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Enzyme-triggered on-demand release of a H_2O_2 -self-supplying $\text{CuO}_2@\text{Fe}_3\text{O}_4$ nanoagent for enhanced chemodynamic antimicrobial therapy and wound healing†

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Nanoagents for chemodynamic therapy (CDT) hold a promising future in the field of antimicrobials, especially copper peroxide (CuO_2) (CP) nanomaterials which have garnered significant attention due to their ability to self-supply H_2O_2 . Nevertheless, the poor stability of CuO_2 remains a critical challenge which restricts its practical application in the antibacterial field. In this study, an advanced nano-antimicrobial system $\text{HA-CP@Fe}_3\text{O}_4$ with enzyme-responsive properties is developed by coating hyaluronic acid (HA) on CuO_2 -loaded iron tetraoxide nanoparticles. The coating of HA not only stabilizes the CuO_2 nanomaterials but also provides responsiveness towards the enzyme hyaluronidase, which is typically secreted by some bacteria. The outer layer of HA in $\text{HA-CP@Fe}_3\text{O}_4$ undergoes decomposition in the presence of hyaluronidase-secreting bacteria, resulting in the release of $\text{CuO}_2@\text{Fe}_3\text{O}_4$. The released $\text{CuO}_2@\text{Fe}_3\text{O}_4$ then self-supplies H_2O_2 and generates reactive oxygen species (ROS) within the infected microenvironment through Fenton and Russell effects, to ultimately achieve effective and precise antimicrobial activity. Simultaneously, the magnetic property provided by Fe_3O_4 allows the substance to be directed towards the infection site. Both *in vitro* and *in vivo* tests demonstrated that $\text{HA-CP@Fe}_3\text{O}_4$ exhibited excellent antimicrobial capabilities at low concentration ($30 \mu\text{g mL}^{-1}$), exceptional biocompatibility and the ability to accelerate wound healing. The findings of this work offer a new and promising approach for targeted and precise CDT.

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1. Introduction

Global health has long been seriously threatened by bacterial diseases.^{1–3} Antibiotics have been the mainstay of conventional treatments, but their overuse and widespread misuse have led to the expression of drug-resistant genes in bacteria,^{4–7} making it increasingly challenging to treat bacterial infections.^{8,9} Significant efforts have been made to develop novel bactericidal strategies that do not rely on antibiotics, aiming to circumvent the drawbacks of conventional antibiotic therapy.^{10–14} Specifically, chemodynamic therapy (CDT), which is based on the Fenton or Fenton-like reaction, stimulates the generation of highly toxic hydroxyl radicals ($\cdot\text{OH}$) from hydrogen peroxide (H_2O_2) to achieve antibacterial activity.^{15–17} CDT is a very

promising antibacterial technique owing to low treatment costs and independence from environmental cues.^{18–20} However, the antibacterial efficacy of conventional CDT nanomaterials against bacterial infections has been restricted by the low concentrations of H_2O_2 in the targeted area.^{21,22} Exogenous H_2O_2 is often required for effective sterilization to resolve this issue, but it can cause damage to healthy tissues during wound healing. Therefore, it is crucial to develop advanced nano-antimicrobial CDT systems that can provide enhanced antimicrobial activity without the need of exogenous H_2O_2 .

Copper peroxide (CuO_2) is an emerging bactericidal material composed of copper ions and peroxy groups. It has the ability to self-supply H_2O_2 and convert it into highly reactive hydroxyl radicals ($\cdot\text{OH}$) for realizing augmented antibacterial treatment.^{23,24} Typically, CuO_2 undergo decomposition in an infected microenvironment, resulting in the formation of Cu^{2+} and H_2O_2 . Subsequently, a Fenton reaction occurs between Cu^{2+} and H_2O_2 , leading to the generation of $\cdot\text{OH}$, which effectively kills bacteria. Despite being an ideal antibacterial material, the practical use of CuO_2 in CDT has been hindered due to its inherent instability. Recently, significant

