Supporting Information

Hydrogel directly assembled from copper metal-organic polyhedron for antimicrobial application

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Experimental Section

Reagents and Characterization

All chemical reagents were obtained from Energy chemical company used without further purification unless otherwise noted. Doubly 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 and transmission electron microscopy (TEM) imaging was performed on a JEM 2100 (TEM, JEOL, Japan). FTIR spectra were recorded in the range 4000–400 cm\(^{-1}\) on a Thermo Nicolet spectroscopy by using KBr pellets. XPS was performed on a K-Alpha X-ray photoelectron spectroscopy. 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. The fluorescent images were obtained on Olympus BX53, Olympus, Tokyo, Japan. The ICP-MS measurement was performed on Agilent 7700x.

Synthesis of Cu-MOP hydrogel

The Cu-MOP was prepared as previous reported. In brief, the mixture of \(\text{H}_2(5\text{-NO}_2\text{-1,3-BDC})\) ((\(\text{H}_2(5\text{-NO}_2\text{-1,3-BDC}) = 5\text{-Nitro-1,3- benzenedicarboxylic acid, } 0.2 \text{ mmol} \) and \(\text{Cu(NO}_3\text{)}_2\cdot3\text{H}_2\text{O} \text{ (0.2mmol) was dissolved in 5 mL solvents } (\text{CH}_3\text{OH:DMF}) =4(\nu):1(\nu)\) 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 \(\text{CH}_3\text{OH} \) several times. Then 30.0 mg Cu-MOP was added in 0.97 mL water and ultrasonic to obtain Cu-MOP hydrogel.

Binding Energy Calculation

To study more about the structure of our hydrogel, we used Packmol software to produce water molecules around the Cu-MOP. In the spherical space of 10 Angstroms from the center of the Cu-MOP, 700 waters are filled.
Next we used MOPAC2016 to optimize and calculate the energy of the water molecule, Cu-MOP and Cu-MOP with waters (PM6-D3H4X) to get the heats of formation, respectively. The enclosure MOPs were shown as follow: A for 5-hydroxyisophthalic acid, B for 5-nitroisophthalic acid and C for 5-bromoisophthalic acid.

**Agar Diffusion Test**

The agar diffusion test was used to visually present the antibacterial activity of Cu-MOP hydrogel according to a previously reported method and the antibacterial property of Cu-MOP hydrogel was compared with the antibiotic levofloxacin. Bacterial suspensions ($10^7$ CFU/ml, colony forming units is abbreviated as CFU) of *E. coli* and *S. aureus* were spread onto nutrient agar plates using a sterile pipette. The concentration of the Cu-MOP hydrogel and levofloxacin was $10 \mu g / ml$ 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.

**Time-Dynamic Antibacterial Test**

A time-dynamic antibacterial test was measured to reveal the antimicrobial effectiveness of the Cu-MOP hydrogel. *E. coli* and *S. aureus* bacterial suspensions ($10^7$ CFU/ml) were inoculated with 1 mg/ml of the Cu-MOP hydrogel. A blank control group was treated without the Cu-MOP hydrogel. Bacteria were sampled from 0 to 480 min at selected time intervals. Briefly, 100 μL 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 24h. 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.
Recycling test

We investigated the change of antibacterial effectiveness of the Cu-MOP hydrogel after using for several times. *E.coli* and *S.aureus* bacterial suspensions (10^7 CFU/ml) were inoculated with 1 mg/ml of the Cu-MOP hydrogel. A blank control group was treated without the Cu-MOP hydrogel. The bacterial solution was incubated for 80 min, and then 100 μL of bacterial solution was taken out and added into 900 μL of sterilized physiological saline. Next 100 μL of the diluted bacterial solution was cultured on the nutrient plates at 37 °C for 24 h and then counted the number of colonies and calculated the antibacterial ratio. Then the collected Cu-MOP hydrogels were washed with sterilized deionized water and employed for the next cycle of antibacterial tests. Each experiment was carried out three times in parallel.

ICP-MS measurement

To investigate the releasing process of copper species from 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 Cu-MOP hydrogel and then the culture medium solution was taken out with the contacting time for the ICP-MS measurement. 200 μL culture medium was taken out and 5 mL sterile water was added in it.

