Supplementary Materials

Charge Microenvironment and Bioactivity of In Situ-Formed PEG-RGD Dual Hydrogel Dressings Promote Wound Healing

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Materials and methods

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Materials and methods

1. Preparation of hydrogels

Materials

Peptide enantiomers $^{L}C^{L}G^{L}R^{L}G^{L}D^{L}G^{L}C$ and $^{D}C^{D}G^{D}R^{D}G^{D}D^{D}G^{D}C$ (L-pep and D-pep) was synthesized through solid-phase peptide synthesis. Briefly, the peptide was assembled on a resin by sequentially coupling amino acids, with Fmoc groups deprotected after each coupling step. The coupling reactions were facilitated using a coupling reagent, such as N,N′-Diisopropylcarbodiimide or 1-hydroxybenzotriazole. After the completion of peptide chain assembly, the peptide was cleaved from the resin using trifluoroacetic acid to ensure proper cleavage and protect the peptide from degradation. The crude peptide was purified using reverse-phase high-performance liquid chromatography, and analyzed using mass spectrometry.

The 4 arm-PEG-maleimide (4-PEG-Mal, 5000 Da) and the PEG grafted with thiol groups at both ends (PEG-SH, 1000 Da) were purchased from Shanghai Peng Sheng Biotechnology Co., Ltd.

Hydrogel preparation

Due to the facile Michael addition reaction between thiol groups (-SH) and maleimides at room temperature, L-pep, D-pep, and PEG-SH can form hydrogels with 4-PEG-Mal at molar ratios of 2:1, 2:1, and 1:2, respectively. The resulting hydrogels are abbreviated as L-RPH (4-PEG-Mal & L-pep), D-RPH (4-PEG-Mal & D-pep), and PH (4-PEG-Mal & PEG-SH). Among these, the mass fraction of 4-PEG-Mal is at least 20%.

For *in vitro*: L-pep, D-pep, PEG-SH, and 4-PEG-Mal were separately dissolved in

phosphate-buffered saline (PBS) and mixed at the appropriate ratios to prepare L-RPH, D-RPH and PH solutions. The ungelled mixture was then added to 96-well plates (20 μL), 24-well plates (120 μL), and 6-well plates (600 μL). After standing for 20 minutes, a thin gel layer formed at the bottom of the wells. Sterile PBS was added to the wells, and sterilization was performed by UV irradiation.

For *in vivo*: L-pep, D-pep, PEG-SH, and 4-PEG-Mal were separately dissolved in sterile PBS. The L-pep/D-pep/PEG-SH and 4-PEG-Mal solutions were sequentially sprayed onto the wound site of the animal model. After standing for 10 minutes, a gel was formed at the wound site and tightly adhered to the tissue.

2. Characterization of hydrogels

^{1}H NMR measurement

^{1}H NMR spectra were recorded on a 400 MHz spectrometer (Bruker, Avance III) at room temperature. L-pep and D-pep were prepared by dissolving 10 mg of the compound in 0.6 mL of deuterated solvent (D_2O) and analyzed using a 5 mm NMR tube. Data were analyzed using Mestrenova software.

Fourier transform infrared spectroscopy (FT-IR) measurement

FT-IR spectra of the samples were recorded using an infrared spectrometer (Nicolet, NEXUS 670). Approximately 20 mg of each sample (dried L-pep, D-pep, PEG-SH, and the corresponding L-RPH, D-RPH, and PH) were finely ground and mixed with potassium bromide (KBr) to form a uniform pellet. The spectra were obtained in the range of 4000-400 cm^{-1}.

Scanning electron microscopy (SEM) imaging

SEM images were acquired using a field emission scanning electron microscope (Hitachi, S-4800). The dried hydrogels were cryo-fractured using liquid nitrogen to obtain cross-sections. Samples were coated with 5–10 nm Au-Pd for enhanced conductivity. Imaging was conducted at an accelerating voltage of 5 kV under high vacuum conditions ($\leq 10^{-3}$ Pa).

Atomic force microscopy (AFM) imaging

AFM measurements were performed using a Scanning electron microscope (Bruker, Multimode 8). Hydrogel solutions were dropped onto a conductive glass, and then dried at room temperature. Data were analyzed using NanoScope Analysis software.

UV-vis spectroscopy and Circular dichroism (CD) spectroscopy measurement

The UV-vis absorption spectra of the L-pep, D-pep, L-RPH and D-RPH samples within the wavelength range of 190 - 400 nm was recorded using a spectrophotometer (Shimadzu, UV-3600) with a step size of 1 nm. The CD spectra within the wavelength range of 190 - 400 nm was obtained using a circular dichroism (Jasco, J-1500) with a step size of 1 nm.

Rheological measurements

Rheological properties of the hydrogels were measured using a rheometer (Anton Paar, MCR302). The three groups of hydrogels (L-RPH, D-RPH, and PH) were prepared into cylindrical shapes with a diameter of 10 mm and a height of 10 mm, and their mechanical properties were tested using a frequency sweep mode with the frequency range of 0.1 Hz to 10 Hz.

Adhesion and tensile strength measurement

Fresh porcine skin was cleaned and fixed on a flat surface. A droplet of ink was added to the hydrogel solution. The hydrogels were then subjected to a range of mechanical deformations, including stretching, twisting, bending, tilting, inverting and water flushing of the skin. The hydrogels' adhesion and tensile strength measurement were recorded using camera.

Porosity measurement

The porosity of hydrogels was evaluated by the liquid displacement method. A certain mass of dry hydrogel was immersed in a certain volume of ultrapure water (V1) for 1 hour. The total volume of the liquid and the hydrogel sample was measured and recorded as V2. Carefully remove the hydrogel again and record the volume of the remaining ultrapure water as V3. The calculation formula for porosity (%) is: porosity = (V1 - V3) / (V2 - V3) × 100%.

Swelling Measurement

The swelling ratio of hydrogels was determined by the gravimetric method. Dried hydrogels were weighed (Wd) and then immersed in PBS, pH 7.4 at 37 °C. At predetermined time points, samples were removed, gently blotted to remove surface water, and weighed (Ws). The swelling ratio was calculated using the following formula: Swelling ratio= (Ws − Wd) / Wd.

Degradation research

The swelling-equilibrium freeze-dried hydrogel (W0) was incubated in PBS, pH 7.4 at 37 ° C. Samples were taken out each day, gently washed with deionized water, freeze-dried and weighed (Wt), and the degradation rate was calculated as follows:

Degradation rate (%)= (W0 - Wt) / W0 × 100%.

3. Cell culture

Mouse macrophages (RAW 264.7) and mouse fibroblasts (NIH 3T3) were cultured in Dulbecco modified eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Gibco). Cells were maintained at 37˚C in a humidified incubator containing 5% CO_2. The culture medium was replaced every 48 hours, and cells were passaged at 80-90% confluency.

All the following cells of cell experiment were seeded in cell culture plates coated with the L-RPH, D-RPH and PH.

4. In vitro experiments

Cell viability

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol (Biosharp). Briefly, cells were seeded at a density of 2 × 10^4 cells per well in a 96-well plate and incubated for 1, 3 and 5 days. Then 10 μL of CCK-8 reagent was added to each well, and the plate was incubated at 37˚C for 2 hours. Absorbance was measured at 450 nm using a microplate reader (BioTek, Synergy H1).

Live/dead cell staining

Cell viability was assessed using the fluorescein diacetate (FDA) and propidium iodide (PI) staining method. Briefly, cells were seeded at a density of 2×10^4 cells per well in 24-well, and incubated for 1, 3 and 5 days. Before staining, the cells were washed with PBS, then 1 ml of prepared FDA/PI staining solution (5 μg/ml FDA, 5 μg/ml PI) was

added to each well, and the plates were incubated at room temperature in the dark for 20 minutes. The living cells were stained green and the dead cells were stained red. Then the staining solution was removed, and the wells were gently washed twice with PBS. Cells were imaged under an inverted fluorescence microscope (Leica, DMi8).

Cell proliferation

Cell proliferation was evaluated using the EdU Cell Proliferation Assay Kit (UElandy). Briefly, cells were seeded at a density of 2×10^4 cells per well in 24-well plates and incubated for 2 days. Subsequently, 10 μM EdU working solution prepared in complete medium was added to the plate. After incubating for 2 hours, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. EdU-labeled cells were stained according to the manufacturer's instructions, and Hoechst was utilized for counterstaining to display the cell nuclei. Fluorescence images were captured using a fluorescence microscope (Leica, DMi8), and the percentage of proliferating cells was determined by computing the ratio of EdU-positive cells (red cells) to the total number of cells (blue cells).