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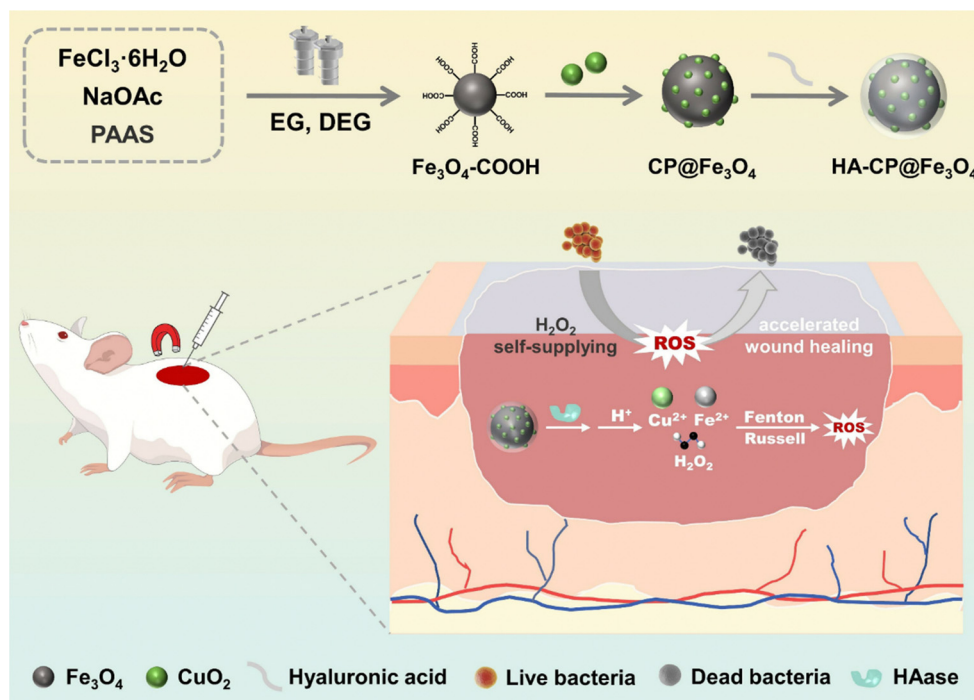
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efforts have been made to improve the stability of CuO_2 by using carriers such as molybdenum disulfide (MoS_2) nanospheres, titanium oxide (TiO_2) nanosheets, and polydopamine (PDA) nanoparticles.^{25–27} However, these nanocomposites lack ability to effectively accumulate at the targeted site and inevitably cause damage to normal tissues. Therefore, it is crucial to select appropriate carriers that not only enhance the stability and utilization of CuO_2 but also minimize harm to healthy tissues.

Meanwhile, iron tetraoxide (Fe_3O_4) has been widely used in antimicrobial applications due to its excellent biocompatibility.^{28,29} Iron ions derived from Fe_3O_4 can generate reactive oxygen species (ROS) through the Fenton effect, thereby exhibiting strong antibacterial properties.^{30–32} In addition, the magnetic properties of Fe_3O_4 have attracted significant attention from researchers. Fe_3O_4 can be effectively combined with other materials to impart magnetic properties to nanomaterials for realizing targeted therapy and/or recycling process.^{33–37} For instance, Liu and colleagues prepared a smart drug delivery system based on iron oxide nanoparticles by utilizing a focal pyroptosis-starvation-chemotherapy strategy.³⁸ Liu *et al.* designed UCNPs/ Fe_3O_4 nanoparticles for photodynamic therapy of cancer.³⁹ Feng *et al.* developed magnetic manganese oxide sweetgum-ball nanospheres for chemotherapy and photodynamic therapy of cancer.⁴⁰ All of these studies have utilized the magnetic properties of Fe_3O_4 to facilitate the aggregation of nanomaterials/drugs at the tumor site, resulting in efficient utilization of materials and enhanced therapeutic efficacy. However, to the best of our knowledge, magnetic aggregation therapy has been rarely studied in the field of antimicrobial

research. Therefore, based on the Fenton effect and the magnetic aggregation property of Fe_3O_4 , we hypothesize that the modification of magnetic nanomaterials can further enhance the utilization rate of CuO_2 and facilitate the dual Fenton effect. Besides, hyaluronic acid (HA) has also been extensively used in medicine due to its excellent biocompatibility. Interestingly, HA can only be degraded by the action of enzyme hyaluronidase (HAase) secreted by some bacteria.^{41–43} Consequently, by coating magnetic nanomaterials exhibiting dual Fenton effect with HA, it is possible to achieve precise control over the release of nanomaterials and further improve the bactericidal rate.

In this study, we successfully developed a robust nanocomposite system, HA-CP@ Fe_3O_4 , with enzyme-responsive properties for enhanced chemodynamic antimicrobial therapy involving self-supplied H_2O_2 (Scheme 1). To prepare HA-CP@ Fe_3O_4 , carboxylated iron tetraoxide ($\text{Fe}_3\text{O}_4\text{-COOH}$) was utilized as a carrier to improve the stability of CuO_2 . This was achieved by leveraging the reaction between carboxyl groups and copper ions to facilitate the *in situ* growth of CuO_2 on the surface of the carrier. Subsequently, the resulting material was coated with HA to prevent the decomposition of CuO_2 and the release of copper ions, resulting in the desired biocompatible nanocomposite, HA-CP@ Fe_3O_4 . Interestingly, HA-CP@ Fe_3O_4 can be aggregated at the infection site under the influence of an applied magnetic field and release CuO_2 @ Fe_3O_4 through destruction of outer HA layer in the presence of target HAase secreting bacteria. In the acidic environment of the infection site, CuO_2 @ Fe_3O_4 then decomposes into Cu^{2+} , iron ions and H_2O_2 . Cu^{2+} and iron ions can induce CDT by converting H_2O_2 to $\cdot\text{OH}$ through the Fenton effect. Simultaneously, Cu^{2+} catalyzes the production of singlet oxygen ($^1\text{O}_2$) for CDT through



