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Supplementary Materials for

ECM-mimetic immunomodulatory hydrogel for methicillin-resistant *Staphylococcus aureus*–infected chronic skin wound healing

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Materials and Methods

Materials

Sodium periodate ($NaIO_4$), and ethylene glycol hydroxylamine hydrochloride were purchased from Aladdin Chemical Reagents Co., Ltd. (Shanghai, China). All chemical reagents were used without further purification. Fluorochrome-labeled monoclonal antibodies CD86 (11-0862-82), CD206 (12-2061-82), F4/80(12-4801-82), were received from eBioscience (San Diego, CA, USA). Primary antibodies to CD31 (ab222783), α-SMA (ab111893), CK-14 (ab181595), IL-6 (ab259341), IL-10 (ab9969), TNF-α (ab109322), TGF-β (ab31013), p-ERK (ab229912), p-JAK1 (ab133666), and β-actin (ab8227), and secondary antibodies (ab150113, ab150075, ab288151) were purchased from Abcam (Cambridge, UK). All experiments were replicated at least three times.

Synthesis and characterization of glucomannan-aldehyde

The oxidized GM, termed as GM-CHO, was synthesized by the following method. Briefly, GM (2 g, 4% w/v) was dissolved in double distilled water, and the calculated amount of sodium periodate in aqueous solution was added dropwise to the reaction mixture. Then the mixture was stirred in the dark overnight at room temperature. The reaction was quenched by adding excess amount of ethylene glycol and dialyzed against water for 5 days. GM-CHO was obtained by freeze-dried, and kept under nitrogen atmosphere before use.

The degree of oxidization was determined by potentiometric titration analysis. The colorimetric hydroxylamine titration analysis was conducted by potentiometric titrator (ZDJ-4B Auto Potential Titrator, INESA Scientific Instrument Co., Ltd, China). Briefly, 100 mg GM-CHO was dissolved in 25 mL of 0.25 M hydroxylamine hydrochloride (pH adjusted to 3) to deplete aldehyde and hemiacetal. After string for 4 h, the mixed solution was titrated with 0.1 M NaOH. The percentage of aldehyde functionality was calculated by:

$$\text{aldehyde functionality (\%)} = \frac{\text{mol of dialdehyde}}{\text{mol of GM}} \times 100\% = \frac{V_{NaOH} \times C_{NaOH}}{m_{GM} / (Mw_{GM}\text{-}18)} \times 100\%$$

The molecular weight of GM-CHO was measured by GPC, which was equipped with PLgel GPC Column (10 μm Mixed-B, Org 300 × 7.8 mm) and RI2000 detector. The FT-IR spectrum of GM-CHO was also recorded by an infrared spectrometer (Elmer Perkin, USA).

Synthesis and characterization of hyaluronic acid-maleimide

HA-maleimide was synthesized by esterification between HA and 3-maleimidopropionic acid. Briefly, HA (2 g, 4% w/v) was dissolved in double distilled water, and the calculated amount of 3-Maleimidopropionic acid in aqueous solution was added dropwise. Then the mixture was stirred at room temperature for 48 h. The product was dialyzed against water for 5 days. HA-maleimide was obtained by lyophilization, and kept under nitrogen atmosphere before use. The degree of esterification was determined by ^{1}H NMR.

The secondary structure of GM-P and HA-P

The secondary structure of GM-P@HA-P hydrogel by circular dichroism (CD) spectroscopy. For CD spectroscopy, the ellipticity of polypeptides and glycopeptide in diluted aqueous solutions (0.1 mg/mL) was measured using a CD instrument (J-810, JASCO, Japan). The samples were incubated at room temperature for 2 h before CD measurement. The UV region was scanned between 190 and 260 nm at 1 nm intervals, using a 1 mm path length quarts cuvette.

Characterization of GM-P@HA-P hydrogel

For test the rheology properties of GM-P@HA-P hydrogel, the hydrogel samples were placed between parallel plates with a diameter of 10 mm and a gap of 1 mm. The measurement of elasticity modulus (G') and viscosity modulus (G'') were carried out over a frequency sweep between 0.1 and 100 rad/s at strain of 1%, a time sweep of 1000 s with 1% strain and 1 rad/s frequency, and a strain sweep from 0.1 to 100 % at 25 ^{o}C.

In vitro antibacterial activity and mechanism

To determine MRSA (ATCC 25923) or *E. coli* (ATCC 21320) killing rate by hydrogels, 100 μL of MRSA suspension was mixed with 100 μL of hydrogel. Bacteria were cultured in constant temperature incubator (37 ^{o}C) for 4 h. Then the bacterial solution (diluted by 10^4 times) was coated on the surface of LB culture medium to determine the antibacterial properties of hydrogel by counting bacterial colony after 24 h.

Hoechst 33342 and PI staining were implemented to determine the ability of GM-P@HA-P to destroy the membranes of MRSA. Briefly, bacterial suspensions (~10^7 CFU/mL) were incubated with GM-P@HA-P for 0.5 h at 37 ^{o}C. After centrifugation for 5 min at 5000 rpm, bacteria were washed with Phosphate Buffer Saline (PBS) for 3 times, and incubated with Hoechst 33342 and PI (5 μg/mL) for 20 min on ice in dark, which was then washed 3 times with cell staining buffer. Then the bacterial suspension was placed on the confocal Petri dish, and observed under confocal laser scanning microscopy (CLSM, LSM710, Zeiss, Germany) using 63× oil-immersion objective.

The cell membrane permeability assay was indicated by measuring the release of cytoplasmic K^+ and *β*-galactosidase from MRSA cells into the culture medium using sodium tetraphenylborate and *β*-galactosidase activity

assay, respectively. For K^+ measurement, MRSA were incubated at 37 ºC for 24 h in LB medium, and MRSA suspensions were incubated with GM-P@HA-P hydrogel. The concentration of K^+ was measured with sodium tetraphenylborate. For *β*-galactosidase determination, MRSA were incubated at 37 ºC for 24 h in Luria-Bertani medium, which were centrifuged at 5000 rpm and the harvested bacteria were incubated in M9 lactose medium at 37 ºC for 8 h. After centrifugation at 5000 rpm for 3 min, bacterial cells were collected, washed 3 times with PBS, resuspended to bacterial concentration ~10^7 CFU/mL with PBS. The cell suspension was mixed with GM-P@HA-P hydrogel, then incubated at 37 ºC. The supernatant was collected over time, and then the optical density of p-nitrophenol was measured using a SpectraMax Plus 96 microplate reader (Thermo Fisher Scientific, USA) at 400 nm. The data was analyzed with GraphPad Prism 7.0. The cell suspension without GM-P@HA-P was used as the control group. The concentration of *β*-galactosidase was calculated according to the standard curve.

For culturing MRSA biofilm, the equal volume of MRSA suspension (~10^7 CFU/mL) and tryptone soy broth (TSB) medium were placed on culture plates to culture at 37 ºC for 72 h. TSB medium was replaced with fresh TSB medium twice. Finally, the TSB medium was removed, and the MRSA biofilm attached on culture plates was obtained. After the MRSA biofilm was obtained, the hydrogels were added to the biofilm for 2 hours. The biofilms were rinsed by PBS five times, and then fixed with 2.5% glutaraldehyde for 2 h at 4 ºC. The samples were dehydrated in alcohol. Finally, the samples were disposed by metal spraying and observed by SEM. The bacterial biofilm of different groups in culture plates were washed with sterile PBS slightly, and then were stained using Live/Dead dye for 15 minutes. The redundant dye was washed by sterile PBS slightly for five times. Finally, the biofilms were observed under a fluorescence microscope.