Apoptosis

Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (Biosharp). Briefly, cells were seeded at a density of 5×10^5 cells per well in 6-well plates and incubated for 2 days. Then, cells were harvested and washed with cold PBS. The cells were incubated with Annexin V-FITC and PI for 20 minutes at room temperature in the dark. Annexin V binds to phosphatidylserine, which is externalized on the surface of early apoptotic cells, while PI stains the DNA of late apoptotic and

necrotic cells. The cells were analyzed by flow cytometry (BD Biosciences, Accrui). Early apoptotic cells were identified as Annexin V-positive/PI-negative, while late apoptotic were identified as Annexin V-positive/PI-positive.

Immunofluorescence assay

Cells were seeded at a density of 2×10^4 cells per well in 24-well plates and incubated for 2 days. Then the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. Following fixation, cells were permeabilized using 0.5% Triton X-100 in PBS for 10 minutes, then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to reduce nonspecific binding. Subsequently, the cells were incubated with primary antibody overnight at 4˚C. After washing with PBS, the secondary antibody conjugated to a fluorophore (Alexa Fluor 488) was applied for 1 hour at room temperature in the dark. Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Fluorescent images were captured using a laser confocal microscope (Zeiss, LSM880). Data were analyzed through ZEN software.

The primary antibodies used in the experiment were as follows: rabbit anti-MRC1 (SAB, 1:200), rabbit anti-iNOS (SAB, 1:200), rabbit anti-FAK (SAB, 1:200), rabbit anti-Vinculin (SAB,1:200), rabbit anti-ILK (SAB, 1:200). The secondary antibody was Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) (1:50).

Flow cytometry

To explore the polarization of macrophages, RAW 264.7 was seeded at a density of 5×10^5 cells per well in 6-well plates and incubated for 2 days. RAW cells treated with

100 ng/ml LPS and 20 ng/ml IL-4 were used as standard samples for M1 and M2 macrophages, respectively. Cells grown in the blank wells served as the negative control. Cells were then harvested and washed with PBS. To assess polarization, the cells were stained with AF488 conjugated anti-CD86 (SAB, 1:200) and APC-cy7 conjugated anti-MRC1 (SAB, 1:200) and were incubated with the antibodies for 30 minutes at 4˚C in the dark. After incubation, cells were washed with PBS and analyzed by flow cytometry (BD Biosciences, Accrui). The proportion of M1 macrophages was determined by CD86 expression, and the proportion of M2 macrophages was determined by MRC1 expression.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was collected after cultured for 2 days by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The purity of total RNA was determined by spectrophotometry using NanoDrop 2000 spectrophotometer (Thermo Fisher). cDNA was prepared from samples with purity reaching the required level (TaKaRa, Perfect Real Time) using the Primer Script RT kit. Quantitative PCR was performed using SYBR Mix (Yeasen) according to the manufacturer's instructions. Data were reported as the fold change of each experimental group relative to the control group, normalized to GAPDH. The primers used for qRT-PCR are shown in the following table.

The primer sequence was shown in the following table:

Gene name	Primers upstream and downstream	Primer sequence(5'-3')
GADPH	Primer-Forward	CCTCGTCCCGTAGACAAAATG

	Primer-Reverse	TGAGGTCAATGAAGGGGTCGT
iNOS	Primer-Forward	CTGTCGCAGCTCCCTATCTT
	Primer-Reverse	TCAGGTTCCTGATCCAAGTGC
CD206	Primer-Forward	GGAGTGGCAGGTGGCTTATG
	Primer-Reverse	CACTGCTCGTAATCAGCCTCC
COL I	Primer-Forward	GAGAGGTGAACAAGGTCCCG
	Primer-Reverse	AAACCTCTCTCGCCTCTTGC
COL III	Primer-Forward	GTGGCAATGTAAAGAAGTCTCTGAAG
	Primer-Reverse	GGGTGCGATATCTATGATGGGTAG
α-SMA	Primer-Forward	GTACCACCATGTACCCAGGC
	Primer-Reverse	GAAGGTAGACAGCGAAGCCA
TGF-β1	Primer-Forward	ACTGGAGTTGTACGGCAGTG
	Primer-Reverse	GGCTGATCCCGTTGATTTCC

RNA-sequencing

NIH 3T3 cells were seeded in 6-well plates and cultured to 80% confluency. Total RNA was extracted using Trizol reagent (Invitrogen) and assessed for concentration and quality using NanoDrop and Agilent 2100 Bioanalyzer (Agilent). PolyA-tailed mRNA was enriched with Oligo beads and fragmented to ~300 bp. cDNA synthesis was performed using random primers and reverse transcriptase. After library construction and PCR amplification, the library size was ~450 bp. The library was sequenced using Illumina's Next-Generation Sequencing with paired-end sequencing. Gene expression was quantified using HTSeq, and FPKM was used for normalization. Differential expression was analyzed using DESeq2 with $|\log2FoldChange| \geq 1$ and P-value < 0.05. GO analysis was conducted with topGO, and results were shown in GO enrichment bar charts.

5. In vivo experiments

Animal surgery

All animal housing and experimental operations were approved by Experimental Animal Research Ethics Committee of Sichuan University (Approval No. KS20240250). Animal experiments were performed according to guidelines determined by the Sichuan University institutional committees for animal welfare. 45 male C57BL/6 mice (18-22g) were housed under a 12-hour light/dark cycle, receiving food and water at regular intervals.

Forty-five mice were randomly divided into three groups: (1) PH group (n=15); (2) L-RPH group (n=15); (3) D-RPH group (n=15). After anesthetizing the mice with a respiratory anesthesia machine, the fur on the dorsal skin was removed using a razor and depilatory cream. After disinfecting the skin with iodine, two symmetrical circular wounds were created on both sides of the dorsal skin using a sterilized 6 mm punch biopsy tool. Finally, the left circular wound of each mouse was used as the experimental side and the corresponding hydrogel dressings were applied respectively. The right circular wound was used as the control side and not treated. Each mouse was housed in a single cage.

Animal sacrifice and tissue processing

All animal experiments were performed in accordance with the ethical guidelines approved by the Institutional Animal Care and Use Committee (IACUC). All mice were euthanized by exposure to an overdose of carbon dioxide, on the 3rd, 7th, and 14th day after surgery, the mice were sacrificed and a whole square tissue including the wound

tissue was excised from the skin wound area at a distance of 5 mm from the wound edge.

For histological analysis, the tissues were immediately fixed in 4% paraformaldehyde for 24 hours at room temperature. Following fixation, the tissues were dehydrated in a series of graded ethanol solutions (70%, 80%, 95%, and 100%), cleared in xylene, and embedded in paraffin. Slides of 5 μm thickness were then cut and mounted on glass slides for subsequent staining and analysis.

Nerve recovery evaluation

On the 28th day postoperatively, the recovery effect of the dorsal skin nerves of the mice was measured.

The thermal pain threshold test: First, the grown dorsal hair was removed, and the wound point was marked. Then, the thermal pain meter was used to irradiate the experimental side, the control side, and the normal skin area of the mouse's dorsal wound. After the mouse made a pain moan, the infrared irradiation of the thermal pain meter was turned off, and the reaction time was recorded.

Chemical stimulation test: 0.01% capsaicin solution (7% v/v Tween 80) was prepared using saline. 6 μL of capsaicin solution was drawn with a microsyringe and subcutaneously injected into each mouse. The number of scratches made by the mice during the 30-minute period was recorded.

Tensile strength measurement of healed skin

The mechanical strength of healed skin was evaluated at day 10 by uniaxial tensile testing. Skin samples were carefully harvested along the scar axis using a scalpel. The

length, width, and thickness of each specimen were measured with a digital caliper. Both ends of the sample were clamped by a mechanical testing machine, and the specimen was stretched at a constant rate of 20 mm/min until failure. The force–displacement curve was recorded, and the ultimate tensile strength was calculated.

Hematoxylin and eosin (H&E) staining

The regenerative skin tissue slides were stained with Hematoxylin for 10 minutes, followed by rinsing in running tap water. The slides were then differentiated in 1% acid alcohol (1% hydrochloric acid in 70% ethanol) for 30 seconds and rinsed again in tap water. After differentiation, the slides were stained with Eosin for 2 minutes, then dehydrated through graded ethanol concentrations (70%, 95%, and 100%) and cleared in xylene. Finally, the stained slides were mounted for microscopic observation (Wisleap, WS-10).