Scheme 1 Schematic diagram of the preparation of the enzyme-responsive nanoplateform HA-CP@ Fe_3O_4 and its application in the study of antibacterial infection *in vivo*.

the Russell effect. Therefore, HA-CP@Fe₃O₄ can be precisely released at the site of infection using the magnetic properties of Fe₃O₄ and the enzyme responsiveness conferred by HA, to ultimately reduce the antimicrobial dose and boost the overall antimicrobial activity of CDT. The outstanding antimicrobial activity of HA-CP@Fe₃O₄ *via* CDT was validated through both *vitro* and *in vivo* studies. This work presents an attractive strategy for developing a powerful antibacterial platform using controlled release in CDT.

2. Experimental section

2.1 Materials

Copper chloride (CuCl₂·2H₂O), 3,3',5,5'-tetramethylbenzidine (TMB), methylene blue (MB), 1,3-diphenylisobenzofuran (DPBF), glutathione (GSH), isopropyl alcohol (IPA), 1,4-benzoquinone (BQ) and L-histidine (His) were provided by Aladdin Chemical Reagent Co., Ltd (China). Poly(vinylpyrrolidone) (PVP), potassium iodide (KI), sodium polyacrylate (PAAS), sodium hyaluronate (HA) and hyaluronidase (HAase) were obtained from Shanghai Macklin Biochemical Technology Co., Ltd (China). Ferric chloride hexahydrate (FeCl₃·6H₂O), anhydrous sodium acetate (NaOAc), diethylene glycol (DEG), ethylene glycol (EG), glutaraldehyde (GA) were provided by Tianjin Kermel chemical reagent factory (China). Hydrogen peroxide (H₂O₂, 30%), potassium permanganate (KMnO₄), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and sodium hydroxide (NaOH) were obtained from Sinopharm Chemical Reagent Co., Ltd. Sodium acetate buffer solution (NaAc buffer) and phosphate buffer solution (PBS) were provided by Shaanxi Zhonghuihecai Biomedical Technology Co., Ltd (China). LB broth and plate counting agar were purchased from Beijing Aoboxing Biotechnology Co., Ltd (China). All analytical reagents are used immediately without any purification. Ultrapure water was used in all experiments.

2.2 Synthesis of HA-CP@Fe₃O₄

Synthesis of Fe₃O₄-COOH: Fe₃O₄-COOH was synthesized by a straightforward one-pot hydrothermal process. In brief, FeCl₃·6H₂O (0.504 g), NaOAc (2 g), and PAAS (50 mg) were added to a mixture of C₂H₅OH/C₄H₁₀O₃ (7/13 mL) and subjected to ultrasonication. Then, the reaction mixture was heated at 200 °C for 12 h. After the completion of reaction, the reaction mixture was allowed to cool followed by washing with ultra-pure water and finally dried at 40 °C to obtain Fe₃O₄-COOH.

Synthesis of CuO₂@Fe₃O₄ (CP@Fe₃O₄): CuCl₂·2H₂O (50 mL, 0.01 M) was added to a reaction flask containing Fe₃O₄-COOH (50 mg) and the mixture was mechanically stirred for 4 h. Afterwards, PVP (3 g), NaOH (50 mL, 0.02 M), and H₂O₂ (1 mL, 10 M) were added sequentially. The reaction mixture was stirred continuously for 30 min followed by washing with ultra-pure water. The product was freeze-dried to finally obtain CP@Fe₃O₄.

Synthesis of HA-CuO₂@Fe₃O₄ (HA-CP@Fe₃O₄): firstly, CuO₂@Fe₃O₄ (50 mg) was dispersed in aqueous CuCl₂·2H₂O

(50 mL, 0.01 M), and stirred for 4 h. Then, HA (50 mL, 0.5 mg mL⁻¹) was introduced, and the reaction mixture was stirred continuously for another 12 h. After washing the product with ultra-pure water and subjected to freeze-drying, HA-CP@Fe₃O₄ was finally obtained. The atomic absorption spectrometer (AAS, AA-6880, Shimadzu Co., Japan) was used to further measure the amount of CuO₂ in HA-CP@Fe₃O₄.