Cytocompatibility of GM-P@HA-P hydrogel

Cells were seeded in 96-well plates (Corning Costa, USA) at a density about 5 ×10^3 cells/well and cultured in 100 μL HDMEM with 10% (v/v) fetal bovine serum (FBS) at 37 ºC for 24 h under humidified condition of 5% CO_2 atmosphere. Then, fresh medium was added into each well with the presence of GM-P@HA-P hydrogel. After culturing for predetermined times, the culture medium was discarded, and 100 μL fresh medium containing CCK-8 was added to the wells. After incubation at 37 ºC for 2 h, the absorbance was measured at a wavelength of 450 nm. The cytotoxicity of GM-P@HA-P is defined as the relative viability that is normalized to that of control group.

Cell viability determined by live/dead assay

Hydrogels were prepared in a 96-well plate with a volume of 100 μL and sterilized prior to use. Then, 100 μL NIH/3T3 cell (CRL-1658, ATCC) suspension was seeded in hydrogel-coated plate with a density of 5×10^3 cells/well. Cells were incubated under a humidified atmosphere containing 5% CO_2 at 37 ºC. At predetermined times, cells were stained by calcien-AM (2 μM) and propidium iodide (4.5 μM), and observed by fluorescence microscope (Leica DMi8).

Hemocompatibility of GM-P@HA-P hydrogel

Fresh red blood cells (RBC) were obtained from rabbit, which was diluted by normal saline. Then 0.2 mL RBC suspension was mixed with 5 mL normal saline (negative control), distilled water (positive control) or GM-P@HA-P hydrogel preheated at 37 ºC for 30 min. The mixtures were incubated for 1 h at 37 ºC, followed by centrifugation (3000 rpm, 5 min). The supernatant was carefully removed and transferred to 96-well plate. The optical density (OD) was determined by spectroscopy at 545 nm. Three replicates were set for each sample. The hemolysis ratio was calculated by hemolysis (%) = [OD value (sample)-OD value (saline)]/[OD value (water)-OD value (saline)] × 100%.

The migration of NIH/3T3 in GM-P@HA-P hydrogel

NIH/3T3 cells with a density 1×10^6 cells /mL were seeded on the 6-well plate. After culturing for 24 h, cell layer was formed on plate. With the guidance of a ruler, the tip of a plastic pipette was used to gently scratch the confluent cells layer to create a scarification. After cultured for predetermined time, the cell migration was observed via optical microscope, and the migration ratio was calculated. Cells cultured in culture plate without hydrogel were used as control.

The tubule formation assay of HUVECS

GM-P@HA-P hydrogel was added into plate and incubated for 30 min at 37 ºC, and HUVECs (PCS-100-013, ATCC) were seeded on the hydrogel-coated plate, and incubated for 24 h. To assess the tube formation, HUVECs were stained with Calcein-AM, which were observed by microscope. The tube formation density was quantified using Image J software.

Macrophage activation by GM-P@HA-P hydrogel

RAW264.7 cells (SC-6003, ATCC) were seeded in 6-well plate at the density of 1×10^5 cells per well, cultured for 24 h. Then GM-P@HA-P hydrogel was added and incubated for 48 h. Cells cultured in medium supplementing with 30 ng/mL IL-4 were used as positive control. After incubation for 48 h, cells were collected by centrifugation and washed three times with PBS. RAW264.7 cells were stained with FITC-labeled anti-CD86 antibodies, PE-labeled

F4/80 antibodies, and APC-labeled CD206 according to the manufacture's guidelines, which was analyzed by flow cytometry (C6, BD, USA).

To observe the cell morphology, RAW264.7 cells were seeded in confocal dishes, treated by different samples for 48 h, then stained with FITC-conjugated phalloidin and 4', 6-diamidino-2-phenylindole (DAPI) to label the F-actin and cell nuclear, respectively. CD206 was used to identify M2 type macrophages. Confocal laser scanning microscopy (CLSM) images were obtained by CLSM (TCS SP511, Leica, Ernst-Leitz-Strasse, Germany).

Reverse transcription-polymerase chain reaction: RAW264.7 cells were treated with GM-P@HA-P hydrogel for 48 h. Then the treated cells were washed with PBS and total RNA was extracted with HR Total RNA kit (OMEGA, R6812-02). The extracted RNA was quantified using a Nano Dro spectra-photometer (Nanodrop2000, Thermo Fisher Scientific, USA). Then reverse transcription of total RNA into cDNA was carried out using GoScript Reverse Transcription System (Promega, A5001). For real-time polymerase chain reaction (PCR) analysis, 50 ng RNA was amplified according to a pre-designed procedure in an IQ5 detection system (Bio-Rad, USA) using SYBR Green Master Mix (Promega, A6001) and 10 μM gene-specific primers. All mRNA expression levels were normalized to the *β*-actin reference gene. The forward a reverse primer sequences were listed as follow: STAT6 (forward: 5'-TCTCCACGCTTCACATTG-3', reverse: 5'-GACCACCAAGGGCAGAGAC-3').

Western blot: RIPA lysis buffer (Beyotime, P1113B) contain 1mM PMSF (Beyotime, ST506) was applied to obtain total cell lysates of RAW264.7 cells treated with GM-P@HA-P hydrogel. After incubation for 5 minutes on ice, the supernatant was collected after 14000 rpm centrifugation. The concentration of protein was quantified by a BCA protein assay kit (Beyotime P0012S). Then the protein was denatured on 95 ^{o}C for 10 min and mixed with loading buffer. Then protein samples were separated by SDS electrophoresis, and transferred onto polyvinylidene difluoride membrane (PVDF, 0.22 μm, Millipore, USA). The PVDF membrane was blocked with 5% nonfat milk for 2 h at room temperature, which co-cultured with primary antibodies (JAK1, ERK, *β*-actin) overnight at 4 ^{o}C. Then the membrane was washed with TBST three times, and incubated with secondary antibodies at room temperature for 2 h. Finally, the blots on the membranes were visualized and captured by the chemiluminescence detection system (ChemiScope 6000 Pro, China). The signal intensity was quantified by Image J software.

Hydrogel degradation in vivo

The degradation of GM-P@HA-P hydrogel was investigated by non-invasive fluorescence imaging. The RB-labeled GM-P@HA-P hydrogel (0.2 mL) was subcutaneously injected into the back of Balb/c mice (female, 8 weeks,

n = 3, Charles River). Fluorescence images were captured, and the fluorescence intensity was quantified at predetermined time points using the fluorescence imaging system (Kodak In-Vivo FX Pro, CT, USA).

In vivo healing of MRSA-infected chronic wounds

Full-thickness defective skin wounds in diabetic SD rats or scald skin wounds in normal SD rats (female, 8 weeks, Charles River) with a round shape and a diameter of 1 cm (diabetic SD rats) or 2 cm (scald skin wounds) were produced on the back. Then, the suspension of MRSA (100 μL, ~10^7 CFU/mL) was inoculated at the wound sites. To confirm the success of infection, bacterial samples were collected from the wound by sterilized cotton swabs at 2 h post-infection or 21 days post-management. Swabs were placed in 1 mL of normal saline solution. Loads of live MRSA in diluted suspensions were investigated by typical streaking plating approach after culturing MRSA on LB agar plates for 24 h at 37 ºC.