Masson staining

The regenerative skin tissue slides were first stained with hematoxylin solution for 10 minutes, followed by rinsing in distilled water. The slides were then stained with Biebrich scarlet-acid fuchsin solution for 15 minutes and washed in distilled water. Differentiation was performed in phosphomolybdic acid for 10 minutes, after which the tissue was stained with aniline blue for 15 minutes to highlight collagen fibers. Following a rinse in distilled water, the slides were dehydrated through ascending ethanol concentrations (70%, 95%, and 100%) and cleared in xylene. Finally, the slides were mounted for microscopic observation (Wisleap, WS-10).

Immunofluorescence (IF) staining

The regenerative skin tissue slides were deparaffinized and rinsed under running water, followed by washing with double-distilled water. Antigen retrieval was then performed using pH 9 EDTA buffer at 98°C for 30 minutes. Goat serum was applied to the tissues and incubated for 20 minutes, after which it was discarded. Next, 100 μl of primary antibody was added to the tissues and incubated at 37°C for 1 hour. After washing with PBS, 100 μl of secondary antibody mixture was applied and incubated in the dark at 37°C for 45 minutes. Following incubation, the slides were washed with PBS three times, each for 2 minutes. The excess liquid around the tissues was discarded and the slides were dried, then 100 μl of prepared DAPI working solution was added. After 5 minutes, the slides were washed with double-distilled water three times, each for 2 minutes, and sealed with an anti-fading agent. The tissue slides were imaged using a slide scanner (Olympus, VS200).

The primary antibodies used in the experiment were as follows: rabbit anti-iNOS (Abcam, 1:500), mouse anti-CD68 (Invitrogen, 1:100), rabbit anti-CD206 (CST, 1:200), rabbit anti-TGF-β1 (Abcam,1:200), mouse anti-α-SMA (Abcam, 1:200). The secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG / Alexa Fluor 647 goat anti-mouse IgG (1:400).

6. Statistical analysis

Data were analyzed using GraphPad Prism 9.5 software. Comparisons between two groups were conducted using unpaired Student's t-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used. A p-value of < 0.05 was considered statistically significant.

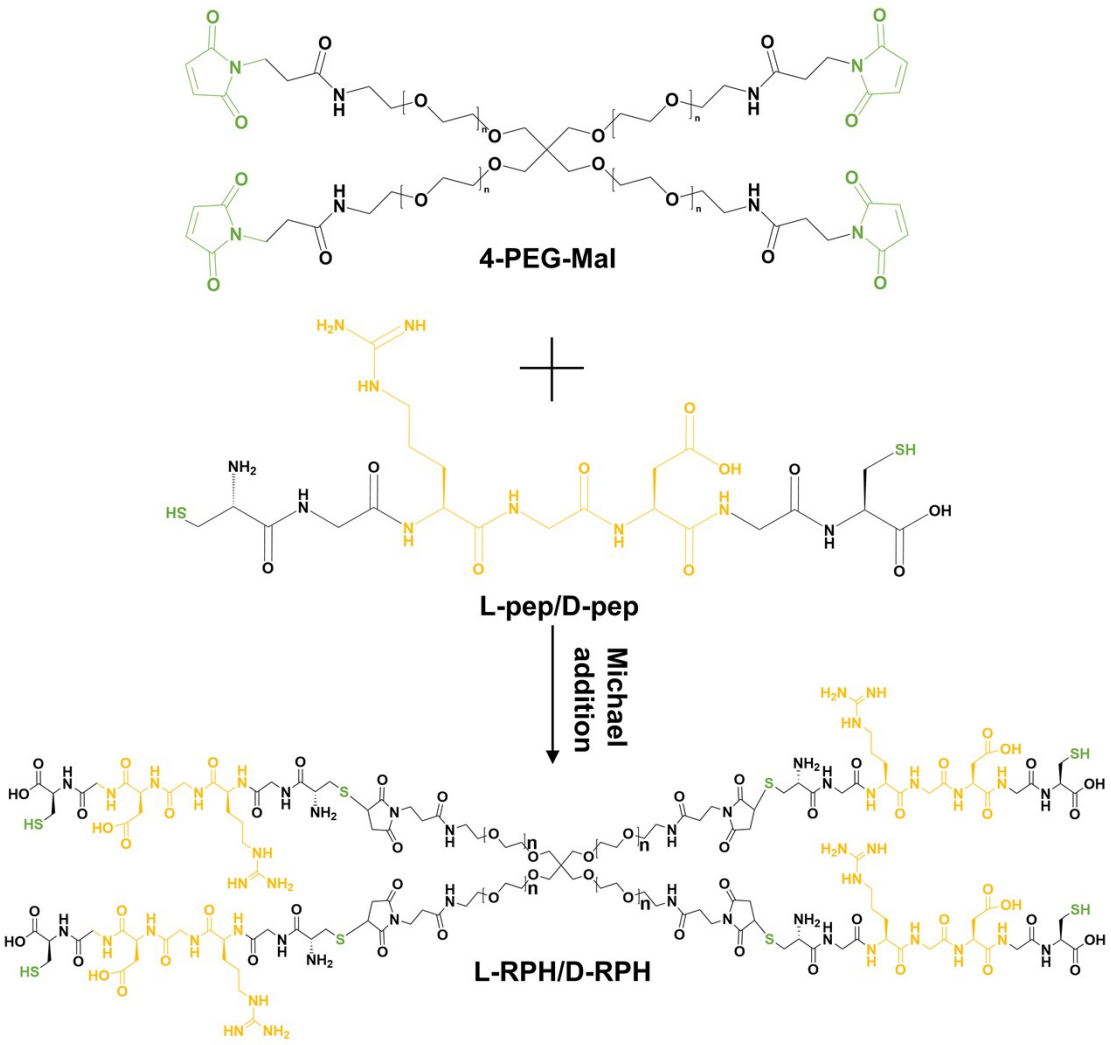

Figure S1. Schematic illustration of the reaction between peptides and 4-PEG-Mal forming a hydrogel via Michael addition.

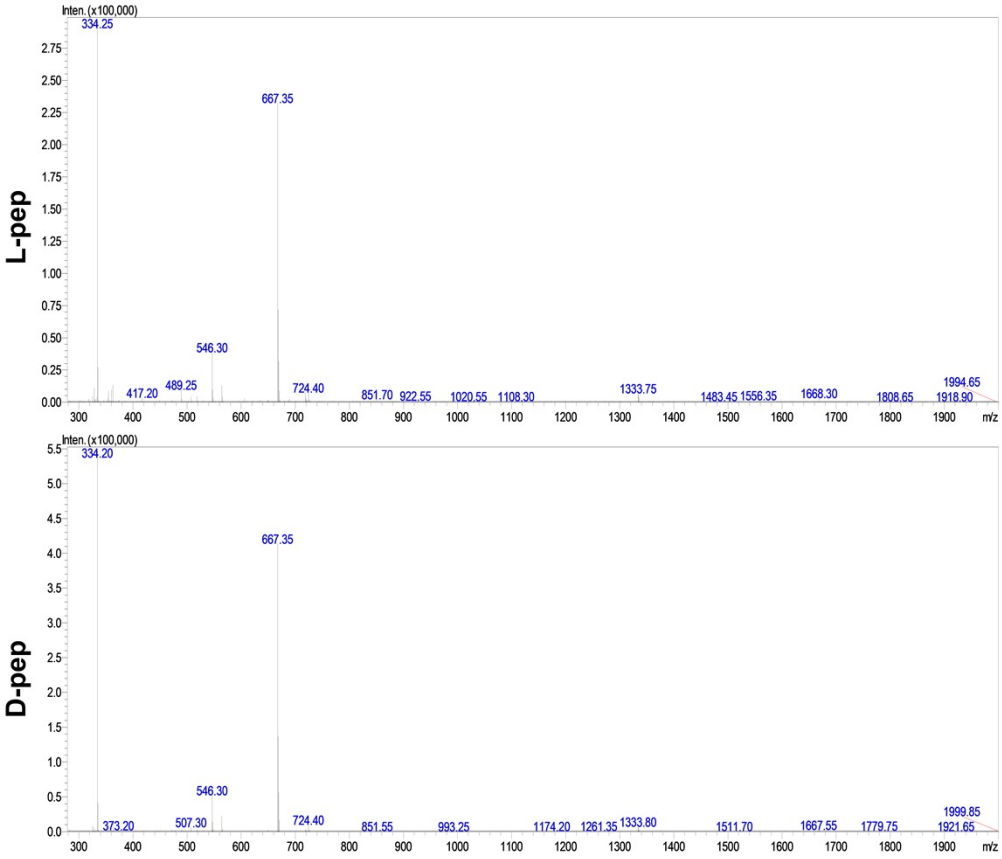

Figure S2. Mass spectrometry (MS) analysis of chiral peptides.