2.3 Monitoring the release of copper ion and H₂O₂ from HA-CP@Fe₃O₄

Release of copper ion: HA-CP@Fe₃O₄ was dispersed in PBS (pH = 7.4) or NaAc buffer (pH = 5.4) in the absence and presence of HAase (150 U mL⁻¹), respectively. The mixture was continuously stirred at 37 °C to simulate the physiological environment. The dialysate was collected after the appropriate time, and buffer solution was replenished. Atomic absorption spectrometer was used to detect the release of copper ions.

Release of H₂O₂: the reaction of H₂O₂ with KI can produce I₃⁻ which has an absorption peak at 350 nm, and can be easily determined by UV-vis spectrophotometer (UV-2450, Shimadzu Co., Japan). Thus, a standard curve can be obtained to quantitatively assess the concentration of H₂O₂ in solution. HA-CP@Fe₃O₄ (200 µg mL⁻¹) was dissolved in PBS (pH = 7.4) or NaAc buffer (pH = 5.4) with or without HAase. After various reaction times, the supernatant was magnetically separated, collected, and mixed with KI (0.5 M). Then, the absorbance of I₃⁻ was measured to determine the amount of H₂O₂. In subsequent experiments with other experimental groups, HAase was added to investigate their activity.

2.4 Determination of catalytic activity

2.4.1 Colorimetric determination of peroxide groups.

KMnO₄ (50 µg mL⁻¹) was dissolved in an aqueous solution of H₂SO₄ (0.1 M), followed by addition of Fe₃O₄, Fe₃O₄ + H₂O₂, CP@Fe₃O₄, HA-CP@Fe₃O₄, or H₂O₂. The UV-vis spectra of the solutions were measured in the range of 400–650 nm after incubation for 10 min.

2.4.2 Determination of Fenton activity. TMB and MB were used as indicators of •OH to evaluate the Fenton activity of HA-CP@Fe₃O₄. Briefly, HA-CP@Fe₃O₄ (200 µg mL⁻¹) was incubated in NaAc buffer containing HAase (pH = 5.4) for 60 min. Oxidized TMB (oxTMB) was then monitored by monitoring its absorbance at 652 nm by UV-vis spectrophotometer. In addition, the generation of •OH was further verified by determining the absorbance of MB at 665 nm, which can be degraded by •OH. HA-CP@Fe₃O₄ (200 µg mL⁻¹) was added to NaAc buffer (pH = 5.4) containing MB (100 µg mL⁻¹), and the absorbance of the supernatant at 665 nm was measured using a UV-vis spectrophotometer every 10 min.

2.4.3 Determination of Russell activity. The solution of DPBF (0.1 mM) in ethanol was added to NaAc buffer containing HA-CP@Fe₃O₄ (200 µg mL⁻¹). The absorbance of the solution at 409 nm was recorded at two-minute intervals using a UV-vis spectrophotometer. Additionally, the electron spin resonance spectroscopy (ESR, EMXplus-10, Bruker Co., Germany) was used to confirm the ROS generation. The trapping agents

2,2,6,6-tetramethylpiperidine (TEMP) and 5,5-dimethyl-1-pyrroline-*n*-oxide (DMPO) were used to trap $^1\text{O}_2$, $\cdot\text{O}_2^-$, and $\cdot\text{OH}$, respectively. Different ROS were identified at room temperature for HA-CP@Fe₃O₄ (1 mg mL⁻¹) by the captured molecules (100 μL , 100 mM). Subsequently, ROS trapping assays were used to assess the effect of these ROS on antimicrobial activity. Isopropyl alcohol (IPA, 10 mM) was used to capture $\cdot\text{OH}$, 1,4-benzoquinone (BQ, 0.1 mM) was used to capture $\cdot\text{O}_2^-$, and L-histidine (His, 1 mM) was used to capture $^1\text{O}_2$.

2.4.4 GSH scavenging assay. GSH (1 mM) was firstly mixed with HA-CP@Fe₃O₄ (200 $\mu\text{g mL}^{-1}$). Then, 100 μL of this solution along with DTNB (20 μL , 5 mM) were added to Tris-HCl buffer (pH = 8.5) at appropriate time, followed by incubation for 30 s. UV-vis spectrophotometer was used to measure the absorption spectrum of the solution.

2.5 Determination of antibacterial activity *in vitro*

The plate counting method was employed to assess the *in vitro* antibacterial activity of HA-CP@Fe₃O₄. In brief, *Staphylococcus aureus* (*S. aureus* ATCC 12228) or *Escherichia coli* (*E. coli* ATCC 8739) were cultured in Luria-Bertani (LB) broth and incubated for 12 h at 37 °C in an incubator with a constant temperature. The bacterial suspensions were diluted with PBS (pH = 7.4) to 1×10^8 CFU mL⁻¹. The experiments were divided into a total of six groups as follows: (1) PBS, (2) H₂O₂, (3) Fe₃O₄, (4) Fe₃O₄ + H₂O₂, (5) CP@Fe₃O₄ and (6) HA-CP@Fe₃O₄. The concentrations of the bacterial suspension, material, and H₂O₂ were 1×10^7 CFU mL⁻¹, 30 $\mu\text{g mL}^{-1}$, and 62.5 μM , respectively. The material solution was mixed with fresh bacterial suspension in a sterile culture tube and co-incubated for 1 h. Following that, 50 μL of the solution was diluted 10^4 times and evenly spread onto the plate. After incubation at 37 °C for 24 hours, the number of colonies on the agar plate was counted and the bacterial viability was determined.