SD rats were randomly divided into three groups (n = 3) and treated with different hydrogels. The wound sties were covered with 3M Tegaderm Film. At predetermined time, wound sites were photographed and measured to calculate the wound closure rate. Hematoxylin % eosin (H&E) and Masson's trichrome staining were used to evaluate the wound healing process Skin samples were fixed in 10% formaldehyde solution, dehydration, and embedded in paraffin. Skin tissues were cut into sections of 5 μm thickness by microtome, which were stained with H&E or Masson's trichrome staining. H&E staining protocol included sequential deparaffinization, alcohol pass, 0.5% hematoxylin staining for 10 min, 0.5% hydrochloric alcohol washing and 0.5% eosin staining for 10 min. Masson's trichrome staining was implemented according to a series of steps including deparaffinization, alcohol pass, and staining by masson blue, reichun/magenta and aniline blue. Slices were observed by optical microscope.

For immunofluorescence staining of CD86, CD206, CD31, α-SMA, and CK14 at the wound sites, tissue sections were dewaxed, dehydrated, and hydrothermal antigen was repaired. Then, the enzyme was inactivated by 3% H_2O_2/methanol solution, and the antigen was recovered with citrate buffer (pH=6.0). Subsequently, 100 μL of goat serum was added dropwise to the slices, which were incubated at room temperature for 20 min. Next, 100 μL primary antibody (diluted 1:100, Abcam) was added dropwise, and the tissue slice was incubated at 37 ºC for 3 h in a wet box. Afterwards, tissue slices were washed by PBS (pH=7.4) for three times with for 5 minutes each. Then incubated with Alexa Flour 488 goat anti-rabbit IgG and Alexa Flour 594 goat anti-mouse IgG (1:500, Invitrogen) corresponding to the primary antibody. Cell nuclei were stained with DAPI, following by washing with PBS (pH=7.4). The images were obtained by fluorescence microscope and quantitatively analyzed by Image J software.

Figures S1 to S21

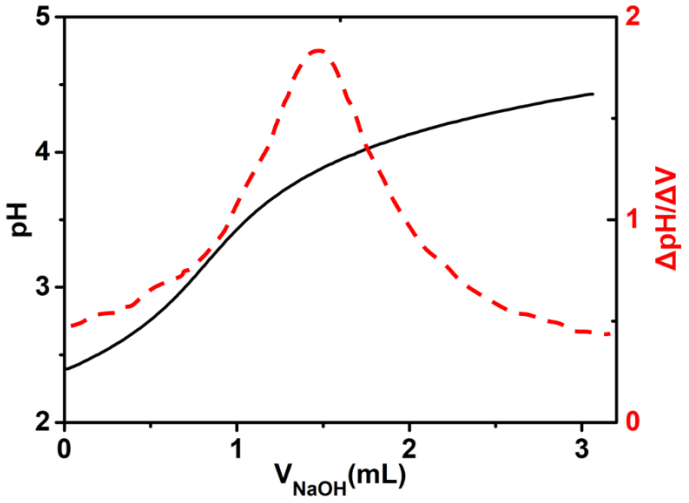

Figure S1. **Colorimetric hydroxylamine titration analysis of GM oxidation.** Black line: GM-CHO titration with hydroxyl-amine. Red dash line: the first derivative of the titration was utilized to determine the equivalence point.

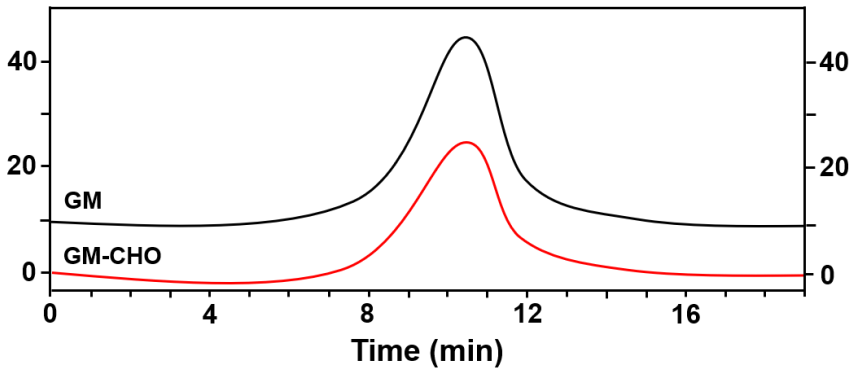

Figure S2. **Gel permeation chromatography profiles of GM (black line) and GM-CHO (red line).**