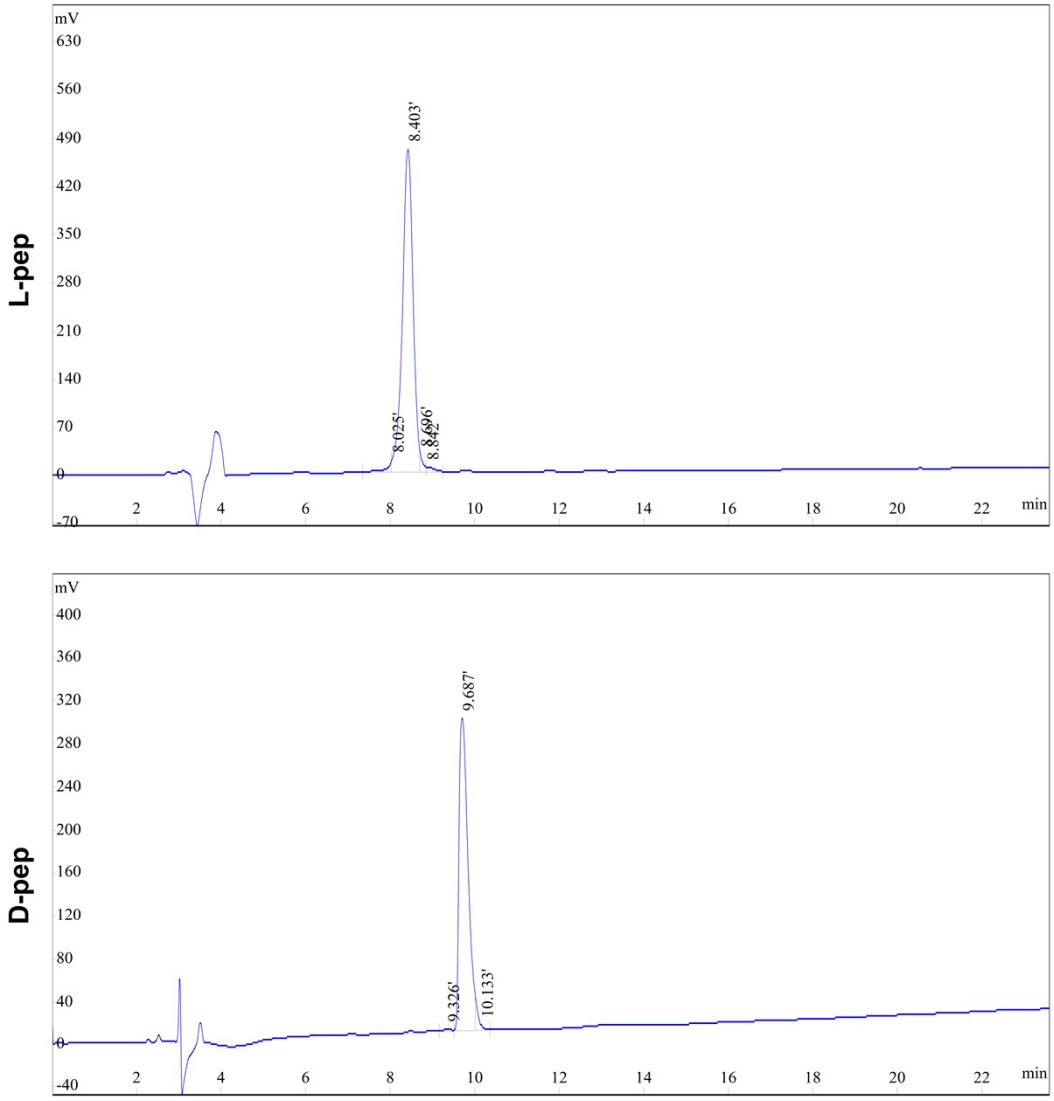

Figure S3. High-performance liquid chromatography (HPLC) analysis of chiral peptides.

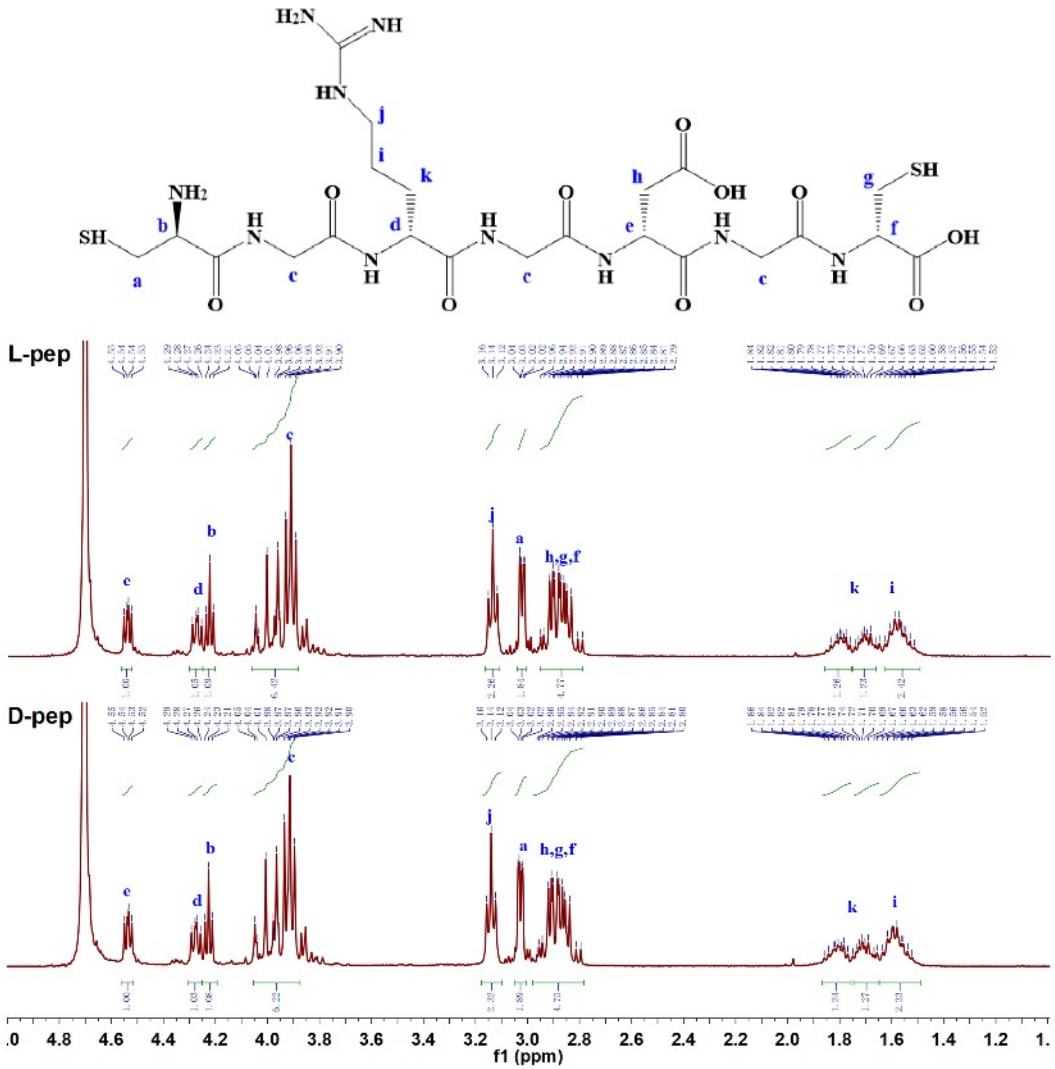

Figure S4. Representative ^{1}H NMR (400 MHz) spectra of L-pep, D-pep.

