Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

This journal is © The Royal Society of Chemistry 2024

Supplementary Information

Enhancing antibacterial properties by regulating valence configurations of copper: a focus on Cu-carboxyl chelates †

QiupingQian[a,b,‡], Jige Chen[d,e,f,‡], Mingming Qin[b,‡], Yu Pei[a], Chunxiu Chen[a], Dongping Tang[b], Pooyan Makvandi[g], Wei Du[e,f], Guoqiang Yang[a,b,f,*], Haiping Fang[c,e,*],Yunlong Zhou[a,b,*]

[a]Joint Center of Translational Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China, gqyang@iccas.ac.cn

[b]Zhejiang Engineering Research Center for Tissue Repair Materials, Wenzhou Institute, University of Chinese Academy of Sciences, Wenzhou 325000, China, zhouyl@ucas.ac.cn

[c]School of Physics and National Engineering Research Center of Industrial Wastewater Detoxication and Resource Recovery, East China University of Science and Technology, Shanghai, 200237, China, fanghaiping@sinap.ac.cn

[d]Shanghai Advanced Research Institute, Chinese Academy of Sciences, Shanghai 201210, China

[e]Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China

[f]University of Chinese Academy of Sciences, Beijing, 100049, China

[g]The Quzhou Affiliated Hospital of Wenzhou Medical University, Quzhou People' 's Hospital Quzhou, Zhejiang 324000, China

[‡]These authors contribute equally to this work.

Contents

1-Methods

1.1 Materials and reagents.

Acrylic acid (AA, > 99%) and Itaconic acid (IA, > 99.0%) were obtained from Macklin. Nutrient broth (NB) and Tryptic soy broth (TSB) were purchased from Beijing Solarbio Science & Technology Co., LTD. AA and IA underwent distillation under reduced pressure prior to use. The 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-arboxanilide (XTT sodium salt, ≈90%), alcohol (≈95%), and PBS buffer were sourced from Aladdin. The Live/Dead Baclight bacterial viability kit was purchased from Thermo Fisher Scientific, while HaCat cells and cell culture fluid were acquired from Shanghai Fuheng Science & Technology Co., LTD. Gibco Cell Culture Reagents and Cell Counting Kit-8 (CCK8) were purchased from Dongren Chemical Technology (Shanghai) Co., LTD. Deionized water (18.2 MΩ cm-1) was obtained from a Milli-Q water-purification System. All other chemicals were used without further purification.

1.2 Cell strains.

The two bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC). The Escherichia coli (*E. coli,* ATCC 8379) and staphylococcus aureus (*S. aureus,* ATCC 12600) were obtained from the Shanghai Bioresource Collection Center (SHBCC). Mouse fibroblast Cells (L929) were used as a mammal cell model.

1.3 Characterization

Fourier Transform Infrared Spectroscopy (FTIR, Tensor II) was utilized for the characterization of various polymers. Scanning Electron Microscopy (SEM, SU8010, Hitachi) and Transmission Electron Microscopy (TEM, FEI Talos) were employed to observe bacterial morphology. X-ray Photoelectron Spectroscopy (XPS, K-Alpha Thermo Fisher Scientific) was applied to characterize the composition of polymers and copper chloride. Gel Permeation Chromatography (Malvern TDA-305) was used to determine polymer molecular weight. The Nuclear Magnetic Resonance Spectrometer (QUANTUM-I-400MHz) was utilized for the characterization of carboxyl copolymers. A Full Wavelength Microplate Reader (ThermoFisher Varioskan LUX) assessed the antibacterial properties and cytocompatibility of polymers and Cu^{2+}. An ATP fluorescence detector was purchased from Shandong Fengtu Internet of Things Technology Co. LTD. ROS levels were monitored using a multimode microplate reader (Thermo Scientific Varioskan LUX). The excitation wavelength was set at 488 nm. ROS production in bacterial cells treated with PAI-Cu was detected using the ROS indicator 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) dye (Beyotime, China).

1.4 Materials synthesis: acrylic and itaconic acid copolymer (PAI)

The thermal polymerization method was employed for the synthesis of the aforementioned polymers. In a concise synthesis procedure, 10 mmol of monomers, including polyacrylic acid (PAA) and polyitaconic acid (PIA), were added to 40 ml of redistilled water. Sodium sulfite and ammonium persulfate, in a 4% mass ratio relative to the monomer, were added and stirred to form a homogeneous solution. For

the synthesis of PIA, sodium hydroxide in an equal mole of itaconic acid (IA), was introduced into the system during free radical polymerization. In the case of synthesizing PAI (molar ratio of AA to IA is 1:1), IA was maintained at 5 mmol. Sodium hydroxide, equivalent to the molar sum of IA and AA, was introduced into the system during free radical polymerization. The solutions were oxygenated with Argon gas for 40 minutes, followed by polymerization at 70 ℃ for 24 hours. For PIA and PAI, the polymerization time was extended to 48 hours. Subsequently, the synthesized polymers underwent dialysis for five days with deionized water and diluted hydrochloric acid solution. All solutions were ultimately freeze-dried.

1.5 Measurements of surrounding copper ions concentration in composite solutions of PAI-Cu.

To determine the concentration of surrounding copper ions in composite solutions of PAI-Cu, a solution of PAI was directly combined with a cupric chloride solution to form the PAI-Cu composite solution. The concentration of the PAI solution was maintained at 100 μg/mL, while the copper ion concentration was set at 100 μM. The mixed solution underwent agitation at 180 rpm for 30 minutes in a constant-temperature bath at 294 K. Subsequently, the solution was subjected to centrifugation and vacuum filtration through a microfiltration system with a pore size of 0.22 μm to eliminate the PAI-Cu complex. The copper concentration in the filtered solution was determined using an Agilent 720ES inductively coupled plasma optical emission spectrometer (ICP-OES).