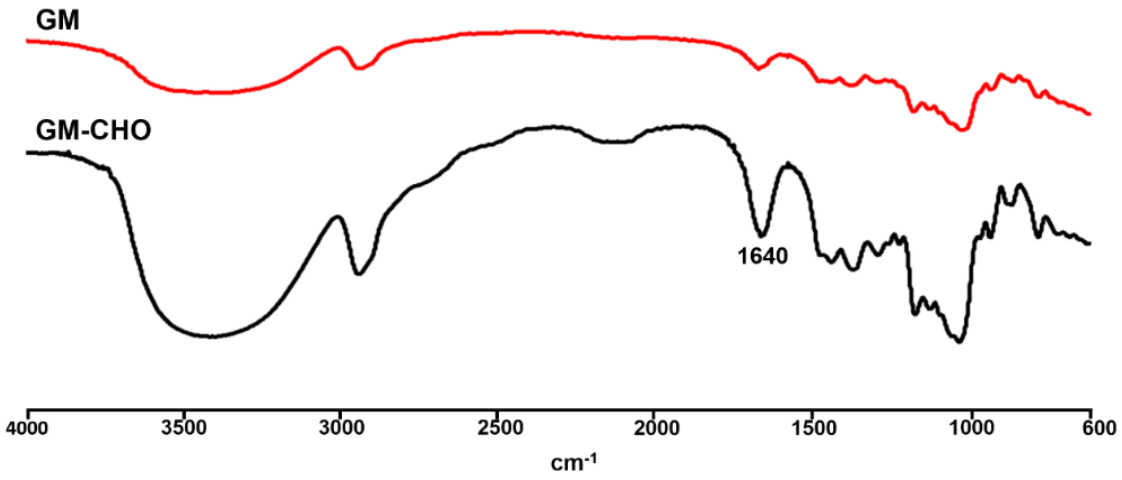

Figure S3. **The FI-TR spectrum of GM (red line) and GM-CHO (black line).**

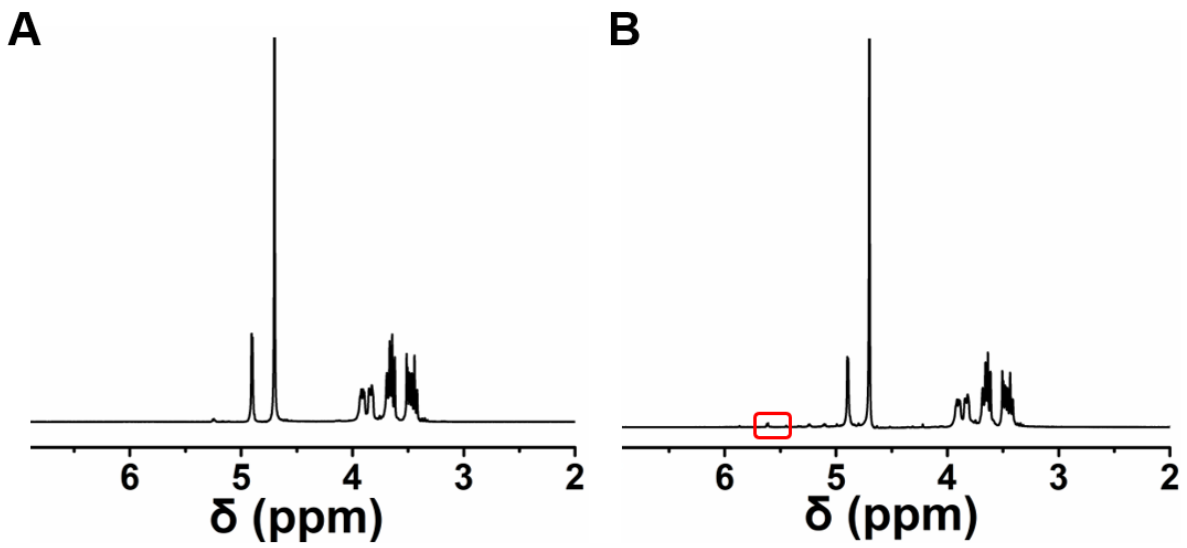

Figure S4. **^{1}H NMR of GM (A) and GM-AMP (B) polymer.** The red box represents the characteristic peak of hydrogen of benzene ring in the AMP.

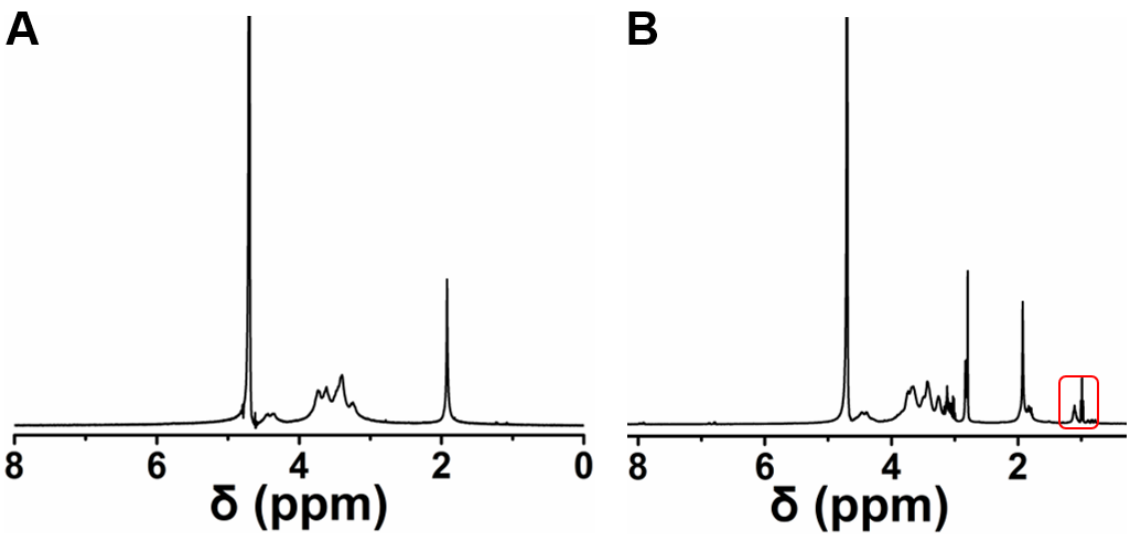

Figure S5. **^{1}H NMR of HA (A) and HA-P (B) polymer.** The red box represents the characteristic peak of hydrogen of methyl in the collagen peptide.

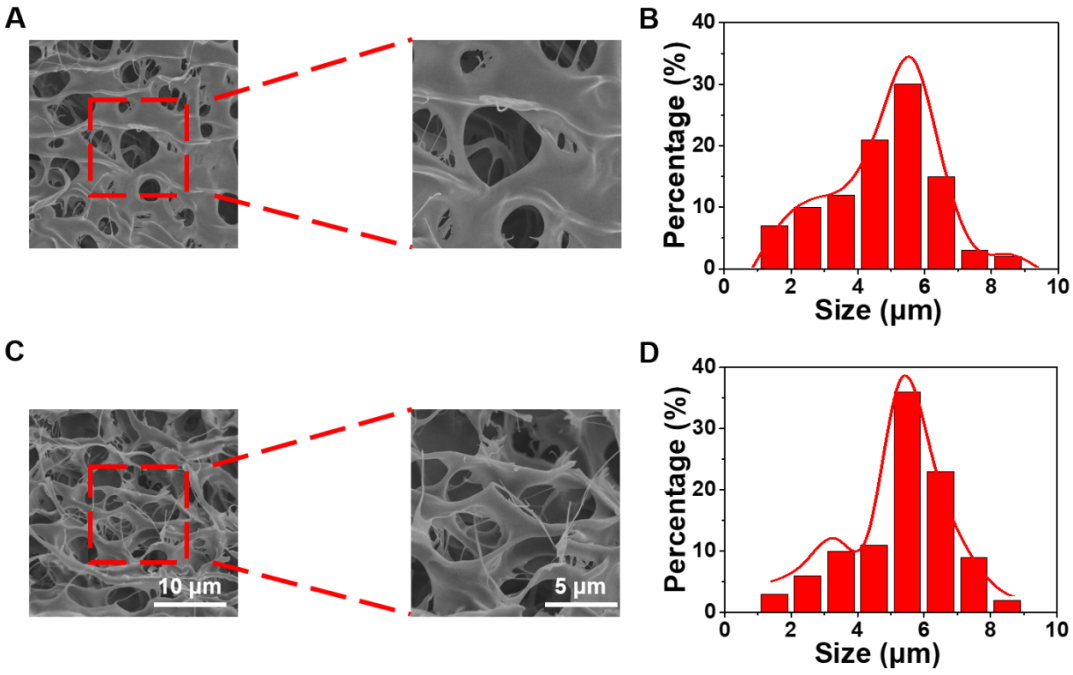

Figure S6. **Structure of the different mass ratio of GM-PHA-P hydrogel.** Representative SEM images and pore size distribution of lyophilized GM-P@HA-P hydrogel with a mass ratio of GM-P to HA-P at 1:2 (**A, B**) or 2:1 (**C, D**).

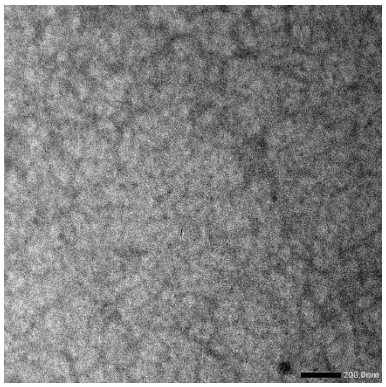

Figure S7. **TEM image of GM-P@HA-P hydrogel.** Scar bar, 200 nm.