ROS generation

As an oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) was applied to identify the existence of the overall ROS levels inside the bacterial cells. This non-fluorescent compound DCFH-DA can be oxidized by cellular oxidants into the highly fluorescent compound 2’7’-dichlorofluorescin (DCF).[^3] *S.aureus* strains were prepared and co-cultured with the sample discs at three different time-points as described above for the antibacterial tests. For staining the adherent bacteria on the samples, the discs were removed and gently washed three times with the sterilized water. Subsequently, bacteria were stained in a 24-well plate with 500 μL DCFH-DA (10 μM) at 37 °C for 30 min and then photographed on a fluorescence microscope (Olympus BX53, Olympus, Tokyo, Japan) with an excitation wavelength of 488 nm and emission wavelength of 535 nm.[^4]

MTT test

The cytotoxicity of the Cu-MOP hydrogel was evaluated by a direct contact method according to ISO10993-5 standard test using L929 cells. Preparation of 96-well plates for cell culture: The L929 cells were counted using the Trypan blue exclusion method and quantified by an automated cell counter. The cells were plated in 96 well flat bottom plates using a multichannel pipette. Each 96-well plate was partitioned into columns in the following way: (1) culture media only; (2) cells incubated in culture medium alone; (3) test cells incubated in culture media with the Cu-MOP hydrogel samples with different concentrations (0.1, 0.5, 1, and 2 mg/ml). All of the conditions
were tested in triplicate wells, and each experiment was repeated three times. Simultaneously, L929 cells were prepared as single cell suspensions for testing by the Cu-MOP hydrogel as described in different test reports accomplishing ISO standards. At $1.0 \times 10^5$ cells per ml of DMEM, 50 µl were plated in each of the 96 wells and incubated under standard culture conditions for the 24 hours. The L929 cell cultures were washed three times with the corresponding culture media. For each washing, 200 µl of culture medium was added to each well, and then the plate was inverted with slightly vigorous shaking to discard the culture medium from each well. When the hydrogel was formed, moderately sterile PBS solution was transferred to the Cu-MOP hydrogel surfaces and rinsed the Cu-MOP hydrogel as described above. Thereafter, the fibroblast was seeded on it at a density of $1.0 \times 10^5$ cells well$^{-1}$. The cells were then incubated up to 72 h after which the cell viability was measured using MTT (5 mg/ml) assay. The results were expressed in cell viability (1day, 2day and 3day, respectively). Background absorbance at 650 nm was subtracted from the readings at 570 nm to obtain the final optical density (OD). The morphology of cells adhered on the Cu-MOP hydrogel was visualized by laser scanning confocal microscopy. All experiments were carried out in triplicate.

$$\text{Viab.} \% = \frac{OD_{570t}}{OD_{570b}} \times 100$$

$OD_{570t}$ is the mean value of the measure optical density of the test sample; $OD_{570b}$ is the mean value of the measure optical density of the blank. The lower the Viab. % value is, the higher the cytotoxic potential of the test item is. If viability is reduced to less than 70 % of the blank, it has a cytotoxic potential. The 50 % extract of the test sample should have at least the same or a higher viability than the 100 % extract; otherwise the test should be repeated.

**Acute toxicity test**

Eight Kunming mice (Experiment center of Quanzhou Medical College, Fujian, China), whose weights were between 20 g and 25 g, were chosen to conduct the experiment that the Cu-MOP hydrogel and physiological saline were injected in mice’s enterocoelia. Then we observed their behaviors and recorded their breathing, movement, defecation, eating, death and so on for 7 days. In the mean time, the weight of the mouse was weighed.

**Observation index of animal reaction in acute toxicity test**

<table>
<thead>
<tr>
<th>degree</th>
<th>reaction</th>
</tr>
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<tbody>
<tr>
<td>none</td>
<td>no toxic reaction</td>
</tr>
<tr>
<td>subtle</td>
<td>Mild symptoms, but no reduction in exercise, difficulty breathing or abdominal stimulation</td>
</tr>
<tr>
<td>medium</td>
<td>Abdominal stimulation, dyspnea, reduced exercise, drooping eyelids, diarrhoea (weight reduced</td>
</tr>
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Exhaustion, cyanosis and tremors, severe abdominal irritation, ptosis of the eyelids, breathing difficulties (sudden weight loss, generally below 15 g)

Skin irritation test

The biological evaluation of Cu-MOP hydrogel was tested by skin irritation, according to ISO10993-11 standard using Kunming mice. First, a total of 8 healthy Kunming mice (20-25 g, Experiment center of Quanzhou Medical College, Fujian, China) were shaved their backs, the depilatory area was 3 cm × 3 cm and then divided into two groups randomly. For group 1, the depilatory areas were treated with Cu-MOP hydrogel. For group 2, the depilatory areas were treated with physiological saline as a negative control. Thereafter, the depilatory areas were changed with Cu-MOP hydrogel or physiological saline every 24 h after cleared. The depilatory areas were examined and photographs were taken every day.