The bacterial morphology was subsequently characterized using a field-emission scanning electron microscope (FE-SEM, GeminiSEM 500, Zeiss, Germany). Following the antimicrobial tests, the bacteria were centrifuged, fixed with 2.5% glutaraldehyde solution overnight, washed with PBS, and then dehydrated in ethanol of varying concentrations (30, 50, 70, 90 and 100%) for 10 min. Finally, 10 μL drops of dehydrated bacterial samples were placed on silica plate, and then analyzed by a field-emission scanning electron microscope (FE-SEM, GeminiSEM 500, Zeiss, Germany).

2.6 Determination of antibacterial activity *in vivo*

Female Balb/c mice (6–8 weeks, Animal Center of Xi'an Jiaotong University) were used for the evaluation of *in vivo* bacteriostatic activity. The hairs from the back of each mice were shaved and the skin was sterilized with 75% ethanol. The mice were anesthetized with isoflurane before creating a circular wound (9 mm diameter) on their back using sterile scissors. A wound model of *S. aureus* infection was established by placing 50 μL of 1×10^8 CFU mL⁻¹ *S. aureus* suspension onto the wound and incubating it for 24 h. The mice were randomly divided into six groups ($n = 4$) as follows: control: Fe₃O₄, Fe₃O₄ + H₂O₂,

CP@Fe₃O₄, HA-CP@Fe₃O₄, and HA-CP@Fe₃O₄ + magnetic(M) groups. All these groups received treatment every 24 h using 100 μL (30 $\mu\text{g mL}^{-1}$) of material solution. Photographs of the three groups of wounds were captured on days 0, 1, 3, 5, and 7, respectively. In addition, the weight of each mouse was regularly monitored using an electronic balance. To evaluate the bacteriostatic effect of different treatments, all mice were euthanized after 7 days of treatment. The wound tissue was collected, and the viable bacteria at the infected site were determined by plate counting method. In addition, the mice were dissected and their wound tissue was soaked in 4% paraformaldehyde aqueous solution. Hematoxylin and eosin (H&E) and Masson reagent were employed to observe the inflammatory response and tissue regeneration of the infected wound. To assess the biosafety of HA-CP@Fe₃O₄, H&E staining was performed on major organs (*i.e.*, heart, liver, spleen, lungs, and kidneys) of mice treated with and without HA-CP@Fe₃O₄. All experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University Health Science Center (Xi'an, China).

2.7 Cytotoxicity and hemolysis assays

The *in vitro* cytotoxicity of various doses of HA-CP@Fe₃O₄ (0, 12.5, 25, 50, 100, and 200 $\mu\text{g mL}^{-1}$) on L929 cells was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. Briefly, L929 cells were incubated in 96-well plates at 37 °C for 24 h. Then, the nanomaterials were added and incubated for another 24 h. Subsequently, the cells were washed three times with sterile PBS, and 50 μL of MTT solution (5 mg mL⁻¹) was added to each well. The supernatant was taken out after 4 h of incubation at 37 °C, and 100 μL of DMSO was added to each well. The absorbance at 470 nm was measured using a microplate reader.

Hemolysis assay was used to determine the blood compatibility HA-CP@Fe₃O₄. After washing the mice erythrocytes using PBS, the collected erythrocytes (0.4 mL) were suspended in PBS (19.6 mL) to ensure even dispersion. Subsequently, 1 mL of the aforementioned erythrocyte suspension was mixed with varying amounts of HA-CP@Fe₃O₄ (1 mL), and the resulting mixture was incubated for 1 h. The hemolysis rate was determined by measuring the absorbance of the supernatant solution (540 nm). Water-treated and PBS-treated groups were used as controls.