1.6 Bacterial culture and Evaluation of antibacterial properties.

The experimental procedure involved two main steps. Initially, 1–2 individual colonies were inoculated into fresh TSB/LB and incubated at 37 °C for 16–18 hours to reach the stationary phase. A 100 μL culture was then diluted 100-fold with fresh TSB/LB and regrown at 37 °C to mid-log phase (OD_{600} = 0.5–0.7). Subsequently, bacterial cells were harvested, washed once with sterile PBS via centrifugation (6000 rpm for 3 min at 4 °C), and adjusted with sterile PBS to ~10^8 CFU/mL. Different water-soluble ligands, copper ions, and their mixture in sterile 0.9% NaCl solution (0.5 mL) were added to 0.5 mL of the adjusted bacterial suspension to achieve a final bacterial inoculum size of ~10^8 CFU/mL. Parallel experiments were conducted in three groups. The resulting mixture was incubated at 37 °C for 1 hour, and the XTT reduction assay quantified the metabolic activity of the treated bacteria. The XTT reduction assay involved freshly dissolving XTT in PBS or NaCl solution at a final concentration of 1 mg/mL. A 400 μM menadione alcohol solution was also prepared. Before each assay, the XTT solution was mixed with the menadione solution at a volume ratio of 20:1. The XTT-menadione solution was added to each well, and the microtiter plates were incubated in the dark for 2 hours at 37 ℃. Following incubation, all groups were subjected to centrifugation at 6000 rpm for 3 min to remove the bacteria, and the color change of the supernatants, resulting from XTT reduction, was measured at 490 nm using a Thermo Scientific Varioskan LUX Multimode Microplate Reader.

Additionally, bacterial cells were harvested, washed once with sterile PBS via centrifugation (6000 rpm for 3 min at 4 °C), and adjusted with sterile PBS to ~10^8 CFU/mL. The water-soluble ligand PAI, copper ions, and their mixture in sterile 0.9% NaCl solution (0.5 mL) were added to 0.5 mL of the adjusted bacterial suspension to achieve a final bacterial inoculum size of ~10^8 CFU/mL. Parallel experiments were conducted in three groups. The resulting mixture was incubated at 37 °C for 0.5 and 1 hour. Subsequently, the ATP fluorescence detector was used to test microbial quantity within 150 s based on the detection of bacteria's adenosine triphosphate (ATP).

Initially, 100 μL of bacteria with 2×TSB/LB at a concentration of 10^3 CFU/mL were cultured with varying concentrations of PAI-Cu. After co-culturing for 12 hours, OD600 testing was conducted, followed by the analysis of MIC_{50}.

1.7 Antibacterial activity enhancement test against *S. aureus* and *E. coli* by colony-forming assays

Bacterial cells were cultivated in TSB/LB medium under shaking at 180 rpm for 18 hours until they reached the stationary growth phase. Subsequently, they were harvested and washed twice with sterile PBS through centrifugation (6000 rpm for 3 min at 4 °C) and adjusted with sterile PBS to ~10^6 CFU/mL. For the bactericidal property assessment, PAI-Cu in sterile 0.9% NaCl solution (0.5 mL) was added to 0.5 mL of the adjusted bacterial suspension to achieve a final bacterial inoculum size of ~10^6 CFU/mL. Parallel experiments were conducted in three groups. To quantify the number of viable bacterial cells, each sample was diluted with standard serial dilution

(~10^4 CFU/mL), and 50 μL of the suspension from each sample was uniformly spread on LB agar plates. The number of bacterial colonies was counted after incubation at 37 ℃ for 18/24 h.

For the Antibacterial Activity Enhancement Test, a reference solution of about 17 μM CuCl was postulated to represent the surrounding copper ions in the PAI-Cu solution. PAI-Cu (PAI 100 μg/mL, Cu^{2+} 100 μM) in sterile 1% medium solution (0.5 mL) was added to 0.5 mL of the adjusted bacterial suspension to achieve a final bacterial inoculum size of ~10^8 CFU/mL. Parallel experiments were conducted in three groups. The quantification of viable bacterial cells followed the same process as described earlier. The antibacterial activity was calculated as the ratio of the colony number in the control experiment divided by the colony numbers in the antibacterial experiments. The enhancement ratio of antibacterial activity (in Fig. 4d and Fig. 4f) was then calculated as the ratio of PAI-Cu antibacterial activity divided by the antibacterial activity of its surrounding copper ions.

Survival rate = Treatment group (colony number)/Control group (colony number)×100%

Antibacterial efficiency = (1-Treatment group (colony number)/Control group (colony number))×100%

1.8 Monitoring the generation of reactive oxygen (ROS)

To examine whether ROS is produced in the process of bactericidal effect by PAI-Cu, fluorescence-based ROS detection assays were conducted. The ROS generation

was probed with DCFH-DA. The treated bacteria (*S. aureus* and *E. coli*) were initially incubated with 10 μmol L^{-1} DCFH-DA in the dark at 37 °C for 60 minutes. Following the co-incubation, the fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

1.9 Monitoring the generation of • OH.

The synthesized PAI-Cu was utilized for generating hydroxyl radicals (•OH) using Electron Spin Resonance (ESR) spectroscopy at room temperature. To initiate •OH generation, PAI-Cu was incubated with an H2O2 aqueous solution, and the resulting •OH was trapped as a spin adduct DMPO/•OH. In brief, ESR measurements were conducted by incubating PAI-Cu/Cu (II) with the H_2O_2 aqueous solution, followed by the addition of 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Subsequently, the ESR spectra were recorded after 2 minutes of incubation.