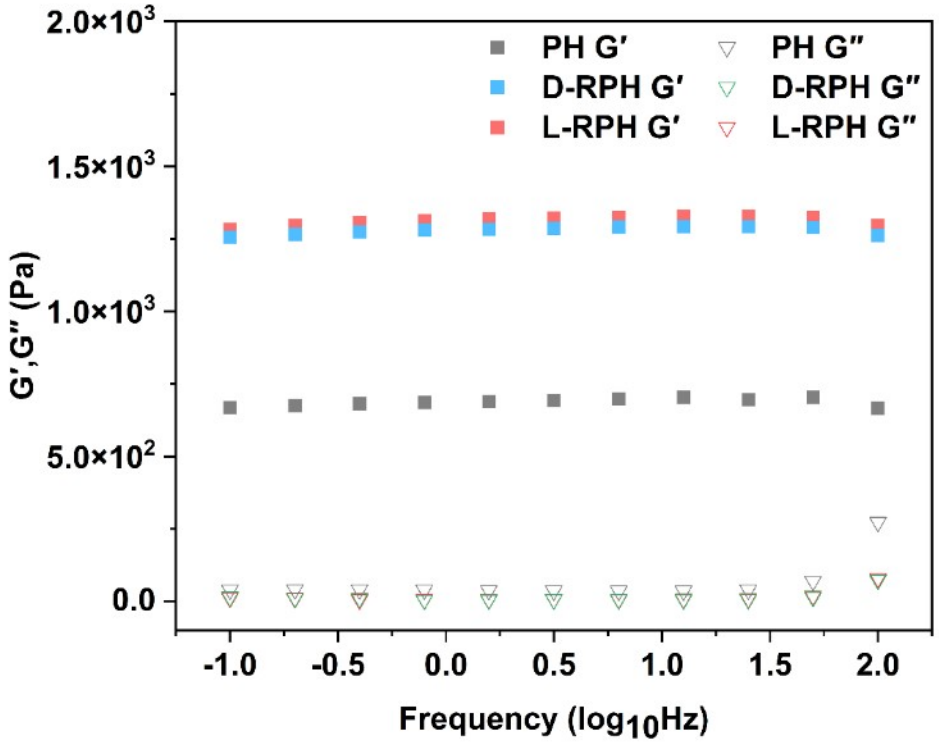

Figure S5. Frequency sweep rheology of hydrogels.

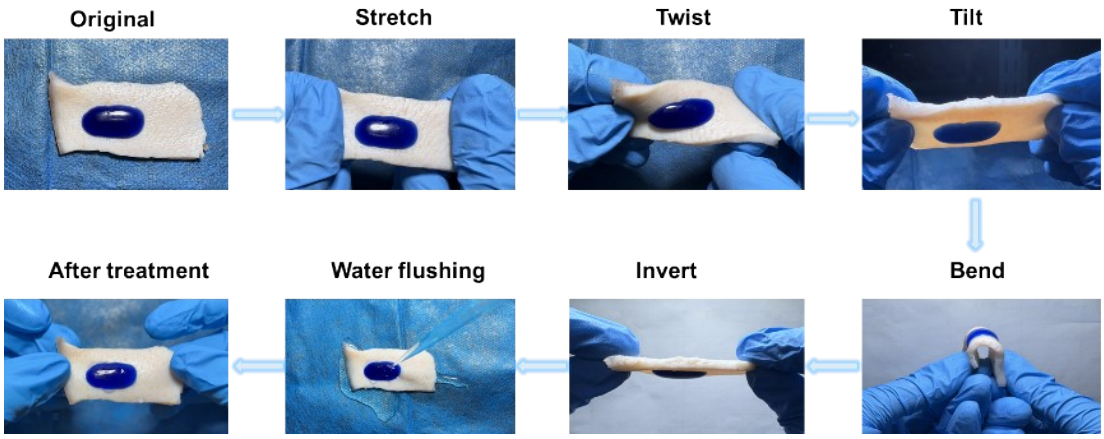

Figure S6. Hydrogel deformation after applied in skin.

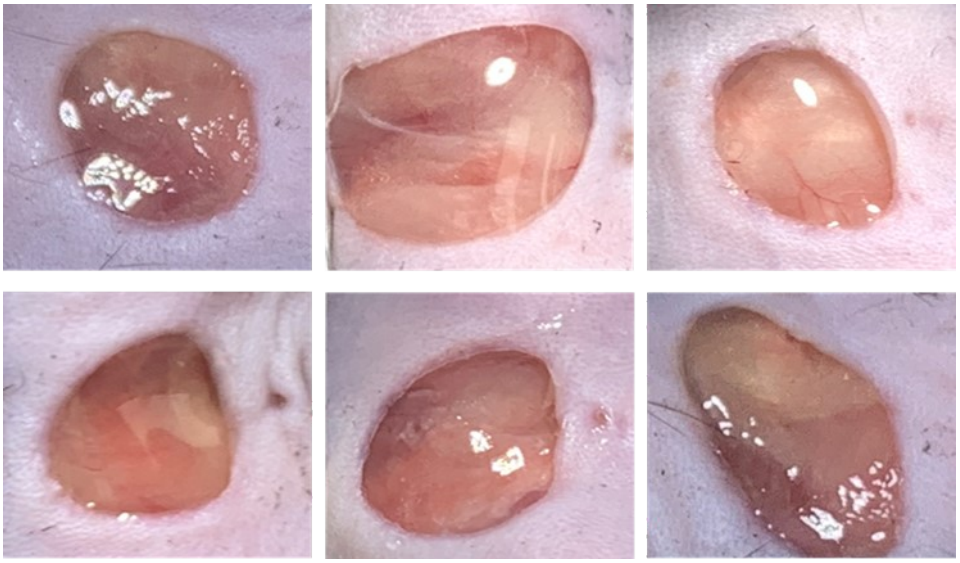

Figure S7. Gelation of L-RDG hydrogel on irregular wounds.

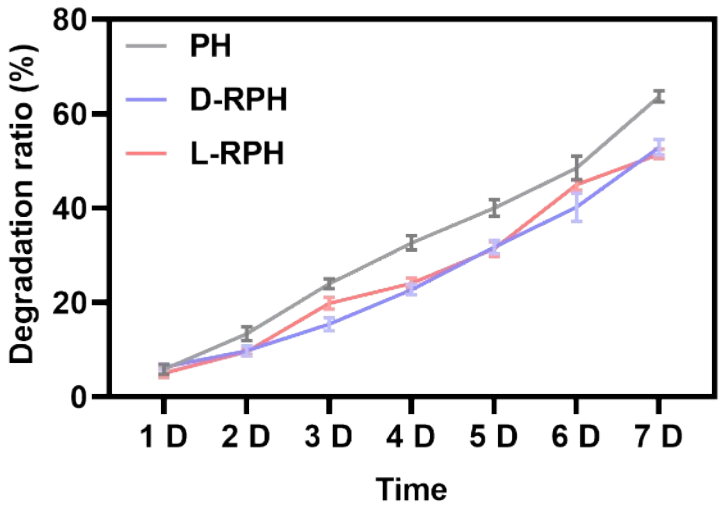

Figure S8. Degradation rates of three hydrogels.

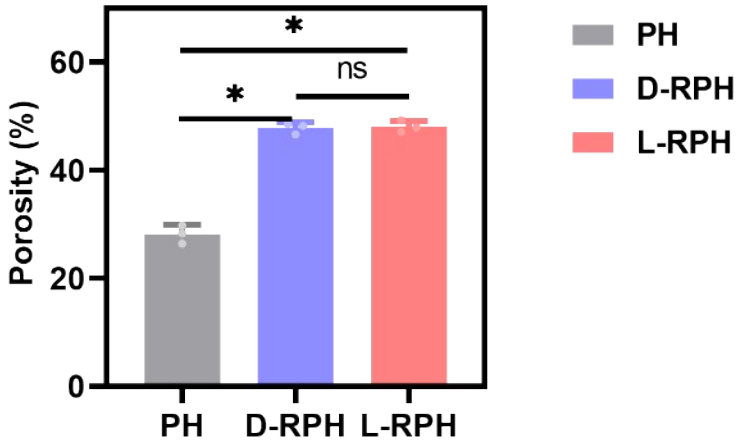

Figure S9. Porosity of three hydrogels.

