Supporting information

Chinese herbal medicine-inspired construction of multi-component hydrogels with antibacterial and wound-healing-promoting function

Chuying Yao,[a] Yue Yuan,[b] Guangyan Du,*[a] Quan Li,*[b] Yutian Ji [c]

[a] College of Materials Science and Engineering, Zhejiang University of Technology, Hangzhou 310014, P.R.China.
E-mail: dugy@zjut.edu.cn

[b] Tianjin Key Laboratory of Therapeutic Substance of Traditional Chinese Medicine, School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine, Tianjin 301617, P.R.China.
E-mail: quanli85@tjutcm.edu.cn

[c] Collaborative Innovation Center for Advanced Organic Chemical Materials Co-constructed by the Province and Ministry, Ministry-of-Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules, College of Chemistry and Chemical Engineering, Hubei University, Wuhan 430062, P.R.China.

Supplementary Note Experimental Section

Materials

Puerarin (PUE), $CaSO_4$, fetal bovine serum (FBS), DMEM, penicillin-streptomycin solution, trypsin cell digestive fluid, yeast powder, tryptone, agar powder were purchased from Hangzhou Bangyi Chemical Co., LTD. All materials are purchased from commercial suppliers.

Gel preparation

The 2 wt% puerarin was dissolved in deionized water and then 0 eqv, 0.125 eqv, 0.25 eqv, 0.33 eqv $CaSO_4$ was added, respectively. The mixture was heated to 90 °C in an oil bath to enable complete dissolution[1], and then naturally cooled to room temperature and a gel will be formed.

Rheological studies

A rheometer (MCR302, Anton paar gmbh, Austria) with a 50 mm plate and 2.0 mm clearance was used for rheological studies. The hydrogel sample should be fully gelatinized at room temperature in advance for rheological testing. The hydrogels were measured at a constant frequency of 1 Hz, with a strain range of 0.01-10% of the scanning modulus and an angular frequency scanning range between 0.01-100 rad/s to measure the storage modulus (G') and loss modulus (G'') with a scanning mode at a temperature of 25 °C. Meanwhile, the shear viscosity was measured when the shear rate was 0.01-1 S^{-1}.

Field emission scanning electron microscope (FESEM)

The morphology of hydrogels was observed by field emission scanning electron microscopy (HITACHI Regulus 8100, Hitachi of Japan, Japan). Prior to observation, the hydrogel was freeze-dried and sprayed with gold. SEM images were collected at a scanning speed of 10 μ s and a resolution of 1536*1024.

Fourier transform infrared spectroscopy (FT-IR)

After freeze-drying, the hydrogel was ground into fine powder by mortar, then pressed with potassium bromide (KBr), and analyzed by Fourier transform infrared spectrometer (Nicolet 6700, United States thermal Nicolai Corporation, USA) in the spectrum range of 4000-500 cm^{-1}.

X-ray diffraction (XRD)

XRD was conducted on a diffractometer (Ultima IV, Rigaku Corporation of Japan, Japan) equipped with Cu-Kα radiation (λ=1.5406 Å) source. The amperage and tube voltage were set at 40 mA and 40 kV, respectively. For each sample, an XRD pattern was collected from 2θ of 3° to 40° with a scanning speed of 4°/min and a step size of 0.02°.

Cytotoxicity test

The prepared puerarin gels were dissolved in PBS buffer solution at the equilibrium of 0.2 g. The swelling-balanced puerarin gel was immersed in DMEM medium (2 mL) at 37 °C for 24 h and removed the supernatant to acquire leach liquor (100 mg/mL) for the cell experiments. L929 mouse fibroblasts were cultured in DMEM medium supplemented with 10 % FBS and 1 % penicillin-streptomycin. L929 cells were seeded at a density of 3×10^3 cells/well in 96-well plates and cultured in a cell culture incubator (37°C, 5% CO_2). After 24 h, the complete medium was replaced with 100 μL puerarin gel leach liquor and incubated at different time points (1, 2 and 4 days). The cells cultured in a complete medium were used as a control. Afterward, the cytotoxicity was detected by CCK-8 kit. The cells were incubated with a complete medium containing 10% CCK-8 for 1.5 h and the absorbance at 450 nm was measured using a microplate reader. The cell viability was calculated by the following equation:

$$\text{Cell viability}=([A]_e-[A]_b)/([A]_c-[A]_b)\times100\%$$

where $[A]_e$ represents the OD_{450} of experimental groups; $[A]_b$ represents the OD_{450} of water without cells; and $[A]_c$ represents the OD_{450} of control groups.

To perform Live/Dead staining, L929 cells were inoculated at a density of 3×10^3 cells per well in 48-well plates. After 24 h incubation, the complete medium was replaced with 200 μL puerarin gel leach liquor to continue the culture for 1, 2, and 4 days, respectively. Then, the medium was removed and cell viability was evaluated by Live/Dead staining assay according to the instructions. The cells were incubated with 100 μL Calcein AM/PI detection working solution for 15 min and subsequently observed under a fluorescent microscope to determine whether cells were live (green) or dead (red).