1.10 Perxidase (POD)-like activity of PAI-Cu.

The peroxidase (POD)-like activity of PAI-Cu was assessed using 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate in the presence of hydrogen peroxide (H_2O_2). In a standard test, TMB (1 mM, DMSO, 20 μL), H_2O_2 (10 mM, 20 μL), PAI-Cu (100 μg mL^{-1}, 1 mM, 160 μL), and copper ions (1 mM) were thoroughly mixed by pipetting at room temperature in a PBS buffer solution (pH 7.2-7.4). Four different concentrations of hydrogen peroxide were obtained by diluting the hydrogen peroxide solution. As the reaction proceeded, a blue color developed, and the absorbance

changes of the reaction mixture at 652 nm were promptly measured in scanning mode to evaluate the POD-like activity (n = 3 for each group).

1.11 Evaluation of redox processes of materials and bacteria by cyclic voltammetry

A 4 mm glassy carbon electrode, prepared through polishing, ultrasonic treatment, and washing, was employed in a sodium chloride solution containing ~10^6 CFU/mL of bacteria along with PAI/Cu (II)/PAI-Cu. Scanning voltammetry was conducted at a scanning rate of 0.04 V/s in the potential range of -0.6 to 0.6 V, and the resulting voltammetric diagram was recorded.

1.12 Nucleic acids and protein leakage from bacteria

The release of nucleic acids and proteins from *S. aureus* and *E. coli* treated with PAI-Cu was quantified to investigate the antibacterial mechanism and discern the contribution of different sterilization mechanisms. The measurement involved assessing the optical density at 260 nm (for nucleic acids) and 280 nm (for proteins) to gauge the leakage of these cellular components.

1.13 Bacterial morphology study by SEM.

Bacterial morphology was studied using Scanning Electron Microscopy (SEM). SEM images were captured to analyze the morphologies of bacteria treated with PAI-Cu. The procedure involved dropping the treated bacteria onto silicon wafers, followed by fixation with 2.5 wt% glutaraldehyde for 4 hours at room temperature. Subsequently, the samples underwent dehydration in a series of ethanol solutions (30 vol%, 50 vol%,

70 vol%, 90 vol%, and 100 vol%, 30 minutes each step). After drying under a stream of nitrogen gas, the samples were prepared by metal spraying and observed using a Hitachi S 8010 instrument at 3.0 kV.

1.14 Bacterial morphology study by TEM.

Transmission Electron Microscopy (TEM) images were captured to examine the morphologies and element distribution of bacteria treated with PAI-Cu. The bacterial cells underwent fixation with 5% glutaraldehyde for 2 hours at room temperature, followed by additional fixation at 4 °C overnight. After being washed thrice with sterile PBS (0.1 M, pH = 7.2), the cells were stained with a 2% osmium tetroxide solution (1 mL) overnight. Subsequently, the sample was rinsed thrice with sterile PBS (0.1 M, pH = 7.2) (30 minutes each, 1 mL), dehydrated in a series of graded ethanol solutions (10, 30, 50, 70, 80, 95, and 100% ethanol in water), and then rinsed thrice with acetone (30 minutes each, 1 mL). For bacterial cell structural integrity assessment, the samples were incubated in a resin solution for 1 hour (with a resin-to-acetone volume ratio of 1:1) and then for 3 hours (with a resin-to-acetone volume ratio of 3:1). For observing the internal structure of bacteria, the sample was embedded in fresh 100% resin for approximately 36 hours. A new batch of resin (750 μL) was added, and the sample was cured at 70 °C for two days. Finally, 70-nm-thick samples were sliced off using an Ultramicrotome equipped with a diamond knife and placed on copper grids for TEM imaging.

1.15. In Vitro Cytotoxicity Assay of PAI-Cu

The cell toxicity of PAI-Cu was assessed using a CCK-8 assay. Briefly, L929 cells were seeded into a 96-well plate at a density of 5000 cells per well. The 96-well plates were then placed in the incubator for 24 h at 37 °C and 5% CO_2. Subsequently, the supernatant was removed, and different doses of Cu(II) and PAI-Cu dissolved in a medium (0, 50, 100, 500 μM Cu(II)) were added to the cells, followed by incubation for 24 h (n = 3). After 24 h, a 10% volume of CCK-8 solution in a serum-free medium was added to the cells. After incubating for 120 min at 37 °C, the absorbance value (λ_{450} nm) of each well was measured using a microplate reader (Varioskan LUX). The cytotoxicity of different treatment groups was calculated using the following equation:

Cell viability%=(As-Ab)/(Ac-Ab) × 100%

Where: As is the absorbance value of the Cu (II)/PAI/PAI-Cu treatment group. Ac is the absorbance value of the blank group without PAI or Cu (II).Ab is the absorbance value of the blank group without cells and PAI or Cu (II).

1.16 In *vivo S. aureus* infected model

All animal experiments were conducted in accordance with the guidelines of the Wenzhou Research Institute of the Chinese Academy of Sciences and were approved by the Institutional Animal Care and Ethics Committee (Approval No. WIUCAS24011004).

