Supporting Information

A biocompatible PAA-Cu-MOP hydrogel for wound healing

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1. Experimental Section

1.1 Materials Characterization of the PAA-Cu-MOP hydrogels

All chemical reagents were obtained from Energy chemical company used without further purification unless otherwise noted. All bacteria were purchased from Guangdong Huankai Microbiology Technology Co., Ltd. Double distilled water was used throughout the experiments. Powder X-ray diffraction (PXRD) was performed on a TTRIII X-ray diffractometer (Rigaku, Japan) with Cu Kα radiation at 40 kV and 200 mA. Scanning electron microscopy (SEM) imaging was performed on a QUANTA200 scanning electron microscope (FEI, USA) operated at 30 kV. FTIR spectra were recorded in the range 4000–400 cm⁻¹ on a Thermo Nicolet spectroscopy by using KBr pellets. Thermogravimetric Analysis (TGA) was performed on a NETZSCH STA 449F3 instrument with the heating rate of 10 °C min⁻¹ under a nitrogen atmosphere. Rheological measurement was on a MRC302 instrument.

1.2 Fabrication of the Cu-MOP embedded hydrogels
The Cu-MOP was prepared as our previously reported.[1] The mixture of H$_2$(5-NO$_2$-1,3-BDC) ((H$_2$(5-NO$_2$-1,3-BDC)= 5-Nitro-1,3-benzenedicarboxylic acid, 0.2 mmol) and Cu(NO$_3$)$_2$•3H$_2$O (0.2 mmol) was dissolved in 5 mL solvents (CH$_3$OH:DMF) = 4(v):1(v) and heated in 25 mL glass vial at 75 °C for 2 days, and then cooled to room temperature. The Cu-MOP was obtained and washed with CH$_3$OH several times. Then mixture of Cu-MOP (30.0 mg), PAA (25.0 mg), glycerol (200 mg) was added in 0.745 mL water, ultrasonic about 3-5 minutes until the mixture were interfused uniformly, afterward, swelled at room temperature about 2-5 hours to obtain PAA-Cu-MOP hydrogel.

1.3 Copper ion release

To investigate the releasing process of copper species from PAA-Cu-MOP hydrogel after contacting with agar culture medium, ICP-MS measurement was performed. 1 mL agar culture medium was used to contact with the PAA-Cu-MOP hydrogel and then the culture medium solution was taken out with the contacting time for the ICP-MS measurement. 200 μL of culture medium was taken out and 5 mL sterile water was added in it.[1]

1.4 Bacteria SEM sample preparation

(1) Bacterial immobilization: The bacterial suspension after interacting with PAA-Cu-MOP hydrogel and Cu-MOP hydrogel was centrifuged at room temperature, 8000 rpm for 1 min. Then, the supernatant was removed and the precipitate was washed with sterile water for three times. Finally, the bacteria was fixed overnight at 4 °C by adding 2.5% glutaraldehyde.

(2) Gradient dehydration of ethanol: The fixed bacteria were centrifuged at 8000 rpm for 1 minute while removing the supernatant and washing precipitate with sterile water for three times. Next, 30%, 50%, 70%, 80% and 90% of alcohol were used to dehydrate bacteria and each step stood for 15 min. At last,
the dehydrated bacteria were resuspended with anhydrous ethanol after washing twice with 100% ethanol with the same conditions as before.[2-3]

1.5 Hygroscopicity and moisture retention test

Hygroscopicity test: The hygroscopicity was evaluated by the percentage of weight change of sample.[4-6] 0.2 g samples were weighted, and placed the samples in desiccators with relative humidity of 81% (saturated (NH₄)₂SO₄) and 43% (saturated K₂CO₃) at 20 ℃ from 0 to h to 312 h.

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\text{Hygroscopic rate (%) = \left( \frac{W_n - W_0}{W_0} \right) \times 100}
\]

\(W_n\) was the weight of sample after placing in desiccators at regular intervals for weighing, \(W_0\) was the weight of sample before placing in desiccator.