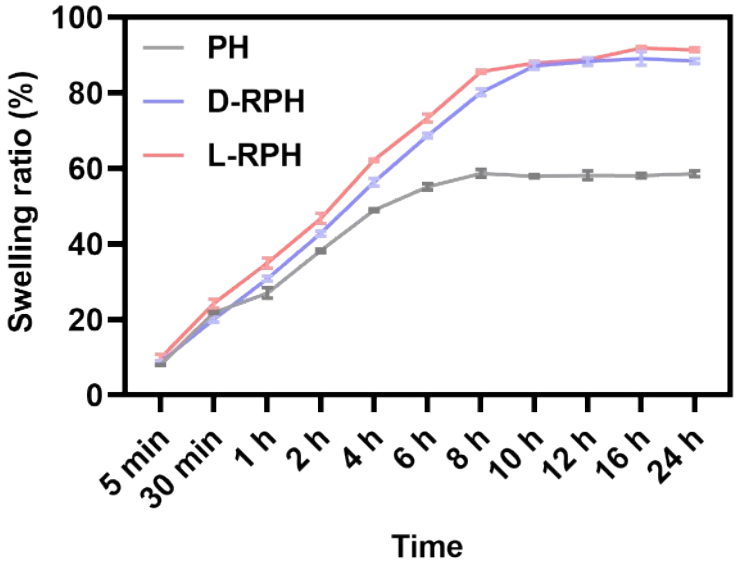

Figure S10. The swelling rate of three hydrogels.

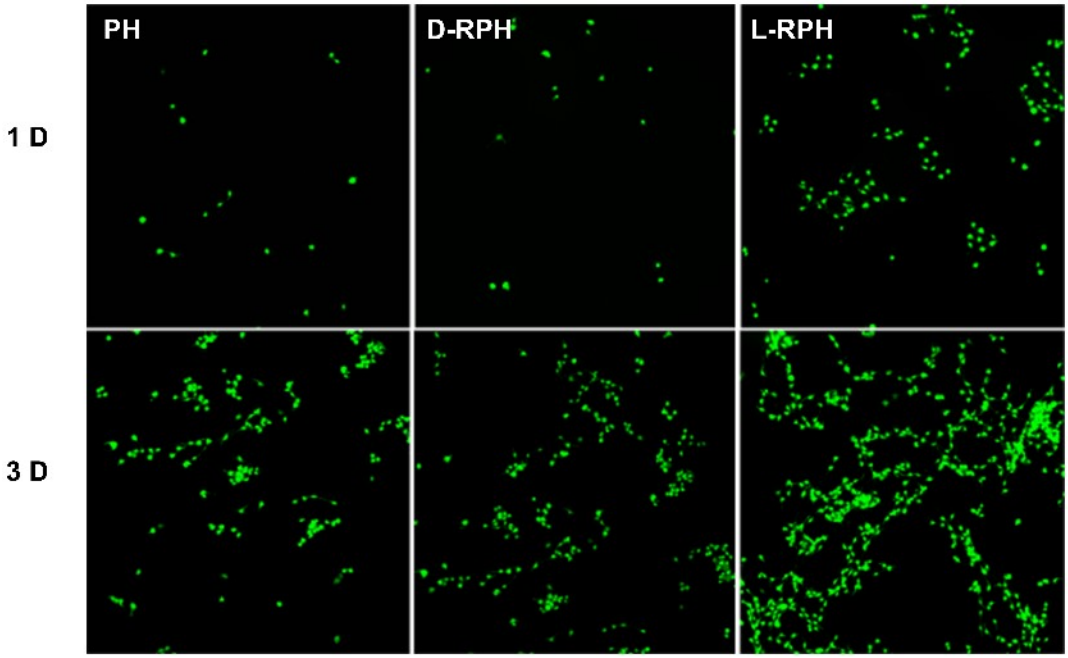

Figure S11. Representative fluorescence microscopy images of fibroblast on the three groups of hydrogels for 1, 3 days and processed with Live (green) and Dead (red) staining.

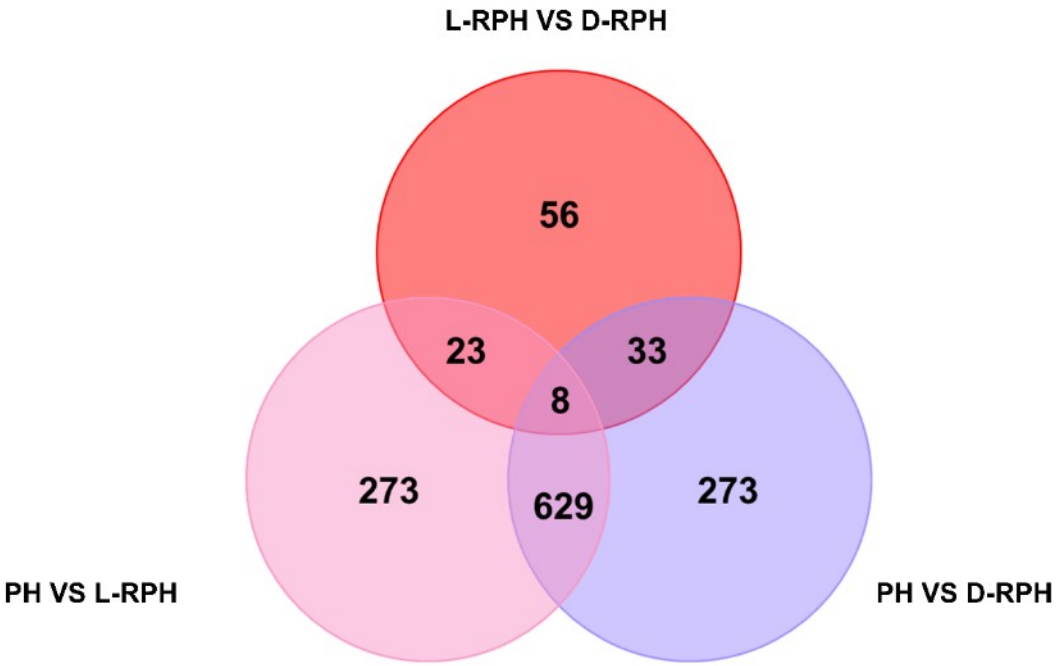

Figure S12. Venn diagram of the number.

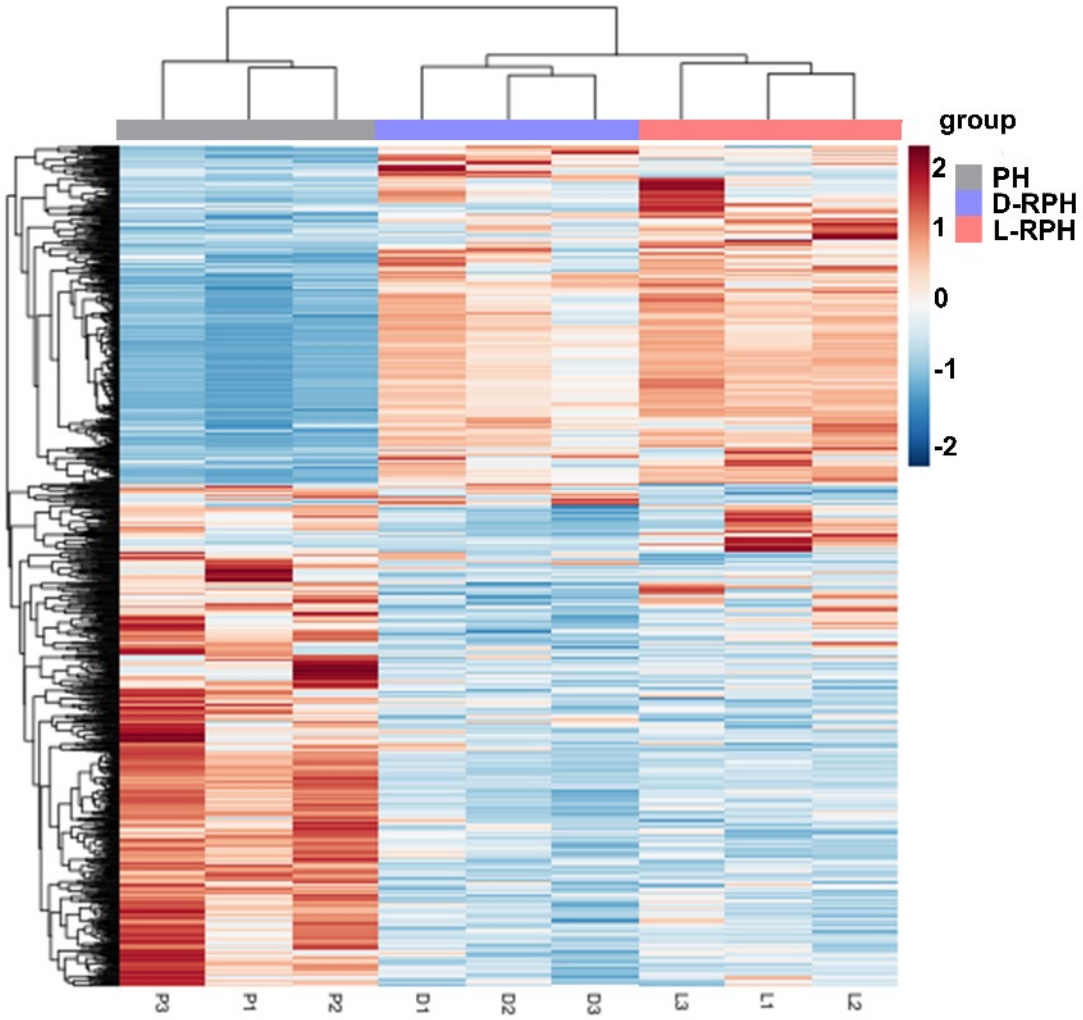

Figure S13. Heatmap of the gene expression differences across three groups.