Eight-week-old Sprague-Dawley (SD) rats weighing 250-300 g were randomly chosen for the wound infection model. An *S. aureus*-infected skin model was

established by inoculating rats under isoflurane anesthesia or chloral hydrate. The rat model of an infected wound involved removing back hairs and creating a full-thickness wound (approximately 10 mm in diameter) on its upper back using a round punch. The wounds were infected with 100 μL of 10^6 CFU/mL *S. aureus* for 24 h to develop the infected wound. The rats were categorized into four treatment groups, where the wounds were treated with PBS, Cu (II), PAI, and PAI-Cu. All treated rats were housed in separate cages and closely monitored during the experimental period. After treatment, wound tissue was dissected and placed in an equal volume of sterile PBS. The number of *S. aureus* was determined by measuring the bacterial load in the infected wound area on day 5. Sterile cotton swabs were used to wipe the wound and washed with a PBS solution of the same volume. The bacterial cell burden on each rat was evaluated by the LB-agar plate dilution method.

Histopathology Assessment: At the end of treatment, histological analysis of S. aureus-infected wound tissue was performed. Firstly, the rats were sacrificed by intraperitoneal injection of Zoletil 50 with 0.3 mg per kg body weight. Secondly, wound tissue and organs from rats in different groups were fixed in a 4% formalin solution and embedded in paraffin. Thirdly, tissues were sectioned into 3 μm thick sections. Fourthly, tissue sections were dewaxed in xylene and underwent gradient dehydration using a graded series of ethanol. Finally, sections were stained with hematoxylin and eosin (H&E) and scanned using Pannoramic NIDI (3D HISTECH). Rat blood was collected from the posterior orbital plexus and stored in an

anticoagulant tube containing sodium citrate. ELISA was performed to test the values of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β).

1.17 Statistical analysis

All experiments were repeated in triplicate, and the results were analyzed with SPSS (version 15). Data are presented as means± standard deviation. Statistical significance differences between group pairs were considered significant at $p<0.05$. The symbols *, **, and *** stand for $p<0.05$, $p<0.01$, and <0.001, respectively.

2-Supplementary figures

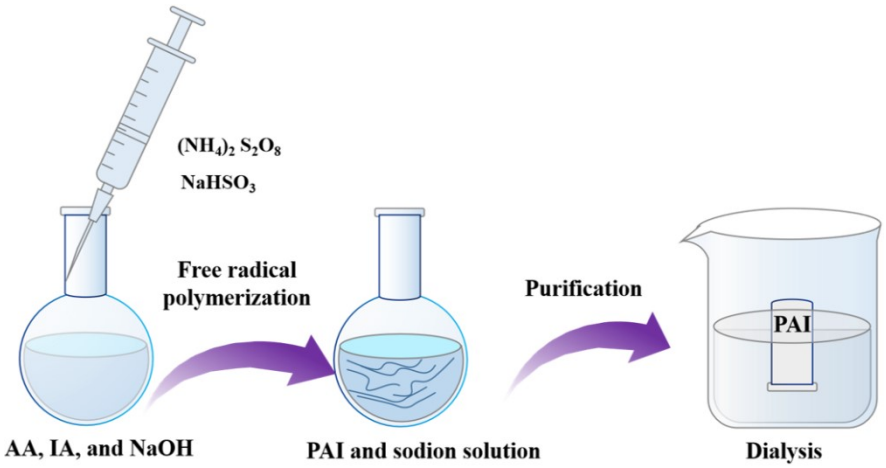

Fig. S1 Schematic Synthesis of PAI.

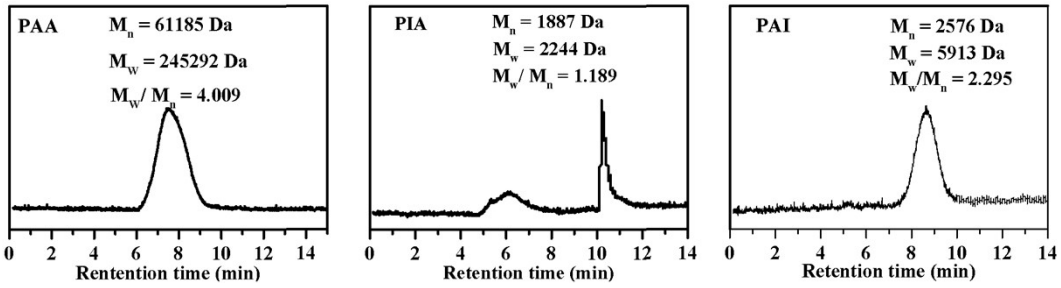

Fig. S2 GPC traces of water-soluble polymers with different functional groups.

