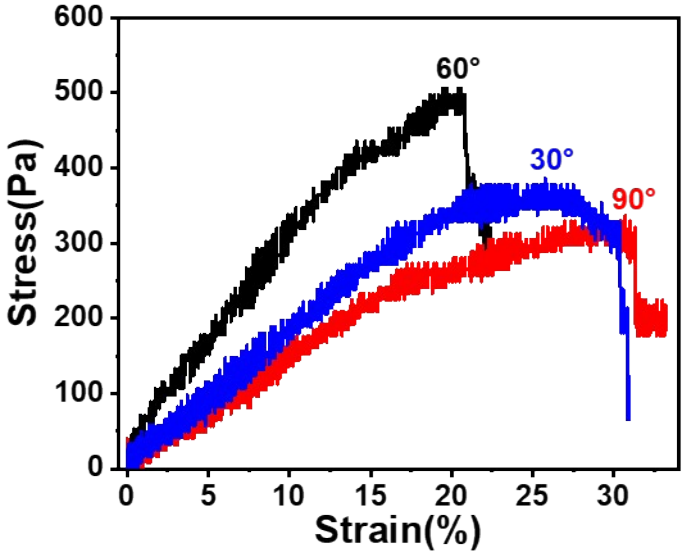

Figure S2. Stress-strain curves of the printed Gel/Alg/HA/PPV patches with 30°, 60° and 90° pore geometry, respectively.

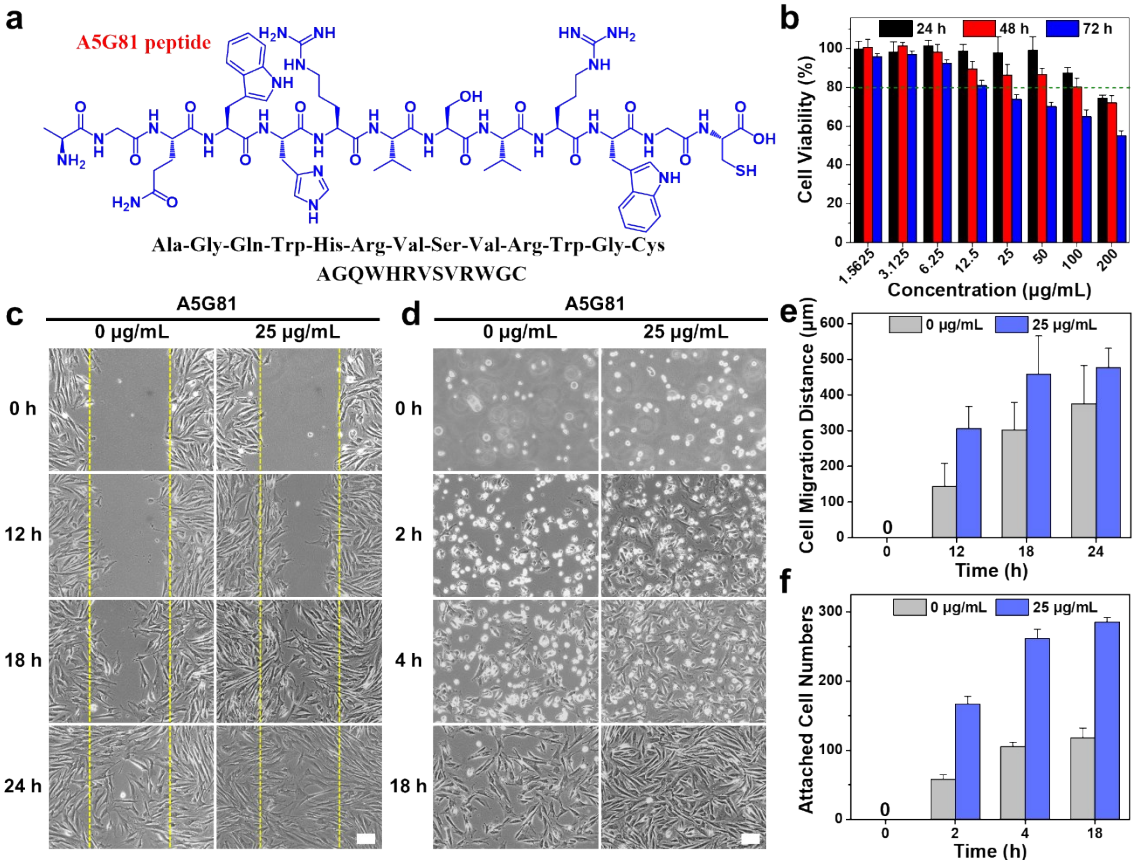

Figure S3. Chemical structure, cytotoxicity and the promoted-cell migration and cell attachment effect of A5G81 peptide. (a) Chemical structures of A5G81 peptide. (b) Cell viability of hMSCs after treated with various concentrations of A5G81 peptide. (c) Optical images and (e) the corresponding statistical histogram of cell migration after treated with 25 μg/mL A5G81 peptide, scale bar: 200 μm. (d) Optical images and (f) the corresponding statistical histogram of cell attachment after treated with 25 μg/mL A5G81 peptide, scale bar: 200 μm.