Hemolysis assay

For hemolysis analysis, 1 mL fresh rabbit blood was diluted with PBS solution and centrifuged at 3000 rpm at 4°C for 5 min until erythrocytes were obtained. The erythrocytes were resuspended to a 2% (v/v) concentration with PBS and mixed with PBS (negative control group), deionized water (positive control group), or different concentrations of gels (experimental group), respectively. After incubation for 2 h in a water bath at 37°C, the erythrocyte suspensions were centrifuged at 3000 rpm at 4°C for 5 min. The absorbance of the supernatant at 541 nm was measured with a microplate reader. The hemolytic percentage (HD) was calculated by the following equation:

$$HD(\%)=(OD_e-OD_n)/(OD_p-OD_n)\times100\%$$

where OD_e, OD_p, and OD_n represent the OD_{541} of the experimental group, positive control group, and negative control group, respectively. According to GB/T16886.4-2003 and related literature, it requires that the hemolytic rate should not exceed 5 % of the national standard.

***In vitro* antibacterial assay**

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and the Gram-negative bacteria *Escherichia coli* (*E. coli*) were selected for this test. *S. aureus* and *E. coli* were cultivated in Luria-Bertani (LB) liquid medium at 37°C with shaking for 12 h. Bacterial density was determined by OD 600 measurements and diluted with LB to a suitable concentration (OD_S = 0.05, OD_E = 0.1). Subsequently, *S. aureus* and *E. coli* solution were added into a 12-well plate and co-incubated with different gels on a shaker at 37°C for 12 and 24 h, respectively. The OD_{600} values of bacterial

suspensions were measured at different time points using a spectrophotometer. In addition, the co-cultured bacterial solution was removed and diluted to an appropriate multiple, then 30 μL of the dilution solution was taken and spread to agar plates. After cultured on a shaker at 37°C for 24 h, the status of bacterial colonies on the plates was recorded with a camera. All tests were repeated three times.

***In vitro* antioxidant assay**

The antioxidant ability of hydrogels was tested by free radical scavenging efficiency for the DPPH and ABTS *in vitro*. Following the DPPH radical scavenging ability assay, 20 mg of the hydrogel was added into 2 mL of ethanolic DPPH detection solution, and the ethanolic DPPH solution was used as a blank control. The mixture was shaken for 30 min at room temperature in the dark and then centrifuged at 1000 rpm for 5 min. The absorbance of the supernatant at 510 nm was measured by a spectrophotometer. For the ABTS radical scavenging ability assay, 20 mg of the hydrogel was placed in 4 mL of the ABTS free radical solution by shaking at 37°C for 30 min in the dark. The mixture was centrifuged at 1000 rpm for 5 min and the absorbance of obtained supernatant was measured at 734 nm. The free radical scavenging rate was calculated using the following formula:

Free radical scavenging rate (%) =(AB_{bla}-AB_{test})/AB_{bla}× 100%

where AB_{bla}, AB_{test} are the absorbances of the blank control group and experimental group, respectively.

***In vivo* wound healing evaluation**

The animal protocols followed the Guide for the Care and Use of Laboratory Animals (Zhejiang University of Technology) and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). Female SD rats aged 7-8 weeks (weight: 200-250 g) were housed at a constant temperature and humidity. After 7 days of acclimatization, the rats were randomized to 6 groups: positive control group (Tegadrem™ film), blank control group (PBS), and hydrogel treatment group (PUE, PCa-1, PCa-2, and PCa-3), The rats were anesthetized by injection of 3wt% pentobarbital sodium, shaved their dorsal hairs and created round full-thickness skin wounds with a diameter of 10 mm. Then, 1 × 10^8 CFU mL^{-1} *E.coli* were inoculated into the wounds and each group of materials was applied post 24 h. The size of the wound site was recorded on 1, 3, 6, and 10 days. The wound closure rate was calculated using the following equation:

Wound closure rate (%) = （S_0 -S_t ）/S_0x100 %

where S_0 and S_t represent the initial and t days post-treatment wound areas, respectively.

Skin wound tissue samples were harvested and divided into two parts on 5 and 10 days. One part of the tissue was performed by H&E staining and Masson's trichrome straining to take histological analysis based on the manufacturer's protocols. Finally, the specimens to be observed were placed under an optical microscope.

Supplementary figure

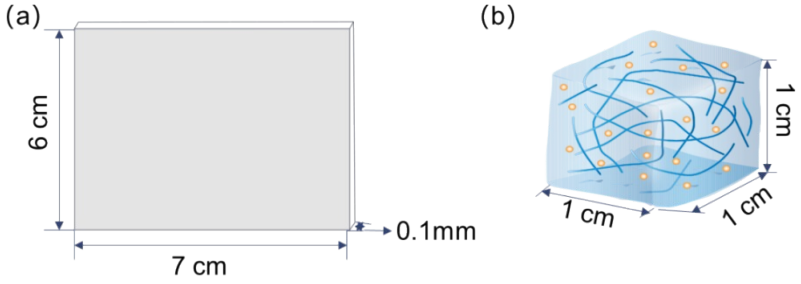

Fig. S1. (a) The size of Tegaderm™ film and the (b) hydrogel.