2.8 Statistical analysis

All data were expressed in the form of mean $\pm$ standard deviation. Statistical analyses were performed by software SPSS via one-way analysis of variance (one-way ANOVA). Significant differences were considered as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Results and discussion

3.1 Preparation and characterization of HA-CP@Fe₃O₄

The synthetic route of HA-CP@Fe₃O₄ is presented schematically in Fig. 1(a). Firstly, Fe₃O₄-COOH nanoparticles were

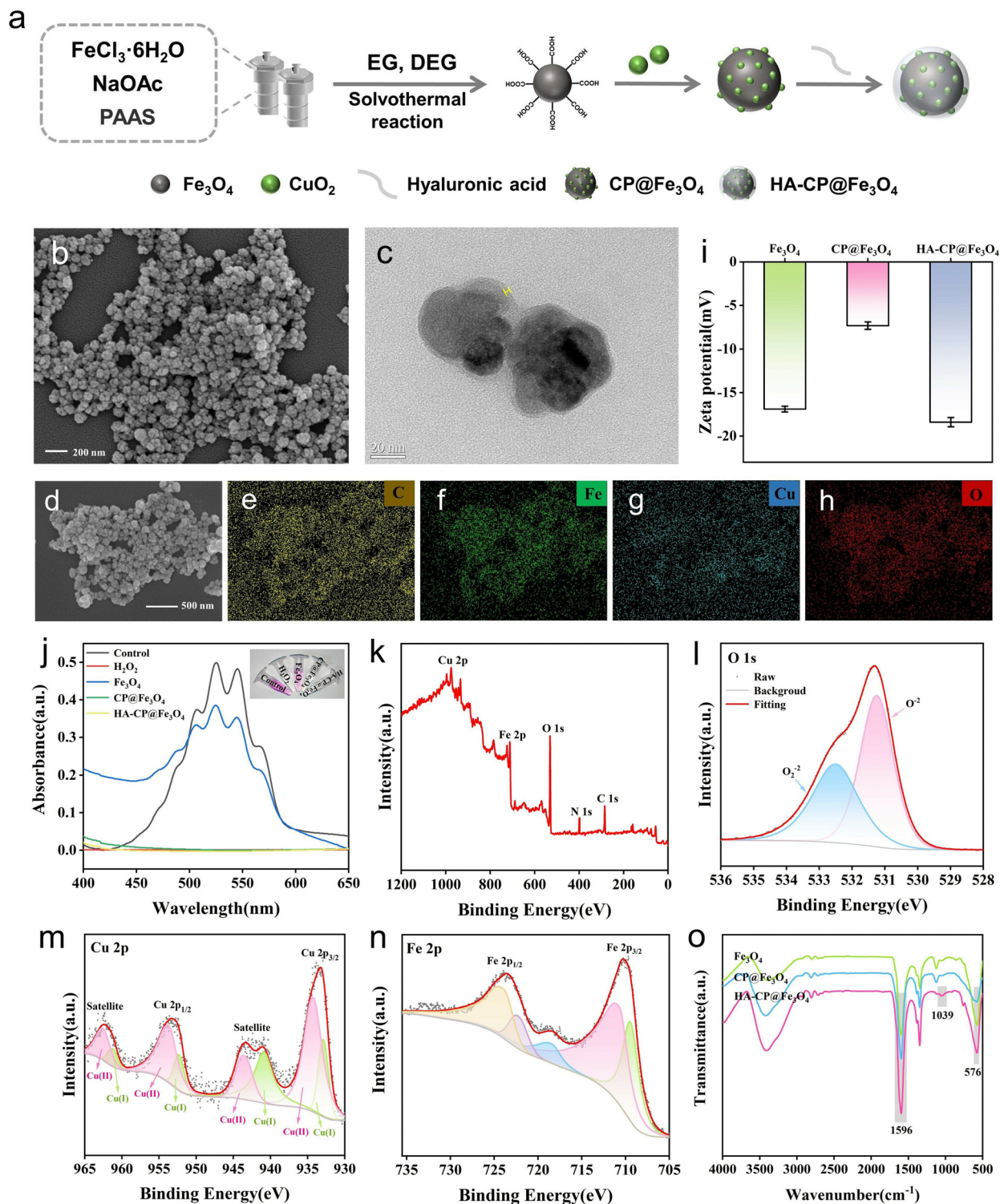


Fig. 1 (a) Schematic illustration of the synthetic route of HA-CP@Fe₃O₄. (b) SEM and (c) TEM images of HA-CP@Fe₃O₄. The yellow lines indicate the thickness of the layers. (d)–(h) EDS elemental mapping images of HA-CP@Fe₃O₄. (i) Zeta potential of Fe₃O₄, CP@Fe₃O₄ and HA-CP@Fe₃O₄. (j) Colorimetric determination of peroxy groups in HA-CP@Fe₃O₄. (k)–(n) XPS spectra of HA-CP@Fe₃O₄. Comparing (o) FTIR spectra of Fe₃O₄, CP@Fe₃O₄ and HA-CP@Fe₃O₄.