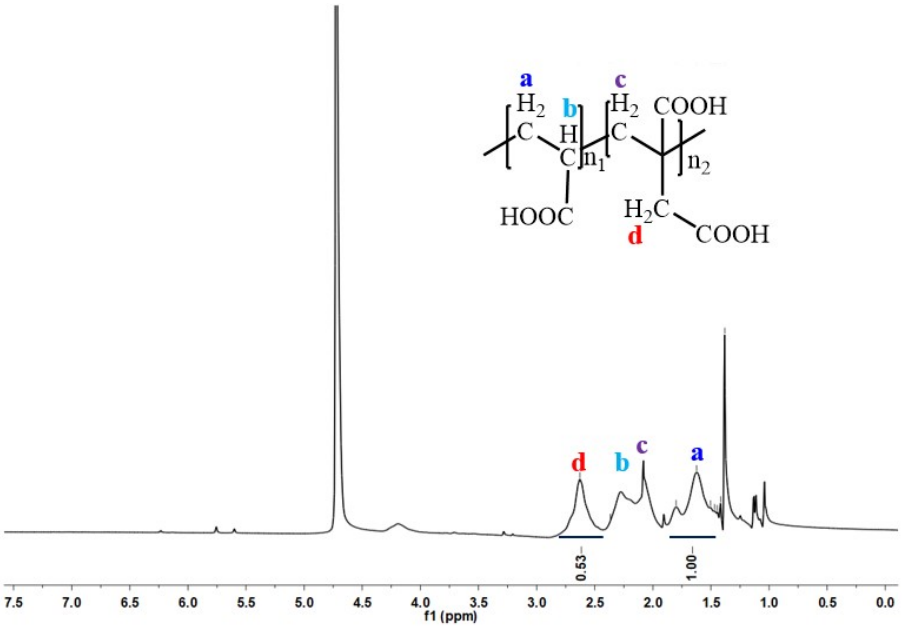

Fig. S3 ^{1}H-NMR spectrum of PAI

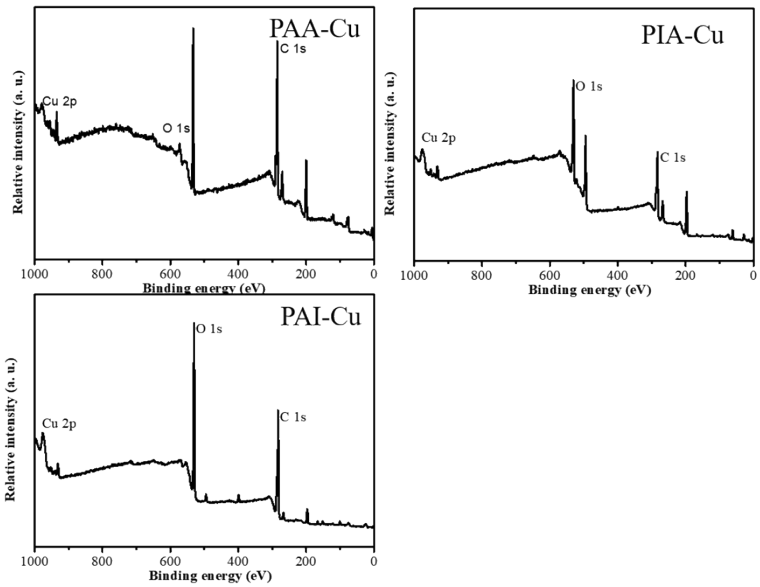

Fig. S4 XPS spectra of PAA-Cu, PIA-Cu and PAI-Cu.

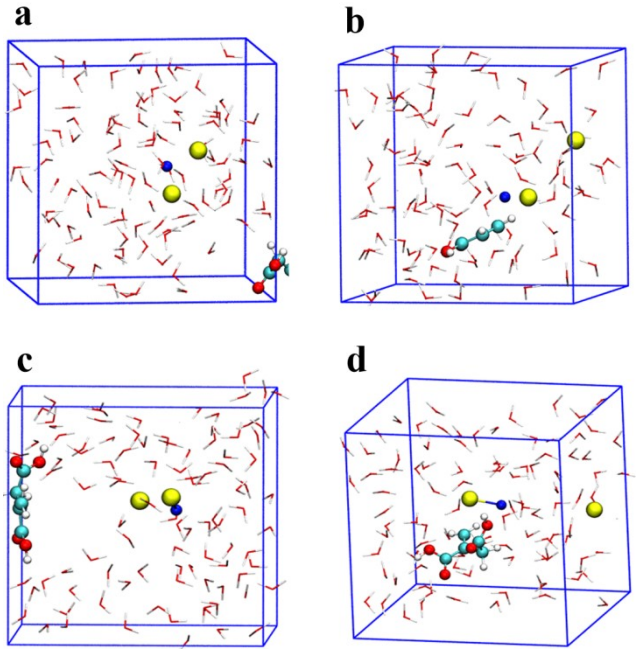

Fig. S5 Two snapshots of the Cu^{2+} state (a) and the Cu^{+} state (b) in the presence of an X molecule (AA) and water molecules. The blue, yellow, cyan, red, and white balls denote calcium, chloride, carbon, oxygen, and hydrogen, respectively. Two snapshots of the Cu^{2+} state (c) and the Cu^{+} state (d) in the presence of a Y molecule and water

molecules. The blue, yellow, cyan, red, and white balls denote calcium, chloride, carbon, oxygen, and hydrogen, respectively.