Moisture retention test: 0.2 g samples were weighted, and added 3 times mass of distilled water, then sealed in a desiccator over silica-gel at 20 ℃ from 0 to h to 312 h.[2-4] The moisture retention ability was evaluated by the percentage of residual water in samples.

Moisture retention rate (%) = \((W_n / W_0) \times 100\).

\(W_n\) was the weight water in sample after placing in desiccator at regular intervals for weighing, \(W_0\) was the weight water in sample before placing in desiccator.

1.6 Agar Diffusion Test

The agar diffusion test was used to visually present the antibacterial activity of PAA-Cu-MOP hydrogel according to a previously reported method. Bacterial suspensions (10⁷ CFU/ml, colony forming units is abbreviated as CFU) of *Escherichia coli* (E.coli), *Staphylococcus aureus* (S. aureus), *Pseudomonas aeruginosa* (P.aeruginosa), *Bacillus subtilis* (B.subtilis) and *Staphylococcus epidermidis* (S. epidermidis) were spread onto nutrient agar plates using a sterile pipette. The content of the PAA-Cu-MOP hydrogel was 167 mg placed by a 6 mm diameter filter paper onto the surfaces of the nutrient plates following
incubation at 37 °C overnight. Inhibition zones were manifested by the obviously transparent halos on the nutrient agar plates and then the diameters of the inhibition zones were measured. Each experiment was carried out three times in parallel.

1.7 Time-Dynamic Antibacterial Test

A time-dynamic antibacterial test was measured to reveal the antimicrobial effectiveness of the PAA-Cu-MOP hydrogel. Bacterial suspensions (10^7 CFU/ml) of *P. aeruginosa*, and *B. subtilis* were inoculated with 1.67 mg/ml of the PAA-Cu-MOP hydrogel. A blank control group was treated without the PAA-Cu-MOP hydrogel. Bacteria were sampled from 0 to 360 min at selected time intervals. Briefly, 100μL of the bacterial solution was taken out and added into 900 μL of sterilized physiological saline and then 100 μL of the diluted bacterial solution was cultured on the nutrient plates at 37 °C for 24 h. At this point, we photographed the bacterial colony on the plates and counted the number of colonies. Meanwhile, the antibacterial rate was measured to assess the antibacterial activity via the following equation: Antibacterial ratio (%) = (number of CFUs in control group − number of CFUs in experimental group)/(number of CFUs in control group) ×100%. Each experiment was carried out three times in parallel.[7]

1.8 Hemolysis Assay

Healthy human blood (2 mL) was donated from a male volunteer. RBCs were collected by centrifugation at 3000 rpm for 10 min, washed with normal saline (0.9% NaCl) for three times, and resuspended using normal saline (100 mL) to prepare 2% erythrocyte solution. Then, 33.4 mg/mL of the PAA-Cu-MOP hydrogel, Cu-MOP hydrogel, PAA, glycerol, and Cu(NO₃)₂·3H₂O were added to the same-volume 2% erythrocyte solution in centrifuge tubes. After incubation at 37 °C for 2 h, the supernatants were obtained through centrifugation at 2000 rpm for 10 min, and transferred to a 96-well plate. The
absorbance at 540 nm was measured by M200 PRO NanoQuant. RBCs in normal saline and distilled water were used as a negative control and a positive control, respectively. The following formula was used to calculate the hemolysis percentage: Hemolysis (%) = (mean of absorbance value of treated group – mean of absorbance value of negative control group)/(mean of absorbance value of positive control – mean of absorbance value of negative control group) × 100%. Each experiment was carried out three times in parallel.

1.9 Wound healing in Vivo

Using a rat model to evaluate the effect of PAA-Cu-MOP hydrogel on wound healing of skin. The animal experiments in the present research were reviewed and approved by the Medical Ethics Committee of QuanZhou Medical College. Sprague-Dawley (SD) male rats (180-250g) were obtained from Shanghai SLRC Laboratory Animal Co. Ltd., China. 15 healthy male SD rats were anesthetized and shaved, and then divided into three groups randomly. A full-thickness round wound (1cm×1cm) was created on the back of each rat. For group 1, the wounds were treated with the physiological saline as a negative control. The group 2 was treated with recombinant human epidermal growth factor as a positive control. For group 3, the wounds were treated with Cu-MOP hydrogel as experimental group. The physiological saline, recombinant human epidermal growth factor and Cu-MOP hydrogel were changed every day. Wound size was measured and recorded with a digital camera every day until the wounds healed completely. Analyze the change of wound area in each group by using image-pro plus.