synthesized by a simple one-step solvothermal reaction. Then, CuO₂ was generated *in situ* onto their surface to acquire CP@Fe₃O₄. Finally, the nanocomposite HA-CP@Fe₃O₄ was

obtained after modification of CP@Fe₃O₄ with HA. Scanning electron microscopy (SEM) reveals that HA-CP@Fe₃O₄ nanoparticles exhibit a coarse spherical morphology with an average

particle size of 80 nm (Fig. 1(b)). According to the dynamic light scattering (DLS) analysis, the size of hydrated HA-CP@Fe₃O₄ was found as 102.03 ± 0.99 nm (Fig. S1, ESI†). The presence of distinct layer on the surface of HA-CP@Fe₃O₄ was confirmed by the transmission electron microscopy (TEM), the thickness of which is about 2 nm. (Fig. 1(c)). On the other hand, the element mapping shows the uniform distribution of C, Fe, Cu, and O in HA-CP@Fe₃O₄ (Fig. 1(d)–(h) and Fig. S2, ESI†), asserting the effective loading of CuO₂. The presence of peroxy groups was further verified by KMnO₄ colorimetric analysis as shown in the Fig. 1(j). After the introduction of HA-CP@Fe₃O₄, permanganate (MnO₄[−]) loses its color under acidic conditions due to the reduction of MnO₄[−] to Mn²⁺ by peroxy groups. In addition, the change in zeta potential observed during the preparation process further affirms the successful modification of CuO₂ and HA onto Fe₃O₄ (Fig. 1(i)). Following modification with CuO₂, the zeta potential of Fe₃O₄ shifted from −16.9 to −7.33 mV, and further changed to −18.4 mV upon loading with negatively charged HA.

The chemical structures and detailed composition of nano-materials were further analyzed by X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and vibrating sample magnetometer (VSM). XPS was employed to analyze the elements and their valence states in HA-CP@Fe₃O₄. As shown in Fig. 1(k), HA-CP@Fe₃O₄ was mainly composed of C, Fe, Cu, and O elements. An apparent N 1s peak was observed, further demonstrating the existence of the poly(vinylpyrrolidone) (PVP) coating. The presence of two O 1s peaks at 531.3 and 532.5 eV (Fig. 1(l)) can be ascribed to C=O and O–O bonds, respectively, confirming the existence of PVP and peroxy groups in the HA-CP@Fe₃O₄ sample. In addition, the Cu 2p XPS spectrum exhibited two main peaks at 953.64 and 934.26 eV and two satellite peaks at 962.53 and 943.56 eV, indicating that a main valence state of Cu in CuO₂ nanodots was positive bivalence. Meanwhile, the Cu 2p spectrum had two main peaks at 932.9 and 952.32 eV accompanied by two satellite peaks at 940.9 and 961.26 eV, suggesting a certain proportion of Cu⁺ possibly due to the decomposition and/or self-reduction of

CuO₂ nanodots during synthesis and storage processes. In addition, the ratio of Cu⁺/Cu²⁺ was 29.9/71.7, indicating that the principal valence state of Cu in HA-CP@Fe₃O₄ is positive bivalence. The XPS spectrum of Fe 2p (Fig. 1(n)) displays two peaks at 710.9 and 724.6 eV, corresponding to the Fe 2p_{3/2} and Fe 2p_{1/2} states, respectively. Besides, the Fe 2p peak further splits into two distinct peaks at 709.4 (Fe(II)) and 711.3 eV (Fe(III)). The proportions of Fe(II) and Fe(III) in the HA-CP@Fe₃O₄ were determined as 36% and 64%, respectively based on the inverse fold product of the Fe(2p_{3/2}) envelope and peak area. Thus, XPS characterization data confirmed the successful preparation of HA-CP@Fe₃O₄. In addition, prepared HA-CP@Fe₃O₄ had a high saturation magnetization (69.14 emu g^{−1}; Fig. S3, ESI†), indicating that HA-CP@Fe₃O₄ has good magnetic properties.

The X-ray diffraction pattern of the prepared samples reveals clear diffraction peaks at 30.26°, 35.54°, 43.24°, 57.12° and 62.76° which corresponds to Fe₃O₄ and are in agreement with the standard cubic spinel Fe₃O₄ phase (JCPDS card 99-0073). (Fig. S4, ESI†) However, no noticeable diffraction peak of CuO₂ was observed in the XRD patterns of CP@Fe₃O₄ and HA-CP@Fe₃O₄. This can be attributed to several factors, including the relatively low copper content (6.43 wt%), ultra-small size, and amorphous nature of CuO₂. By using FTIR spectroscopy, it was possible to locate the characteristic peak of Fe–O in Fe₃O₄ at around 576 cm^{−1}. Typical peaks at 1596 cm^{−1} and 3410 cm^{−1} are attributed to tensile and bending vibrations of O–H. (Fig. 1(o)) Additionally, a notable absorption peak at 1039 cm^{−1} was observed exclusively in HA-CP@Fe₃O₄, which corresponds to the C–O–C asymmetric stretching vibration. All these observations confirm the successful modification of HA and provide additional evidence for the successful synthesis of HA-CP@Fe₃O₄.