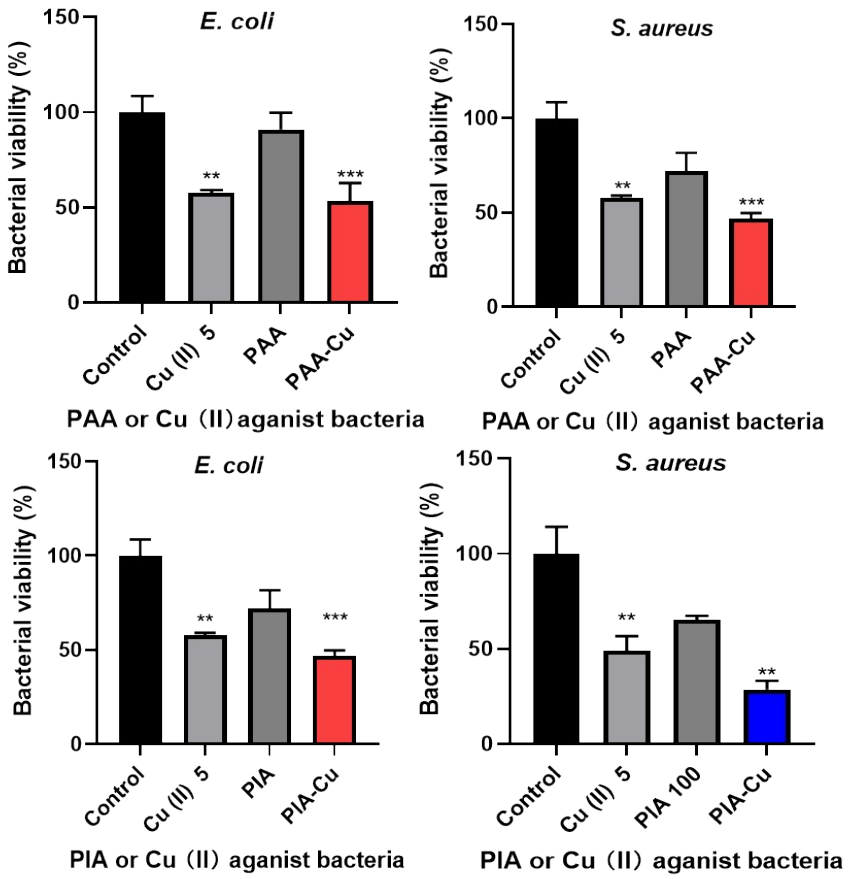

Fig. S6 Histograms of the PAA, PIA, or Cu (II) against *E. coli and S. aureus*.

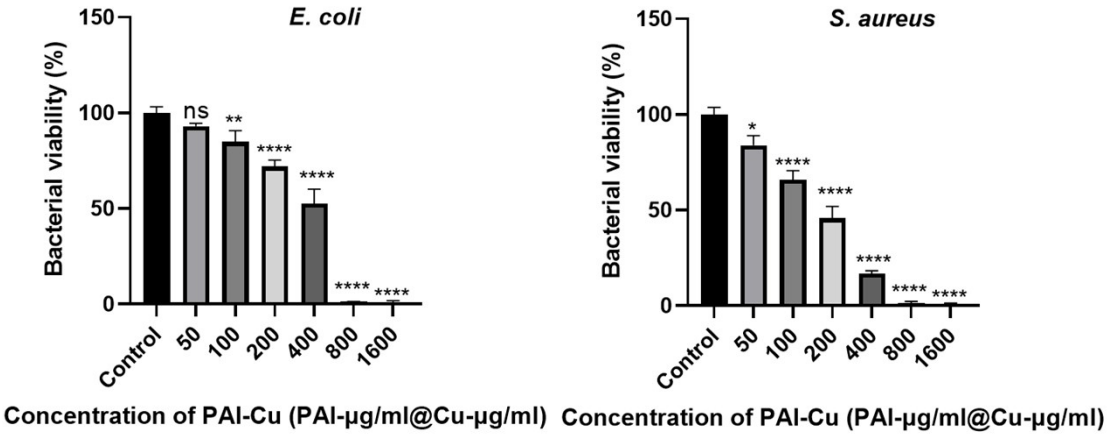

Fig. S7 Histograms of the PAI-Cu against *E. coli* and *S. aureus* for MIC (Minimum Inhibitory Concentrations,12 h culture).

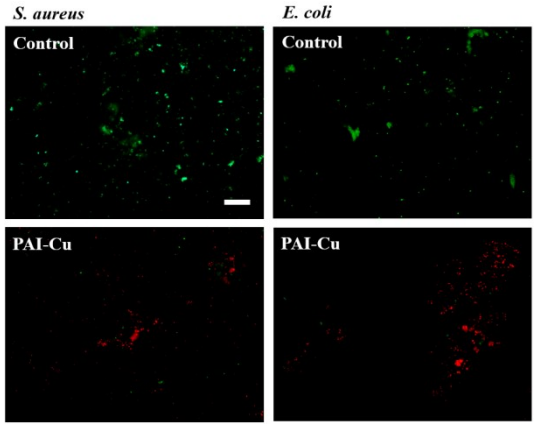

Fig. S8 Viability fluorescent staining of *S. aureus* and *E. coli* observed by fluorescence microscope. Scale bar: 50 μm.

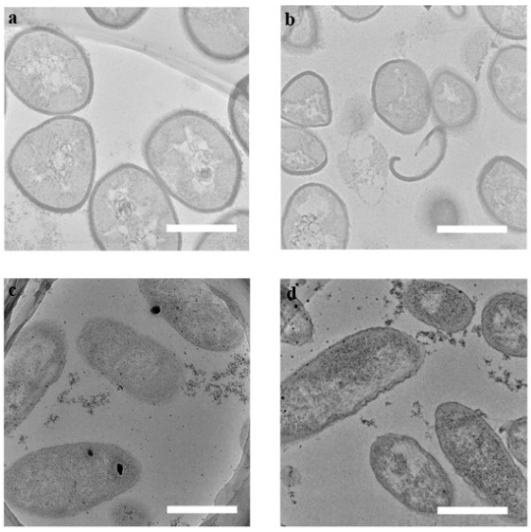

Fig. S9 Bacterial ultrathin section of TEM images by PAI-Cu treatment, **a**, *S. aureus* after co-culture with PBS solution. **b**, after PAI-Cu treatment. **c**, *E. coli* after co-culture with PBS solution, **d**, after chelate treatment, scale bar: 500 nm.