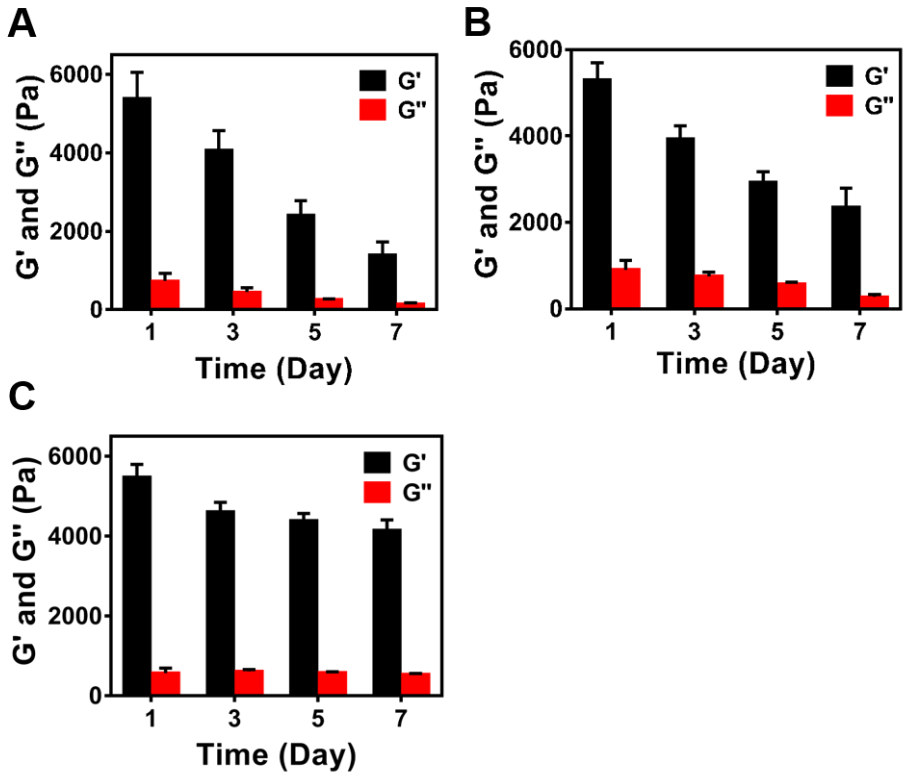

Figure S8. **Modulus variation of the GM-P@HA-P hydrogel in acidic condition (pH ≈ 6).** (**A**), MMP-9 (**B**) or alkaline condition (PBS, pH ≈ 8) (**C**) as a function of time.

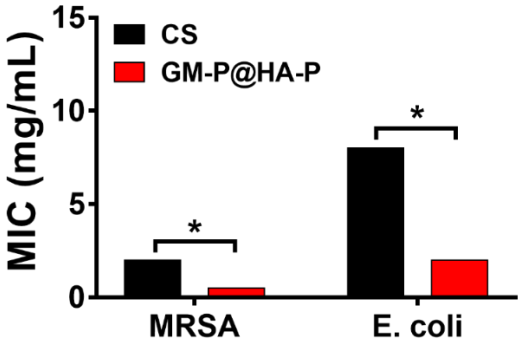

Figure S9. **Minimum inhibitory concentration (MIC) of CS, and GM-P@HA-P.** Statistical difference between indicated groups was analyzed by Student's t-test, * $P < 0.05$.

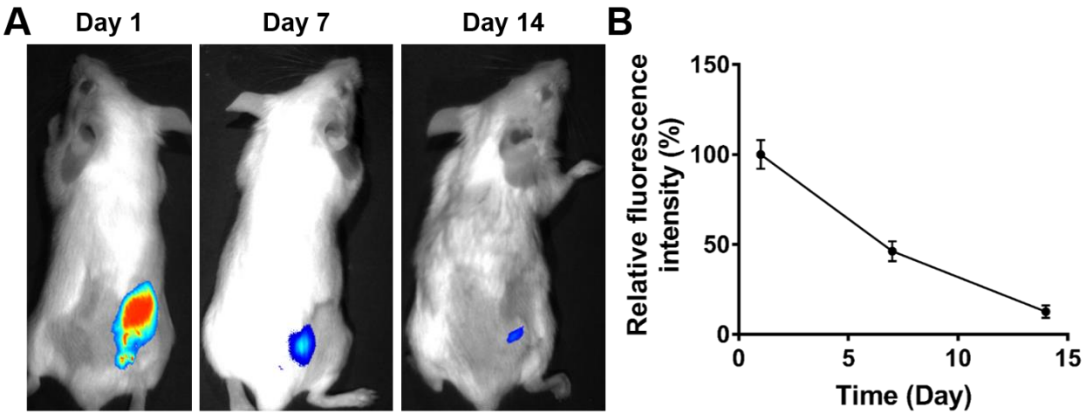

Figure S10. **In vivo GM-P@HA-P hydrogel degradation.** (**A**) Time dependent fluorescent images of mice received subcutaneous injection with rhodamine (RB) labeled GM-P@HA-P hydrogel. (**B**) The fluorescence intensity of RB at different time points after injection.

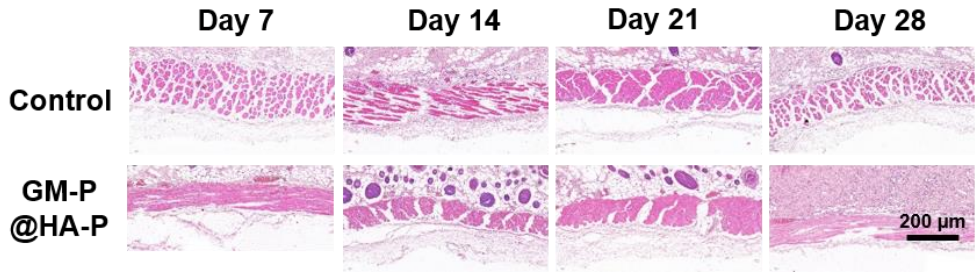

Figure S11. **H&E-stained sections of tissues from the subcutaneous location of hydrogel injection at different time.**

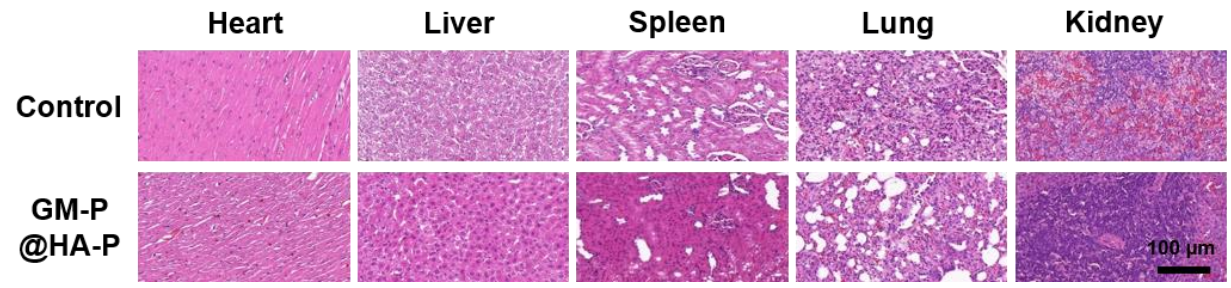

Figure S12. **H&E-stained tissue sections of major organs at 4 weeks post GM-P@HA-P hydrogel administration.**