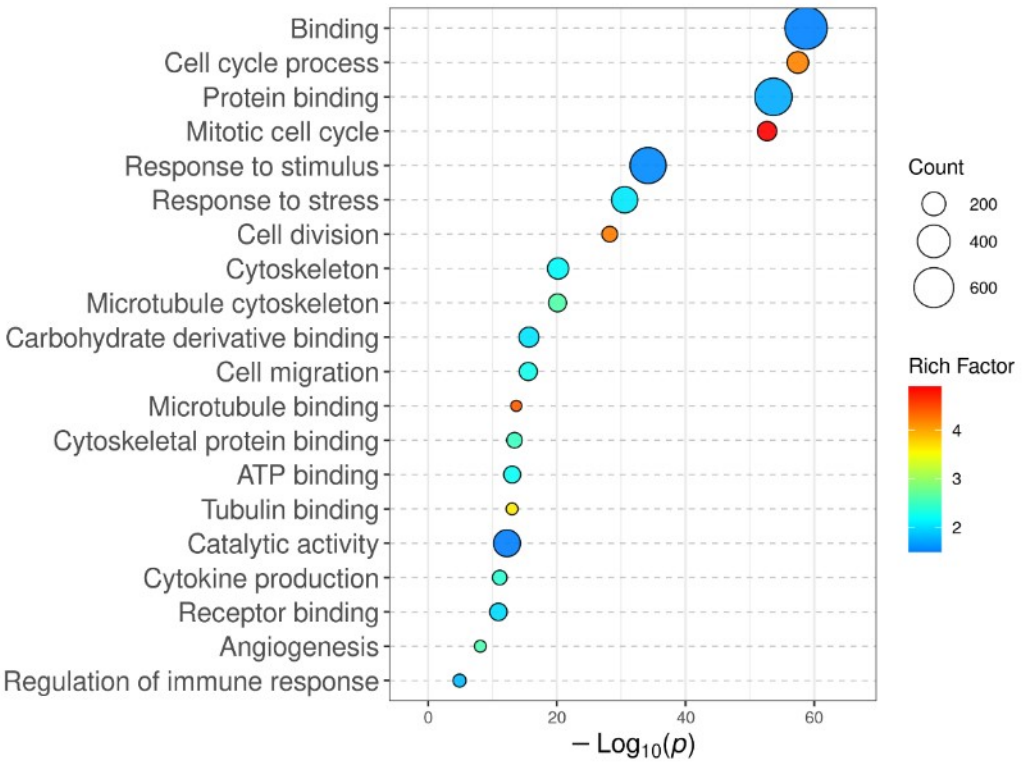

Figure S14. Top 20 enriched GO in NIH 3T3 cells cultured on PH compared with those on L-RPH

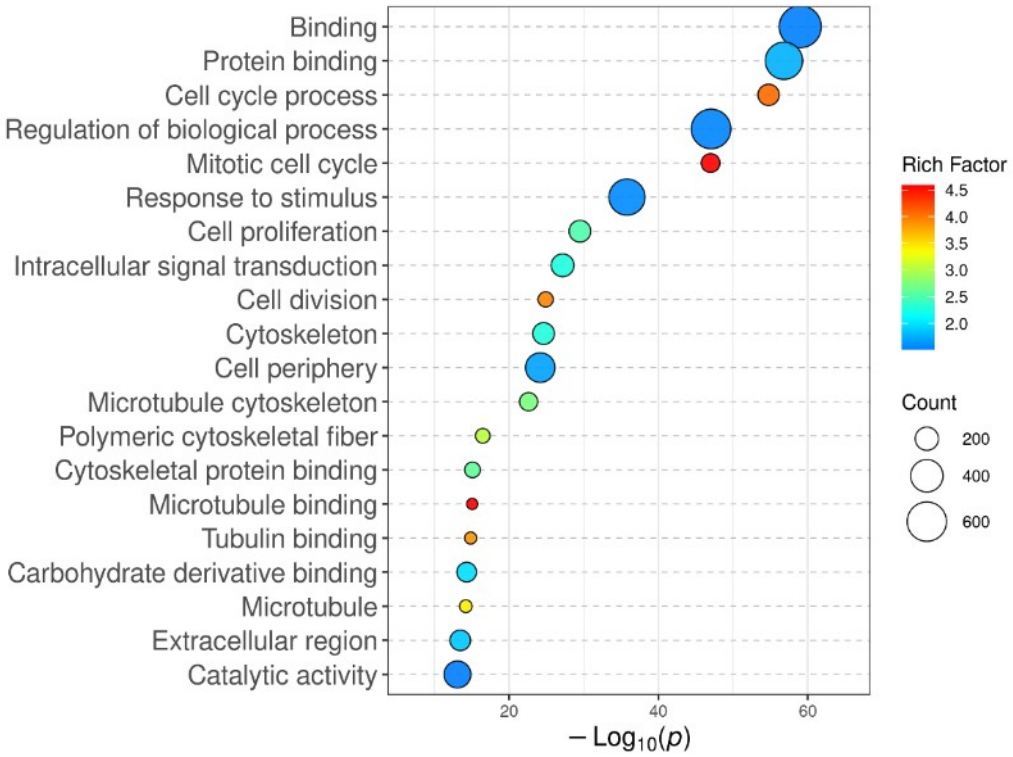

Figure S15. Top 20 enriched GO in NIH 3T3 cells cultured on PH compared with those on D-RPH.

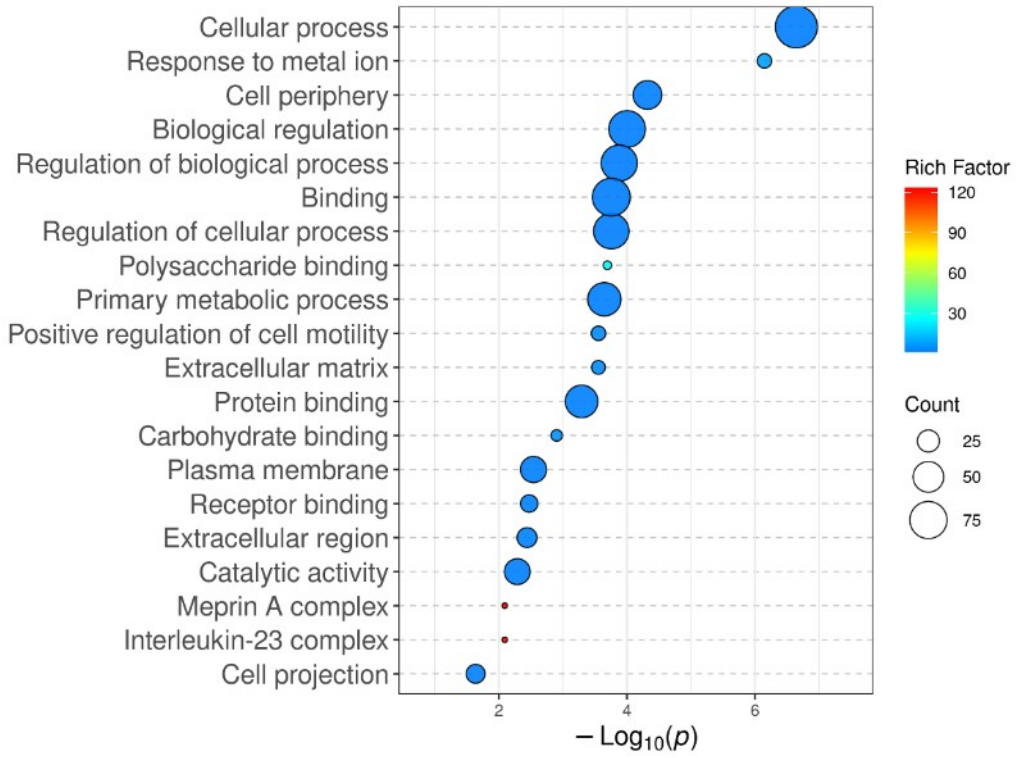

Figure S16. Top 20 enriched GO in NIH 3T3 cells cultured on L-RPH compared with those on D-RPH.