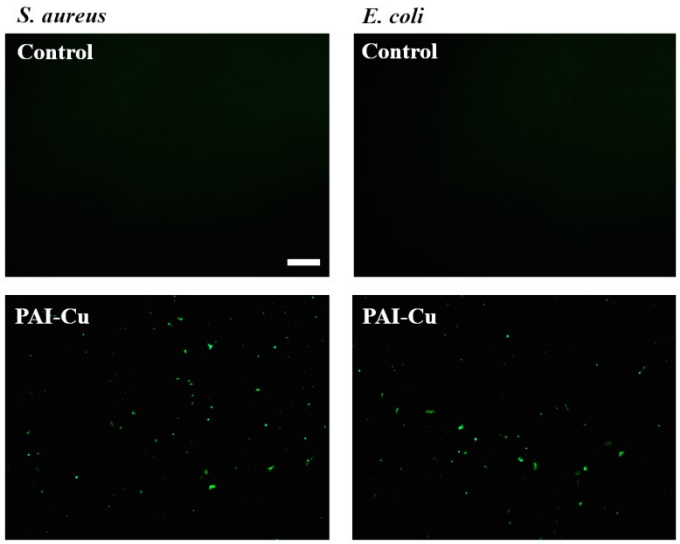

Fig. S10 Fluorescence microscope images of ROS generation in *S. aureus* and *E. coli* treated with PAI-Cu，Scale bar: 50 μm.

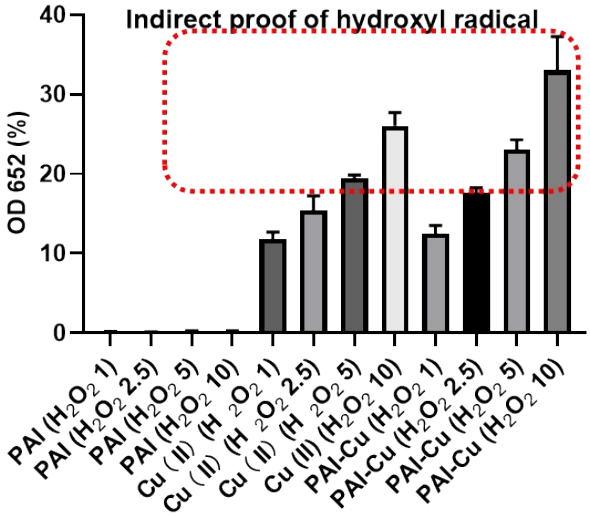

Fig. S11 The peroxidase-like activity of different concentrations of H_2O_2 in the presence of Cu (II) or PAI-Cu.

We infer that PAI is essential for transforming Cu (I) species in the stable system, which can be carried out through the coordination between Cu (I) and the carboxyl

group on the molecular chain of PAI. Therefore, cyclic voltammetry (CV) was selected to study the effect of bacteria and PAI on the redox process of Cu (II) on the electrode surface. As shown in Figure 5f-h, divalent copper ions in the electrode can easily obtain electrons and become Cu (I) or Cu (0). At the same time, Cu (0) can be oxidized to Cu (I) and further oxidized to PAI in a Cu (II) solution, which can directly form a chelate compound with Cu (II) (formula 1). Whether there are bacteria or not, the reduction potential of Cu (II) is reduced, which is more conducive to the stability of Cu (I) species in the system.

$$PAI + Cu^{2+} \rightarrow PAI{-}Cu\ (II) \quad (1)$$

$$O_2 + e^{-} \rightarrow \cdot O_2^{-} \quad (2)$$

From Fig. 2g, there are a large number of apparent low valence copper in the XPS spectrum of PAI-Cu, which directly shows that it can improve the stability of Cu (I) species. The multivalent Cu of Cu (I)/Cu (II) coordinated with the carboxyl group in PAI participates in the active site of promoting redox reaction. The active site originally absorbs dissolved O_2, and then converts Cu (I) into Cu (II), reducing O_2 to $\bullet O_2^-$ (another part comes from O_2 to obtain electrons from the bacterial surface) or hydrogen peroxide (COOH in PAI can provide a large amount of H^+, which is more conducive to the timely conversion of $\bullet O_2^-$ to hydrogen peroxide, meanwhile, when PAI-Cu interacts with bacteria, physiologically relevant antioxidants in bacteria are catalyzed to produce the H_2O_2 in the presence of O_2 [14-16], as shown in Equations 2 and 3.

$$2 \cdot O_2^- + 2H^+ + e^- \rightarrow H_2O_2 \quad (3)$$

Under appropriate conditions, electrons in the system based on $\bullet O_2^-$ are transferred to Cu(II) species and PAI-Cu, which recovers to a univalent state in the catalytic process.

$$PAI{-}Cu\ (II) + \cdot O_2^- \rightarrow PAI{-}Cu\ (I) + O_2 \quad (4)$$

It can be seen from Equation 4 that low-priced copper Cu (I) or PAI-Cu (I) can directly catalyze hydrogen peroxide to generate a large number of hydroxyl radicals, as shown in Equation 5.

$$PAI{-}Cu\ (I) + H_2O_2 \rightarrow PAI{-}Cu\ (II) + \cdot OH + OH^- \quad (5)$$

Furthermore, the specific catalytic reaction process can be seen from the schematic diagram in Fig. S12.