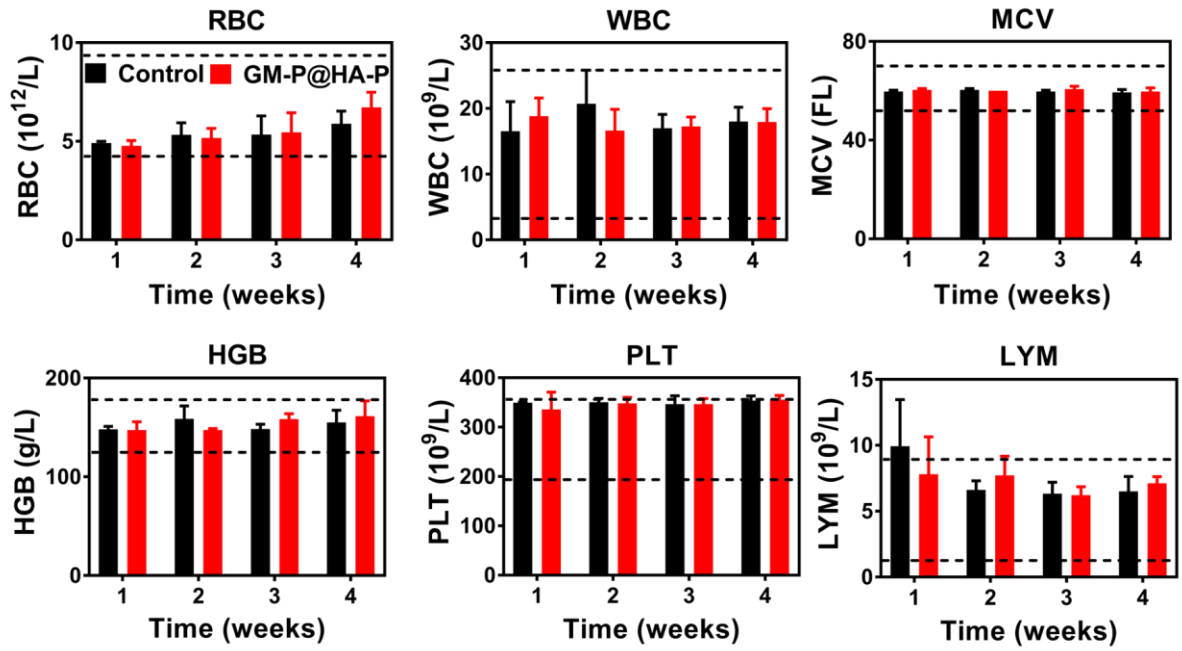

Figure S13. **Blood parameters at various time points after GM-P@HA-P hydrogel injection.** Data are shown as mean ± SDs (n=5). WBC, White Blood Cell; LYM, Lymphocyte; RBC, Red Blood Cell; HGB, Hemoglobin; MCV, Mean Corpuscular Volume; PLT, Platelets. Dotted lines represent the normal range of indicators.

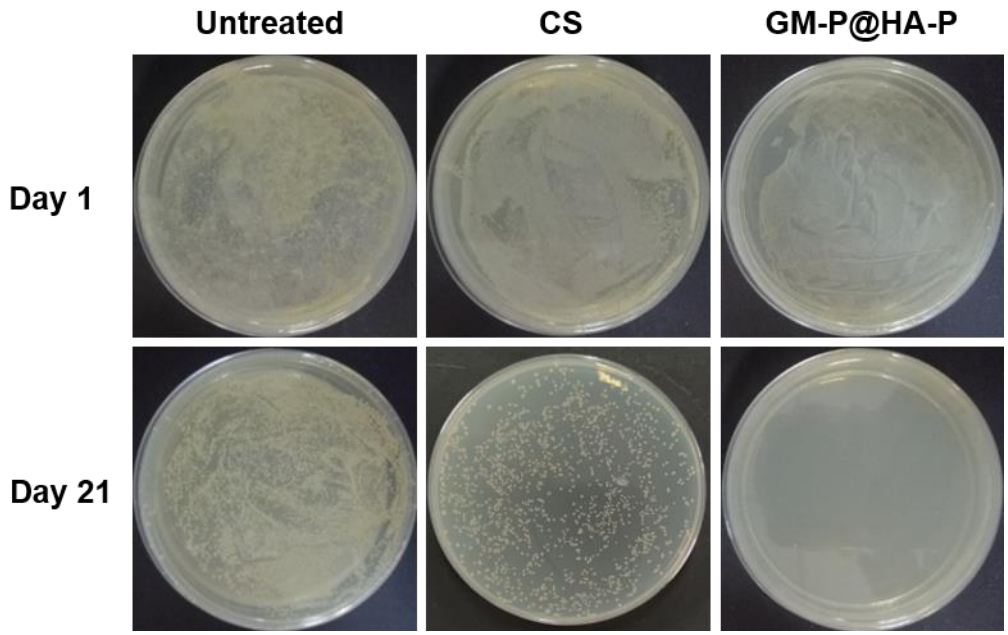

Figure S14. **Digital pictures of bacterial colony collected from MRSA-infected diabetic wounds before (day 1) and after (day 21) treatment.**

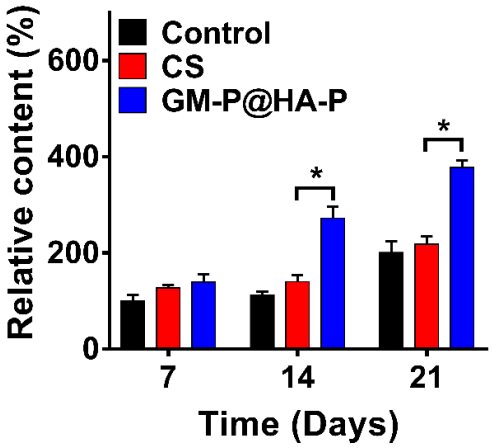

Figure S15. **Quantitative analysis of relative content of collagen at different times after treatment.** Data are shown as mean ± SDs (n=3), * $P < 0.05$, Student's t-test.

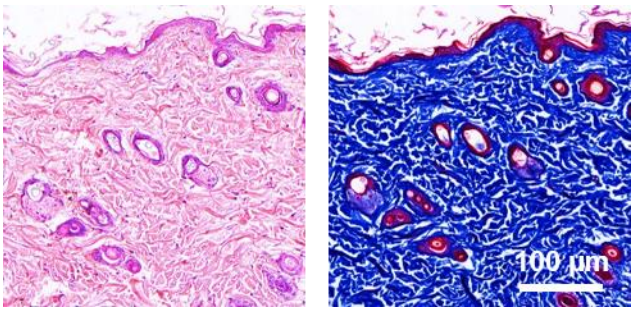

Figure S16. **H&E and Masson's trichrome staining of tissues sections of normal skins.**

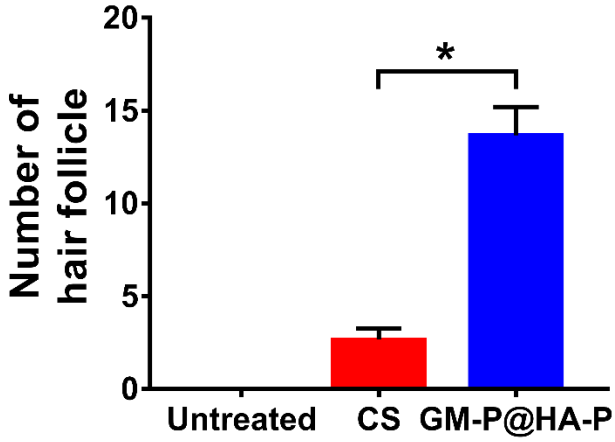

Figure S17. **Quantification of regenerated hair follicles on Day 21.** Data are shown as mean ± SDs (n=3), * $P <$ 0.05, Student's t-test.