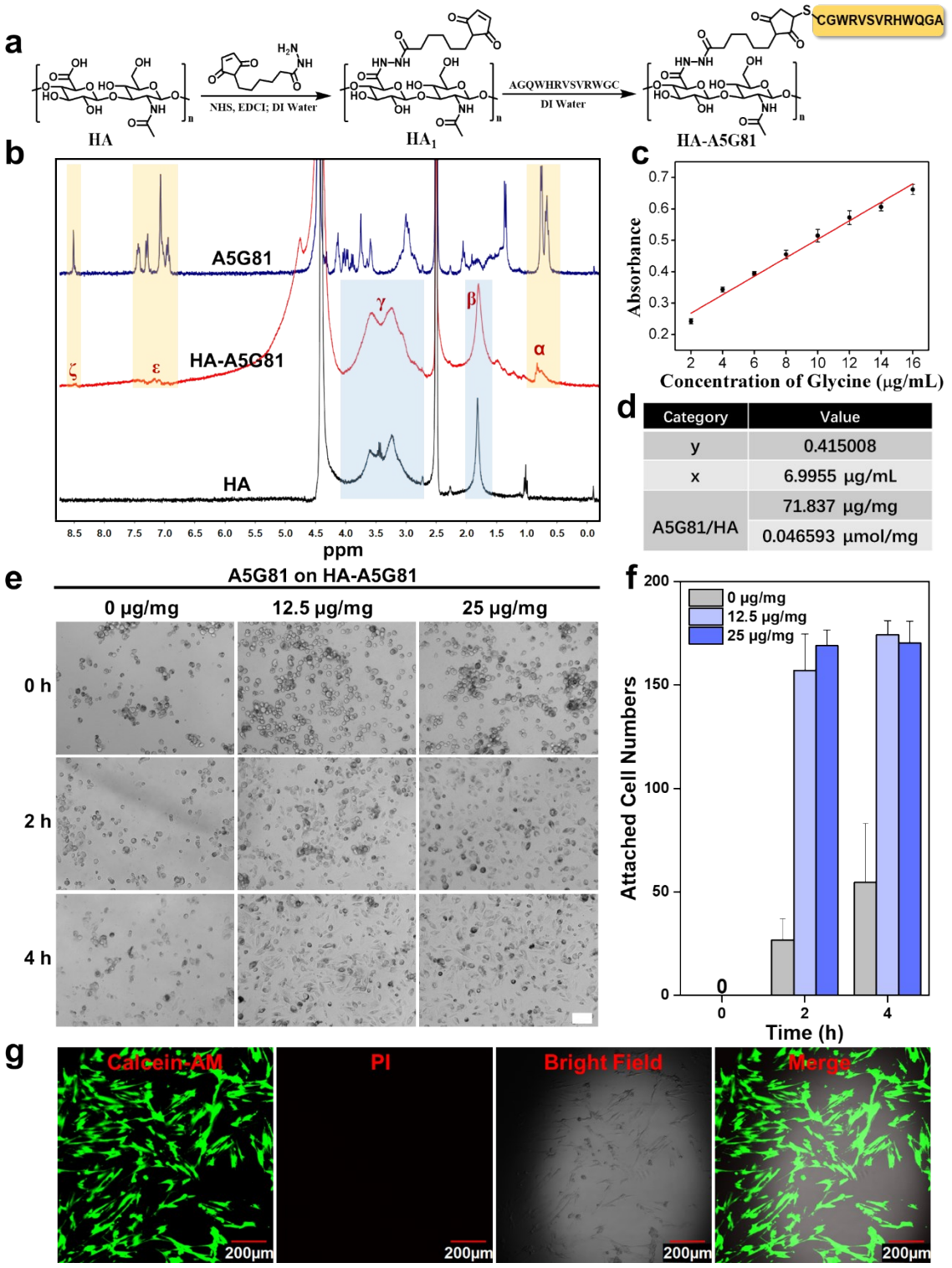

Figure S4. Synthesis and characterization of HA-A5G81 as well as the cell attachment-promoted of Gel/Alg/HA-A5G81/PPV skin patches. (a) Synthesis route of HA-A5G81. (b) ^{1}H-NMR spectra of A5G81, HA-A5G81 and HA in D_2O, respectively. (c) Standard curve of TNBS assay for quantification of A5G81 on HA-A5G81. (d) Table of values for A5G81

modified rate on HA-A5G81. (e) Digital images and (f) the statistical histogram of promoted-cell attachment on Gel/Alg/HA-A5G81/PPV skin patches, scale bar: 200 μm. (g) Live/dead cell CLSM images of hMSCs on Gel/Alg/HA-A5G81/PPV skin patches, scale bar: 200 μm. The fluorescence of calcein-AM and PI were collected at the range of 500-540 nm (λ_{ex}: 488 nm) and 570-650 nm (λ_{ex}: 559 nm), respectively.

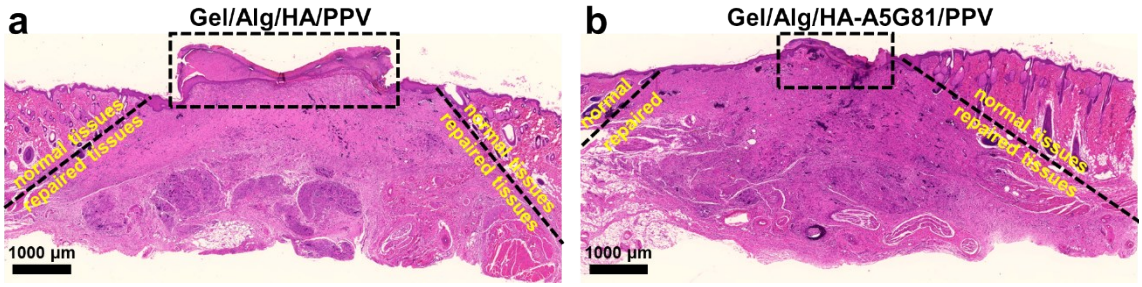

Figure S5. H&E Staining of the wound sites with treatment with Gel/Alg/HA/PPV and Gel/Alg/HA-A5G81/PPV skin patches at 19 d post wounding, respectively. The unhealed wound width was indicated with dash square while the boundary of normal and repaired tissues was also indicated with dash line.