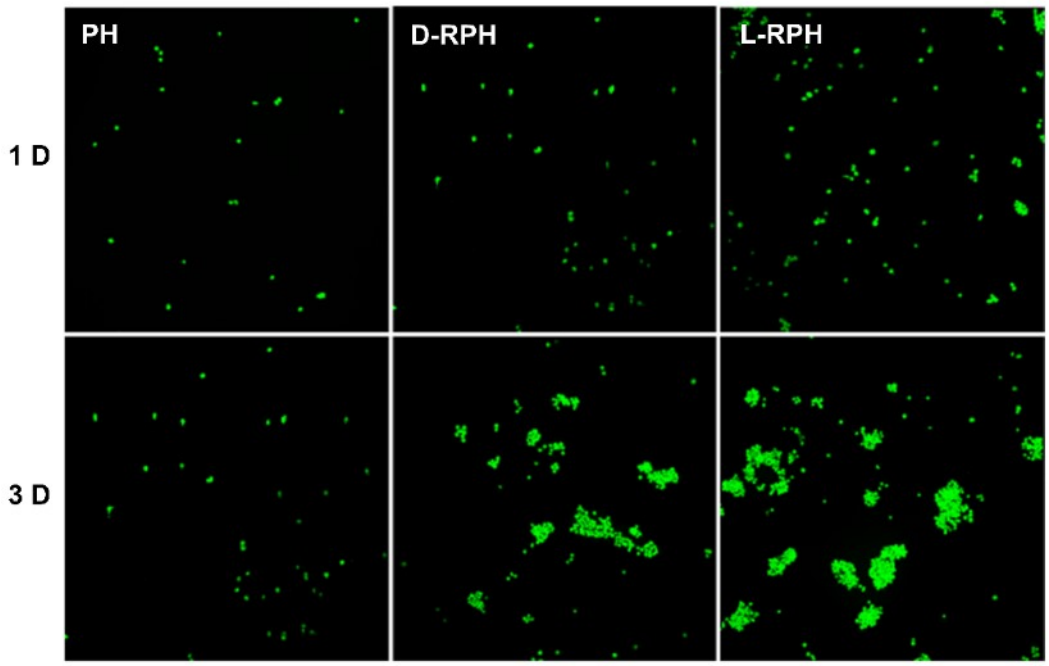

Figure S17. Representative fluorescence microscopy images of macrophage on the three groups of hydrogels for 1, 3 days and processed with Live (green) and Dead (red) staining.

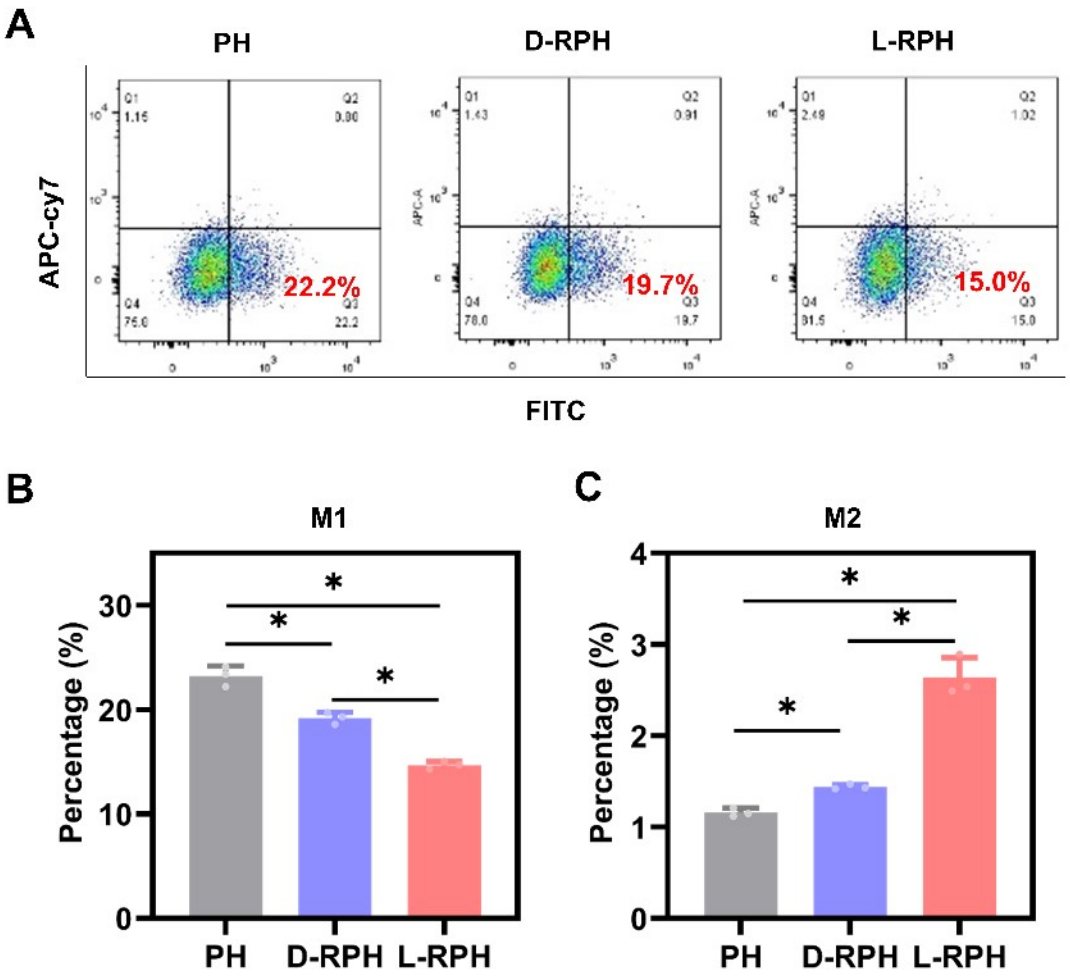

Figure S18. Macrophage polarization characterization. (A) M1 macrophages and M2 macrophages were test by flow cytometry. (B, C) Quantitative statistical percentage of M1 and M2 macrophages.

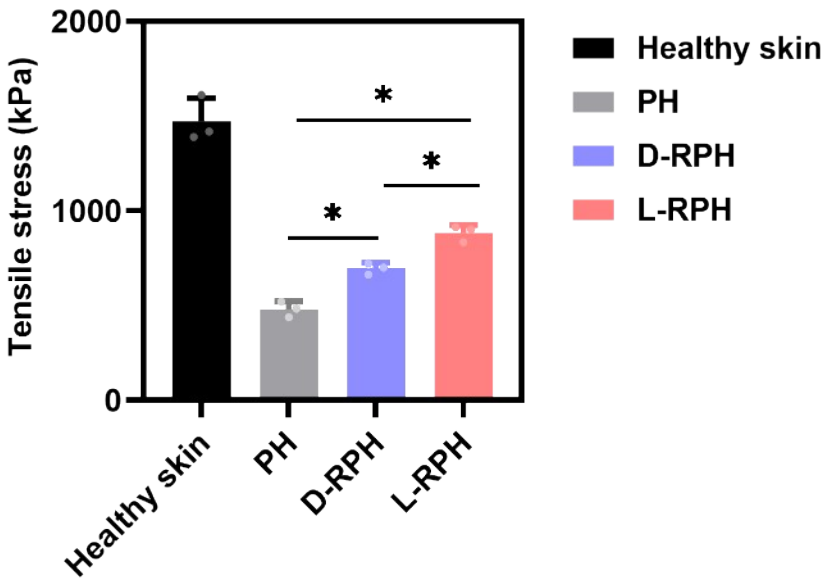

Figure S19. Tensile stress of regenerated skins at day 10.