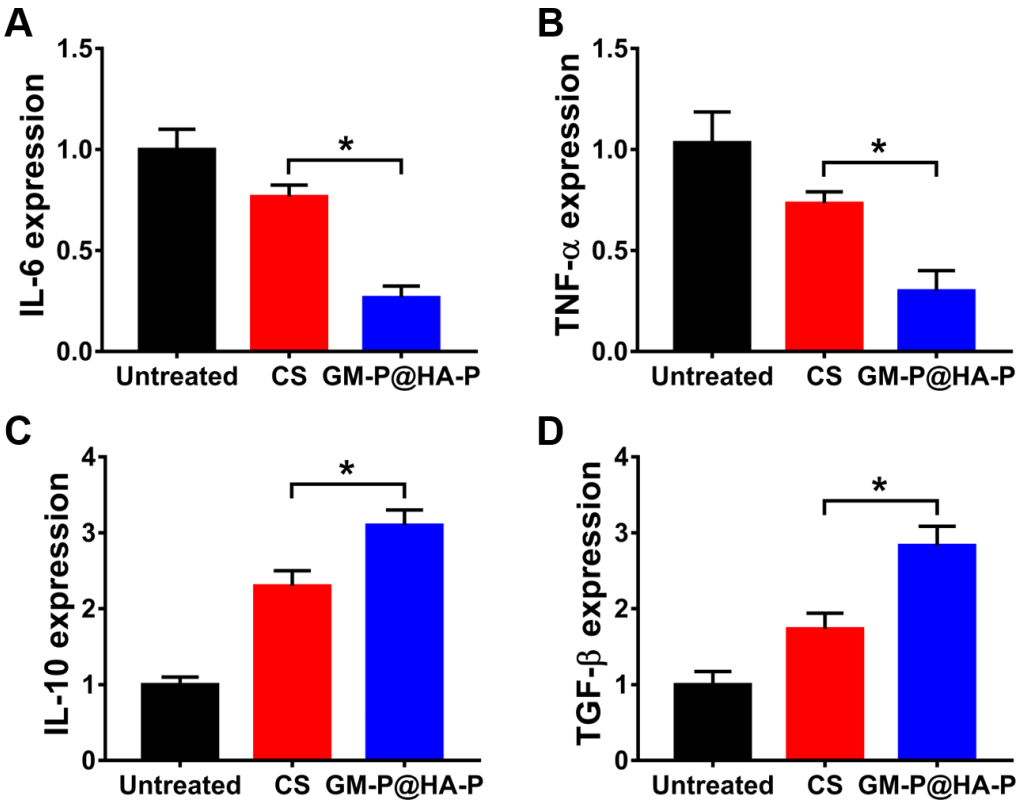

Figure S18. **Quantification of the relative content of IL-6 (A) and TNF-*α* (B), IL-10 (C), and TGF-*β* (D).** * $P <$ 0.05, Student's t test.

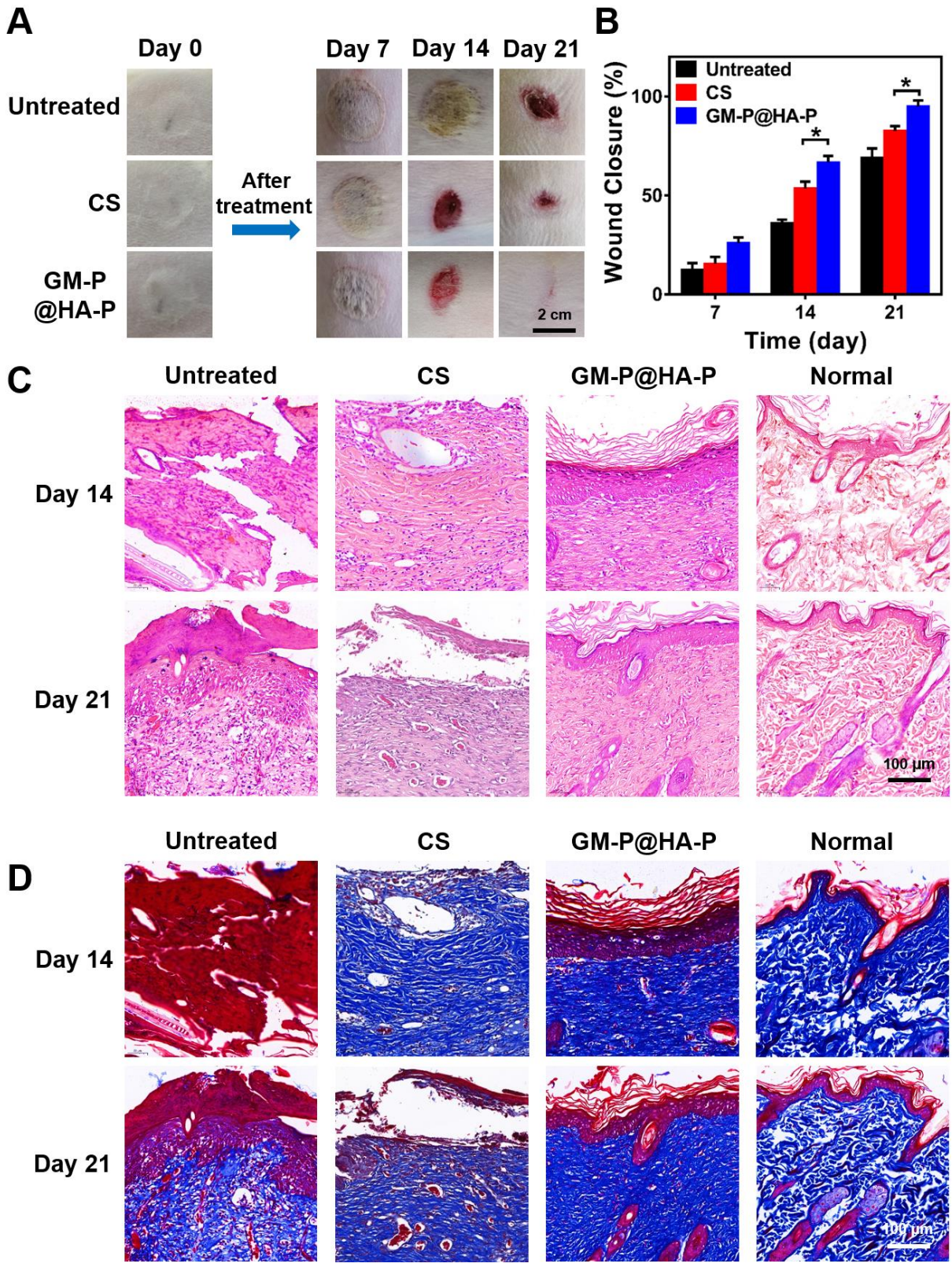

Figure S19. **GM-P@HA-P hydrogel promoted MRSA-infected scald wound healing in SD rats.** (**A**) Representative images of wounds during wound healing process. (**B**) Wound closure rate at different times after wound

care. Data are shown as mean ± SDs (n = 3), * P < 0.05. (**C**) H&E staining of tissues of skin collected from wound areas at day 14, 21. (**D**) Masson's trichrome staining of tissue sections of skin collected from wound areas at day 21. The label of "normal" represents undamaged skin. Scale bar, 100 μm.