The scoring system of skin reaction

<table>
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<tr>
<th>The type of skin reaction</th>
<th>Irritation intensity</th>
<th>score</th>
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<td>0</td>
</tr>
<tr>
<td>Slight</td>
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<td>1</td>
</tr>
<tr>
<td>Erythema</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obvious</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Serious</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Reddish erythema with scab</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Edema</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Obvious, the area≤1mm</td>
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<td>2</td>
</tr>
<tr>
<td>Serious, the area≥1mm</td>
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<td>3</td>
</tr>
<tr>
<td>Edema, the area≥1mm</td>
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Statistics Analysis

Statistical Analysis was performed using the SPSS 19.0 software. The results were shown as mean (average) ± SD (standard deviation). The statistical techniques employed for analysis were T-test and one-way ANOVA.
Figure S1. Rheological measurements of the Cu-MOP hydrogel: (A) 3% content Cu-MOP hydrogel stocked for 12 days; (B) 6% content Cu-MOP hydrogel for 8 days.

Analysis: The mechanical strength of the Cu-MOP hydrogel can be modulated by increasing the amount of the Cu-MOP in water and the rheological measurement of the higher content Cu-MOP hydrogel was conducted (Figure S1B). The result indicated that the storage modulus and loss modulus increased after increasing the concentration of Cu-MOP in water, suggesting the mechanical strength of the hydrogel can be modulated by facilely adjusting the amount of Cu-MOP.

Analysis: The stability of Cu-MOP hydrogel in water was tested by rheometry measurement and the result indicated that when Cu-MOP hydrogel was stocked for 12 days, its storage modulus and loss modulus were larger than the ones of as-prepared hydrogel. This result may due to the evaporation of some water and induced stronger interactions in the Cu-MOP after long time storage. Hence, the Cu-MOP hydrogel in water has a good stability.

Figure S2. PXRD patterns of the Cu-MOP, Cu-MOP hydrogel and Cu-MOP hydrogel after a week.
Figure S3. The TGA spectrum of the Cu-MOP and Cu-MOP hydrogel.

Figure S4. (A) FTIR spectrum of the 5-Nitroisophthalic acid, Cu-MOP and Cu-MOP hydrogel. (B) The XPS spectrum of the Cu-MOP and Cu-MOP hydrogel.
Figure S5. (A) The FTIR spectrum of the 5-Nitroisophthalic, Cu-MOP and Cu-MOP hydrogel, respectively. (B) The FTIR spectrum of the Cu-MOP hydrogel and the Cu-MOP hydrogel stocked in room temperature after a week.

Figure S6. The narrow XPS spectra of Cu-MOP and Cu-MOP hydrogel (A) C 1s; (B) O 1s; (C) N 1s; (D) Cu 2p.

Analysis: X-ray Photoelectron Spectroscopy (XPS) measurement of Cu-MOP and Cu-MOP hydrogel was further measured to study their chemical composition and chemical states. As shown in the survey spectrum, Cu-MOP
and Cu-MOP hydrogel had the same elementary composition (Figure S4B). Binding-energy peaks were observed for Cu 2p3/2 and Cu 2p1/2 at 934.8 and 954.5 eV, respectively, which are typical values for Cu(II). Moreover, the two shakeup satellite lines were observed at 944.1 and 963.0 eV were characteristic of Cu(II) with the d⁹ configuration in the ground state, thus indicating the paramagnetic chemical state of Cu(II) which further confirm that Cu²⁺ is coordinated with O atoms of 5-Nitroisophthalic acid (Figure S6D). The peak at 531.9 eV of O1s can be deconvoluted into two peaks of -O- (532.8 eV) and -N=O (531.8 eV) (Figure S6B).

Figure S7. The SEM image of the Cu-MOP hydrogel stocked in room temperature after a week.
Figure S8. (A) Plate photographs of *E. coli* on agar plate treated with Cu-MOP hydrogel (point 1), Cu(NO$_3$)$_2$$\cdot$3H$_2$O(point 2), antibiotic levofloxacin (point 3) and 5-Nitroisophthalic acid (point 4). (B) The fluorescence microscope images of *E. coli* alone stained with Acridine Orange and Ethidium Bromide (AO/EB). (C) The fluorescence microscope images of *E. coli* after contacting with the Cu-MOP hydrogel stained with AO/EB. (D) Plate photographs of *S. aureus* on agar plate treated with Cu-MOP hydrogel (point 1), Cu(NO$_3$)$_2$$\cdot$3H$_2$O (point 2), antibiotic levofloxacin (point 3) and 5-Nitroisophthalic acid (point 4). (E) The fluorescence microscope images of *S. aureus* alone stained with AO/EB. (F) The fluorescence microscope images of *S. aureus* after contacting with the Cu-MOP hydrogel stained with AO/EB. Green fluorescent color demonstrated live cells, whereas red fluorescent color depicted dead cells with destroyed cell membrane. Scale bar: 20 μm.
Figure S9. Antibacterial activity of Cu-MOP hydrogel measured by agar plate tests compared with levofloxacin and Cu$^{2+}$ (A) Gram-negative bacteria E.coli; (B) Gram-positive bacteria S.aureus (n=3, *$P$ < 0.05, **$P$ < 0.01). The antimicrobial circle diameter of Cu-MOP and levofloxacin against E.coli and S.aureus were significantly affected by the time ($P$ < 0.01); the antimicrobial circle diameter of Cu$^{2+}$ against E.coli was affected by the time ($P$ < 0.01), but antimicrobial circle diameter of Cu$^{2+}$ against S.aureus was not significantly affected by the time.
Figure S10. The antibacterial lasting time of Cu-MOP hydrogel to E. coli: (A) inhibition zones images, (1) is water and (2) is the Cu-MOP hydrogel; (B) histogram data from the plate (n=3, There was no significant difference between each two groups, and the antimicrobial circle diameter was not significantly affected by the time); and S. aureus: (C) inhibition zones images, (1) is water and (2) is the Cu-MOP hydrogel; (D) histogram data from the plate (n=3, There was no significant difference between each two groups, and the antimicrobial circle diameter was not significantly affected by the time).
Figure S11. (left) The amount of Cu released from the Cu-MOP hydrogel to the agar plate. (middle and right) The pictures of the degrading properties of Cu-MOP hydrogel.