The price of a typical Tegadrem™ with a volume of 0.42 cm^3 is 1.8 RMB. The price of a typical gel with a volume of 1 cm^3 is estimated at approximately 0.21 RMB (the price of puerarin is 11 RMB/g, and that of $CaSO_4$ is 0.26 RMB/g).

Therefore, the cost of a gel is significantly lower than the Tegadrem™ film. And the procedure of gel preparation is also much easier than the film.

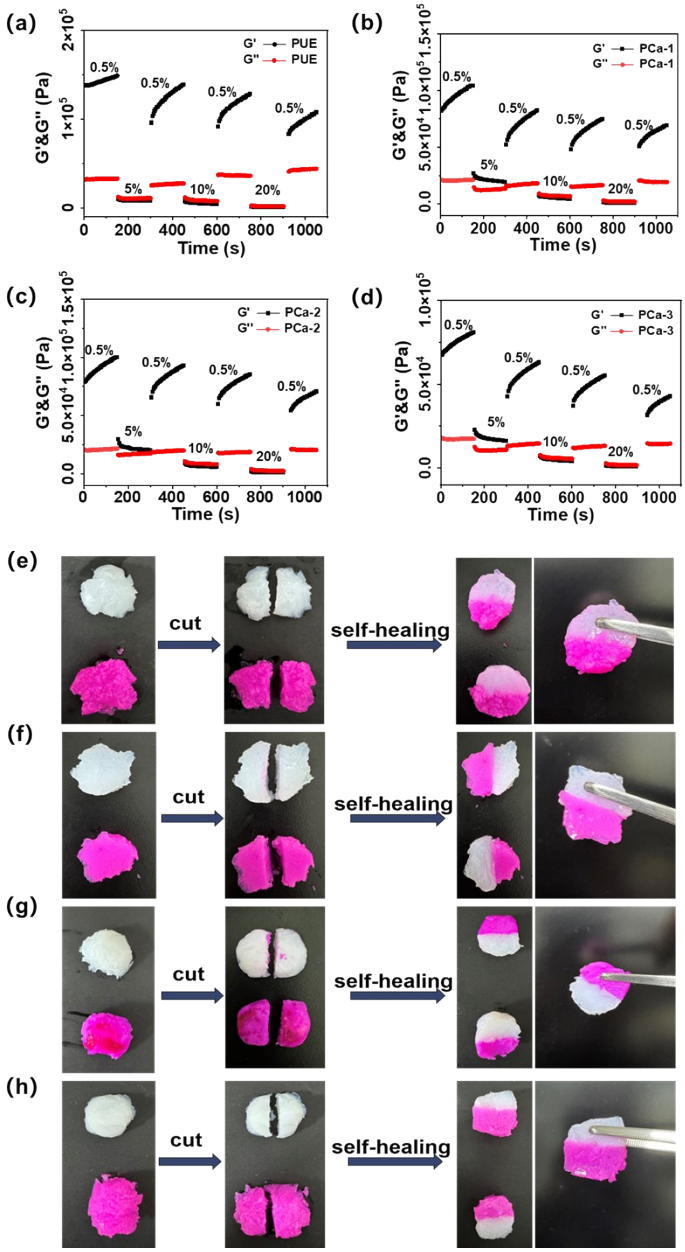

Fig. S2. Continuous step strain testing of a) PUE, b) PCa-1, c) PCa-2, and d) PCa-3 hydrogels. Illustration of self-healing properties of hydrogel e) PUE, f) PCa-1, g) PCa-2, and h) PCa-3. A very small amount of Rhodamine B (~1% molar ratio of PUE) was added to the gel for visual contrast.

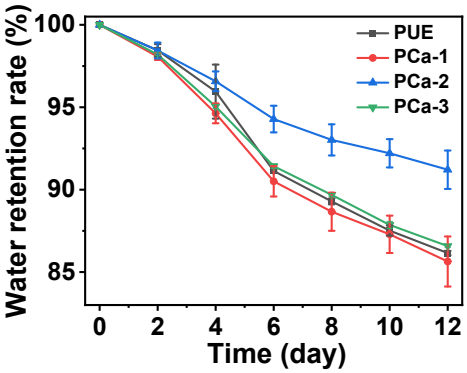

Fig. S3. Water retention test of PUE, PCa-1, PCa-2, and PCa-3 hydrogels.

References

1. H. D. Guo, Y. Jiang, X. C. Shangguan, Q. F. Zhang, *Food Sci. Tech-brazil*, 2014, **39**, 229-232.