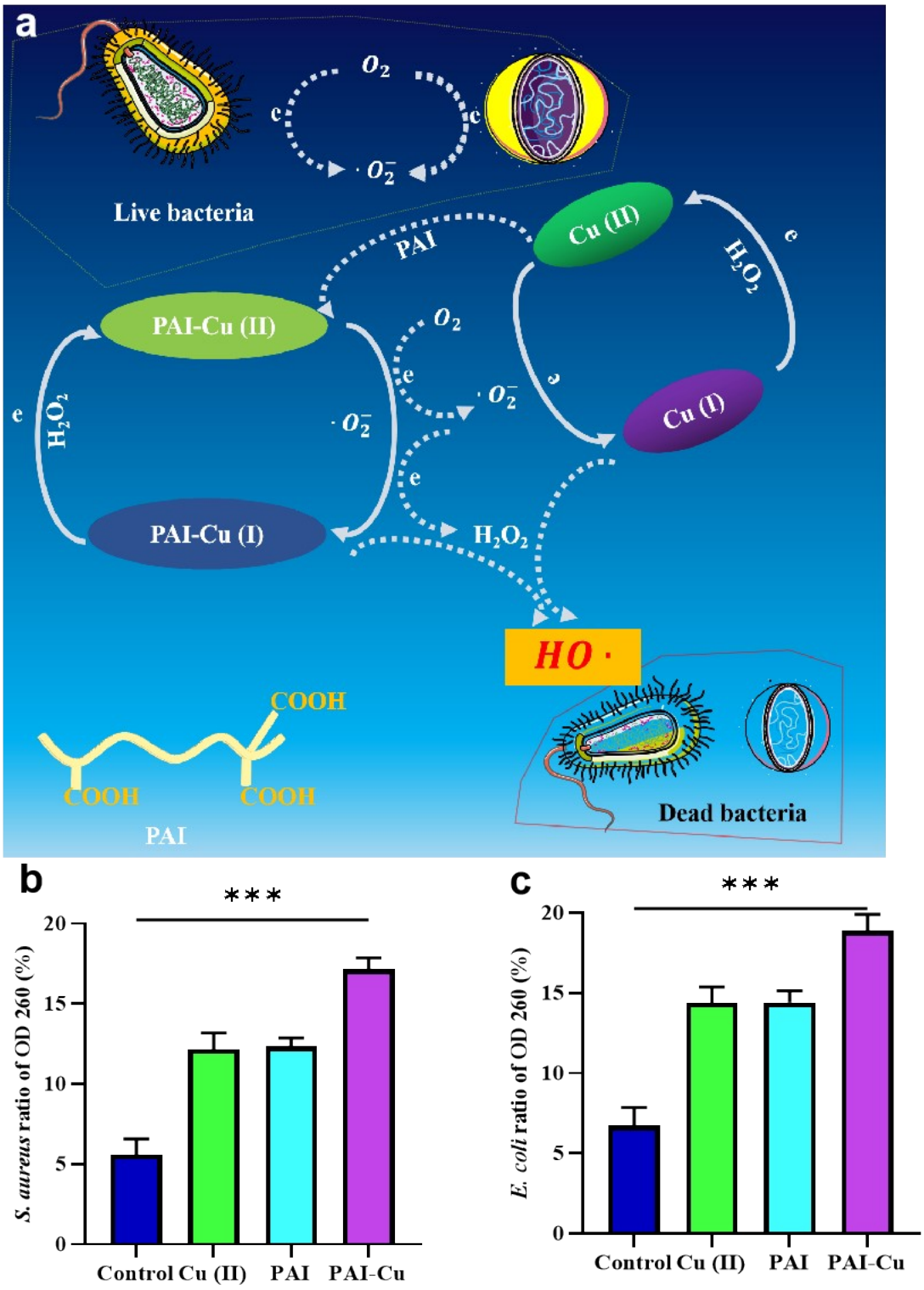

Fig. S12 (a) The schematic illustration for the catalytic mechanism of PAI-Cu. Nucleic acids from PAI-Cu treated *S. aureus* (b) and *E. coli* (c) by measuring the optical density at 260 nm.

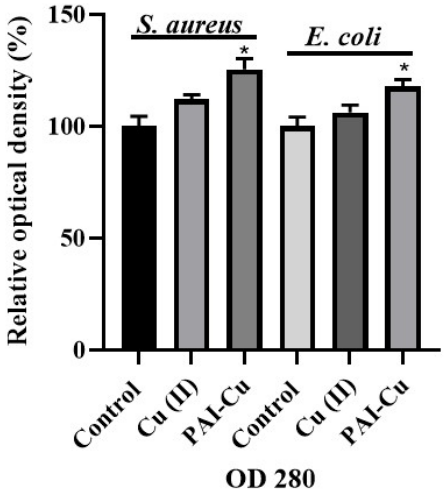

Fig. S13 Leakage of proteins from PAI-Cu treated bacteria by measuring the optical density at 280 nm. *p＜0.05

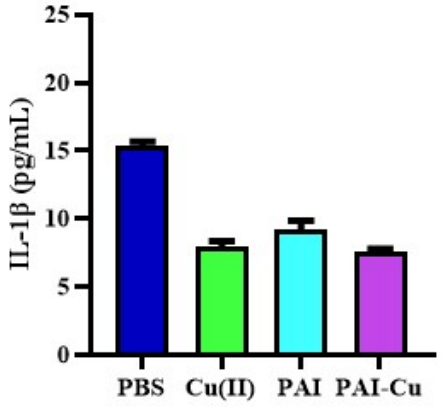

Fig. S14 Changes in IL-1β levels in serum at 8th-day therapy in rats.

3-Supplementary table 1:

Materials	Cu （mg/L）		
Cu (II)	6.36	6.41	6.31
Supernatant liquid of PAI-Cu	1.09	0.93	1.16

Table S1 The concentration of surrounding copper ions detected by ICP-OES.