Wound healing rate = ( mean of initial wound area - mean of current wound area) / mean of current wound area × 100%

Animal experiments were reviewed and approved by the Animal Investigation Ethics Committee of Quanzhou medical college.
1.10 Procoagulant ability test

Mouse liver bleeding model was used to investigate the procoagulant ability of PAA-Cu-MOP hydrogels in vivo. 16 healthy Sprague-Dawley (SD) male rats (180-250g) were obtained from Shanghai SLRC Laboratory Animal Co. Ltd., China. Firstly, 20% chloral hydrate was injected to anesthetize SD rats. Then the liver were dissected and punctured to bleed by using an 18G needle. The preweighed filter paper was placed on the bleeding position of liver. At the same time, about 15-20 mg PAA-Cu-MOP hydrogels were daubed on the bleeding position. The weight of the filter paper with blood was measured at every 30 s interval, and the blood loss was calculated for 120 s until the liver stopped bleeding. The stopped bleeding time of without any treatment, recombinant human epidermal growth factor and hydrogel matrix (PAA-glycerol - hydrogel) were measured as control groups.[9] All experiments were carried out three times in parallel. Animal experiments were reviewed and approved by the Animal Investigation Ethics Committee of Quanzhou medical college.

1.11 Histologic analysis

The wound tissues postoperation were collected from each group of rats on day 7 and day 14. The major organs including the heart, liver, spleen, lung and kidney were excised from each group of rats on day 14. All these samples were fixed with 10% formalin to prepare the slides. After staining with Hematoxylin and Eosin (H&E), the histological images were acquired on an optical microscope (Nikon Eclipse T1-SR).

1.12 Immunohistochemistry of microvessels with CD31

The slices embedded the wound tissues post-operation were deparaffinized in xylol and hydrated to lessen the concentration of alcohol. The slices were maintained in EDTA buffer (pH 9.0) and heated in a microwave oven for antigen retrieval. Then the slices were rinsed in phosphate-buffered saline (PBS, pH 7.4). To block endogenous peroxidase activity, the slices were treated with 3% hydrogen peroxide for 25
min and rinsed in PBS (pH 7.4) followed by an incubation with 3% albumin from bovine serum (BSA) for 30min. Then the slices were incubated with a goat anti-rat primary antibody (dilution 1:100) for CD31 at 4 °C overnight. Subsequently, the slices were incubated with goat anti-rabbit secondary antibody (dilution 1:200) labeled with horseradish peroxidase (HRP) at room temperature for 50 min, and rinsed in PBS (pH 7.4) afterwards. Staining was performed with diaminobenzidine (DAB), counterstaining was performed with hematoxylin. The positive cells were stained as dark brown. The histological images were acquired on an optical microscope (CIC, XSP-C204). The number of microvessels was determined by image-pro plus. All antibodies were diluted with PBS (pH 7.4). The steps needed to be rinsed using PBS for per process 3 times, and each time was 5 minutes.

1.13 Ki-67 immunofluorescence assay for cell proliferation

The slices were deparaffinized by heating for 60 min at 60 °C and soaking in xylene, and rehydrated to lessen the concentration of alcohol. Antigen retrieval was performed with citrate buffer (pH 6.0) at 100 °C for 10 min, after that, it was slowly cooled down to room temperature. Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide for 10 min at room temperature and the slice was rinsed in PBS (pH 7.4). Antigen was blocked with 10% normal donkey serum for 30min at room temperature. Then the slices were incubated with a goat anti-rat primary antibody (dilution 1:200) for Ki-67 at 4 °C overnight and a donkey anti-goat secondary antibody (dilution 1:400) labeled with Cyanine 3 (Cy3) at room temperature for 60 min, then rinsed in PBS (pH 7.4). Counterstaining was performed with 4’6-diamidino-2-phenylindol (DAPI) for 10 min at room temperature. The immunofluorescence images were acquired on a fluorescence microscope (Nikon Eclipse T1-SR).