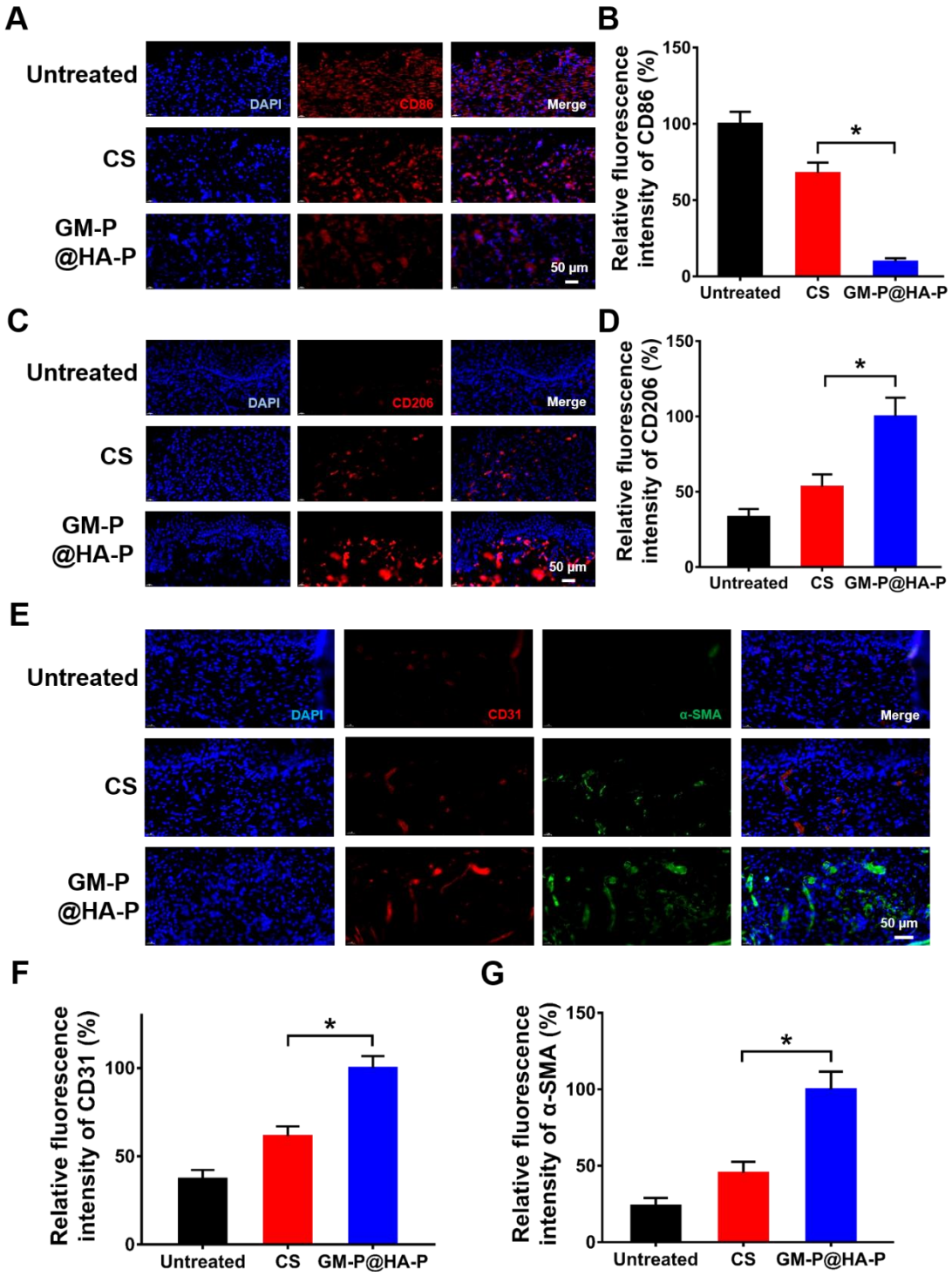

Figure S20. **GM-P@HA-P hydrogel induced macrophage polarization and improved angiogenesis during scald wound healing.** (**A**) Representative immunofluorescence images of regenerated scald wound tissues stained by CD86 immunostaining on day 21 post wound healing. Scale bar, 50 μm. (**B**) Quantification analysis of relative fluorescence intensity of CD86 immunostaining. (**C**) Representative immunofluorescence images of regenerated wound tissues stained by CD206 immunostaining on day 21 post wound healing. Scale bar, 50 μm. (**D**) Quantification analysis of relative fluorescence intensity of CD206 immunostaining on day 21 post wound healing. Scale bar, 50 μm. (**E**) Representative immunofluorescence images of regenerated wound skin tissues stained by CD31/α-SMA immunostaining on day 21 post wound healing. Scale bar, 50μm. (**F, G**) Statistical data of relative fluorescence intensity of CD31 (**F**) and α-SMA (**G**) immunostaining on day 21 post wound healing. * $P < 0.05$.

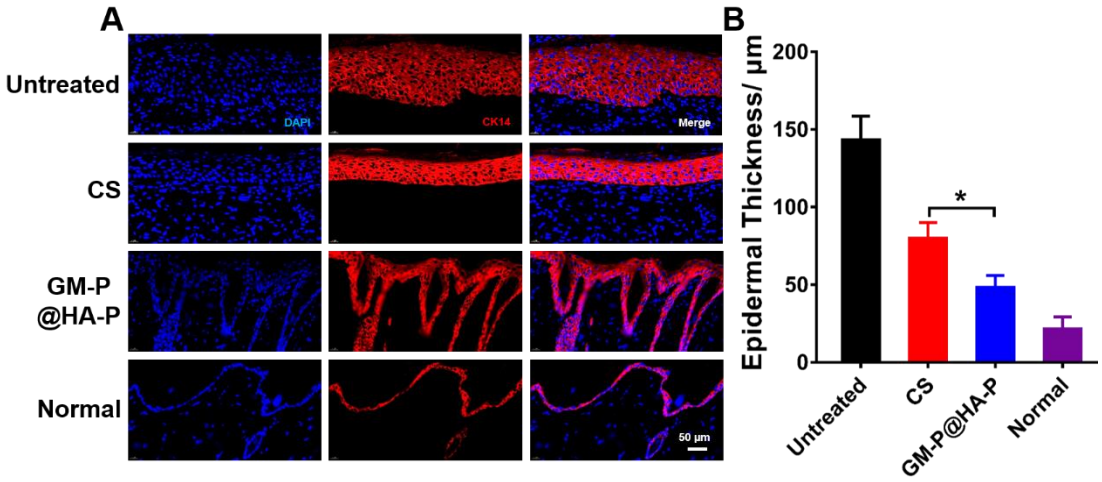

Figure S21. **GM-P@HA-P hydrogel promoted the epidermal regeneration.** (**A**) Images of immunohistochemistry staining for CK 14 of scald skin wound tissues. The cells stained with CK 14 in red, and the nuclei were counterstained with DAPI in blue. Scale bar, 50 μm. (**B**) The epidermal thickness measured and quantified by staining intensities of CK 14 in different groups, while the normal tissue acted as the positive control group. * $P < 0.05$.