Analysis: The released copper ions in cultural medium can attach with bacteria’s membrane to damage its integrity and permeability through the electrostatic interaction. Then, the copper ions may cause fracture of bacterial membrane or other damages to bacteria which will kill them. Moreover, the valence state changes will highly promote the production of ROS, which cause the bacteria dead\cite{6}.

Figure S12. SEM images of E.coli: (A) normal E.coli; (B) E.coli after acting with Cu-MOP hydrogel. SEM images of S.aureus: (C) normal S.aureus; (D) S.aureus after acting with Cu-MOP hydrogel.
Figure S13. (A) The characteristic UV adsorption spectrum of DNA at 260nm. (B) The characteristic UV adsorption spectrum of β-galactosidase at 420nm.
**Figure S14.** DCFH-DA assay for measurement of intracellular ROS production: *E. coli* (A) negative contrast with *E. coli* alone; (B) experiment group with Cu-MOP hydrogel in the test. *S. aureus* (C) negative contrast with *S. aureus* alone; (D) experiment group with Cu-MOP hydrogel in the test. Scale bar: 50 μm.

Analysis: It has been reported that copper and copper complexes have been widely used in antibacterial field with the advantage of broad antimicrobial spectrum and low toxicity [6-11]. At the same time, copper is also an important and necessary trace element with its biological functions. It is a cofactor of some enzymes including cytochrome oxidase, superoxide dismutase and so on, which were involved in many important physiological processes such as energy metabolism, cell signaling and gene expression [12]. In addition, it also has been reported that copper ions could stimulate proliferation of human umbilical artery, vein endothelial cells and mouse osteoblasts [12,13]. However, the excess of copper ions are considered to be cytotoxic [14,15]. On the other hand, the reactive oxygen species (ROS) generation causes a series of damages such as protein denaturation, lipid peroxidation [14,15]. Nevertheless, ROS has been identified as an important chemical mediator in the regulation of signal transduction processes involved in cell growth and differentiation. ROS has been shown to stimulate cell proliferation of a variety of preparations including smooth muscle cells, fibroblasts, amnion cells and aortic endothelial cells [16-19]. Moreover, copper ion activated the signaling pathways of MAPK-ERK, PI3K-Akr to promote cell proliferation by increasing ROS [19,20]. Hence, based on the structure of MOP [21], the Cu-MOP hydrogel could control the release rate of copper ion to remain a nontoxic concentration. Overall, the phenomenon is not conflicting.
Figure S15. The MTT experiment was measured by ISO10993-5 standard test (L929 cells) to detect the toxicity of the Cu-MOP hydrogel to animal cells (n=8, *p < 0.05, **p < 0.01, ***p < 0.001).

Figure S16. The acute toxicity test of Cu-MOP hydrogel was examined on Kunming mice.
Figure S17. Skin stimulative experiment of the Cu-MOP hydrogel was detected on Kunming mice to research its damage on skin. Up: control group; Down: experiment group.

Table S1. The theoretical binding energy calculation data of three MOPs, respectively.

<table>
<thead>
<tr>
<th>Substituent group</th>
<th>Formation heat without water (KJ/Mol)</th>
<th>Formation heat with water (KJ/Mol)</th>
<th>Binding energy (KJ/Mol)</th>
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<tbody>
<tr>
<td>-Br</td>
<td>-1.75×10^4</td>
<td>-2.12×10^5</td>
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<tr>
<td>-NO_2</td>
<td>-1.82×10^4</td>
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<td>-OH</td>
<td>-2.26×10^4</td>
<td>-2.15×10^5</td>
<td>4.54×10^4</td>
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References