1.14 Related genes expression
All samples were collected from each group of rats on day 7 and day 14 for real time PCR to determine the relative mRNA expression level. The total RNA was extracted using Trizol according to the manufacturer’s instructions. 1 µg of RNA was used to synthesize cDNA using a reverse transcription reagents kit. The quantitative real-time PCR was carried out following the protocol and conducted with Real-time PCR system. The temperature profile as follow: at 95 °C for 10 minutes, then 40 cycles at 95 °C for 15 seconds and at 60 °C for 60 seconds. Vascular endothelial growth factor (VEGF) and tissue growth factor-β1 (TGF-β1) were aimed to assess the wound healing process. All primer sequences were listed as following:

β-actin (GenBank accession No. NM_031144.3),

forward 5’- TGCTATGTGCCCCTAGACTTCG-3’,
reverse 5’-GTTGGCATAGAGGTCTTTACGG-3’.

VEGF (GenBank accession No. NM_031836),

forward 5’- CAATGATGAAGCCCTGGAGTG-3’,
reverse 5’-GCTCATCTCTCTATGTGCTGG-3’.

TGF-β1 (GenBank accession No. NM_021578.2),

forward 5’-GGCGGTGCTCGCTTTGTA-3’,
reverse 5’-TCCCGAATGTGCTTACACGTA-3’.
Figure S1. (A) PXRD patterns of the PAA-Cu-MOP hydrogel. (B) The TGA spectrum of the PAA-Cu-MOP.

Figure S2. (A) The micro morphology of the PAA-Cu-MOP hydrogel. (B) The SEM image of selected area of measured mapping image. (C-F) Mapping images of C, N, O, Cu.
**Figure S3.** Hygroscopicity retention of the PAA-Cu-MOP hydrogel under relative humidity of 81%.

**Figure S4.** The amount of Cu released from the PAA-Cu-MOP hydrogel on the agar plate.
Figure S5. (A) The antibacterial circle of the PAA-Cu-MOP hydrogel and Cu$^{2+}$ hydrogel to P.aeruginosa. (B)(C)(D) The antibacterial ratio of PAA-Cu-MOP hydrogel to P.aeruginosa and B.subtilis, *P<0.05, **P<0.01.

Figure S6. The SEM image of (A) native E.coli; (B) E.coli contacting with PAA-Cu-MOP hydrogel; (C) native S.aureus; (D) S.aureus contacting with PAA-Cu-MOP hydrogel.
Figure S7. The antibacterial efficiency of PAA-Cu-MOP hydrogel on five bacteria.

Figure S8. Hemolysis activity of PAA-Cu-MOP hydrogel.
**Figure S9.** H&E stained tissue sections shown for the organ tissues (heart, liver, kidney, lung, and spleen) from the SD rat treated with PAA-Cu-MOP hydrogel on day 14 in comparison with the healthy SD rat.

**Figure S10.** In vivo assessments of the PAA-Cu-MOP hydrogel for wound healing. Wound healing ratio for treating with physiological saline, recombinant human epidermal growth factor and PAA-Cu-MOP hydrogel, n=5, *P < 0.05, **P < 0.01.
**Figure S11.** (A) Histological images of tissues stained with H&E after treating on day 7. (B) Histological images of tissues stained with H&E after treating on day 14. Black triangle: blood vessel; green oval: fibroblast; black square: collagen fiber; black rhombus: hair follicle; purple hexagon: fat cell; black pentagon: sensory corpuscles (scale bar: 10 μm, magnification: 200×).

**Figure S12.** Immunofluorescent images of tissues on day 14 from wound (red, Ki-67 cell positive; blue, cell nucleus; scale bar: 10 μm, magnification: 200×)
Figure S13. Procoagulant ability of PAA-Cu-MOP Hydrogel, n=3, *P < 0.05, **P < 0.05, ***P < 0.001.

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