3.2 Enzyme-responsive release of copper ions and H₂O₂

Flame atomic absorption spectrometer (FAAS) was employed for monitoring the release of copper ions. As indicated in the Fig. 2(a), significant amount of copper ions were released from HA-CP@Fe₃O₄ when HAase was present in NaAc buffer

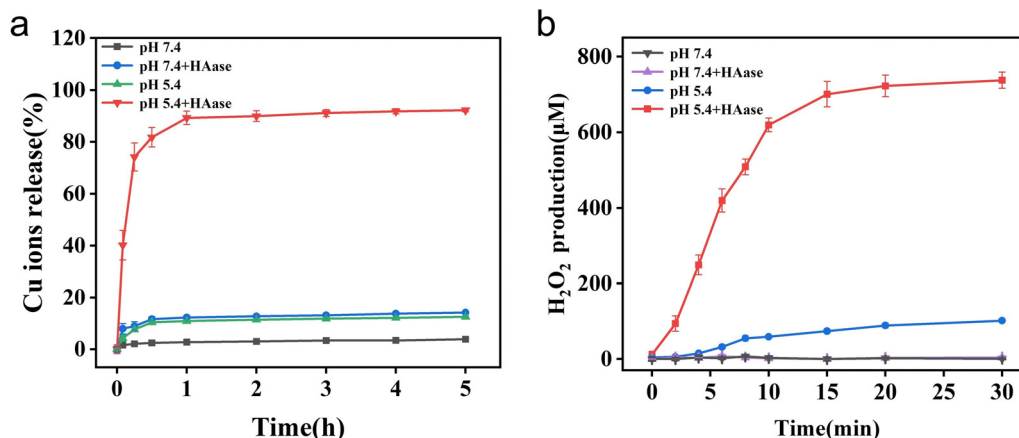


Fig. 2 The release of (a) Cu²⁺ ions and (b) H₂O₂ from HA-CP@Fe₃O₄ under different conditions.

(pH = 5.4), and the equilibrium was achieved in about 1 h. In contrast, when HAase was absent, only 13.4% of copper ions were released which can be attributed to the protection of copper ions by HA. These observations illustrate the enzyme-responsive nature of copper ion release within HA-CP@Fe₃O₄ system. To confirm the absence of copper ion leakage in a neutral environment, the release of copper ions was also examined in PBS (pH = 7.4). As anticipated, the release of copper ions could reach about 15.8% in the presence of HAase, whereas the release of copper ions in the absence of HAase was negligible. Thus, the coating of HA not only prevents copper ions from producing toxic side effects on normal tissues, but also ensures its precise and controlled release at the weakly acidic infection site. These results support the enzyme-responsive behavior of copper ion release in the HA-CP@Fe₃O₄ system.

Under acidic conditions, CuO₂ can undergo decomposition into Cu²⁺ and H₂O₂, and the amount of H₂O₂ released is directly correlated with the amount of ROS generated. In this study, KI was utilized as a probe to detect and quantify H₂O₂ released by HA-CP@Fe₃O₄ under various conditions (Fig. S5, ESI†). The reaction between KI and H₂O₂ leads to the formation of I₃[−], which exhibit a characteristic absorption peak at 350 nm. As depicted in Fig. 2(b), HA-CP@Fe₃O₄ produced up to 738 mol L^{−1} of H₂O₂ when incubated with HAase in NaAc buffer (pH = 5.4), whereas only minimal amounts of H₂O₂ was formed in the absence of HAase. On the other hand, the production of H₂O₂ was negligible in PBS (pH = 7.4). These findings confirm (1) the capability of material to produce H₂O₂ autonomously as well as (2) its ability to trigger H₂O₂ release in response to specific enzymatic activity. Thus, HA-CP@Fe₃O₄ with both autonomous H₂O₂ production and enzyme-responsive H₂O₂ release properties could be a potential nanoagent for precise and augmented CDT.

3.3 Catalytic activity of HA-CP@Fe₃O₄

The ability of synthesized materials (Fe₃O₄, CP@Fe₃O₄ and HA-CP@Fe₃O₄) to generate •OH radicals was investigated using a commonly used probe 3,3',5,5'-tetramethylbenzidine (TMB). After the oxidation process, the colorless TMB was transformed into blue oxidized TMB (oxTMB). The results indicate that Fe₃O₄ can oxidize the TMB indicator in the presence of H₂O₂ (Fig. 3(a)). Additionally, both CP@Fe₃O₄ and HA-CP@Fe₃O₄ were able to catalyze the oxidation of TMB to blue oxTMB with a distinctive absorption peak at 650 nm in the absence of H₂O₂, demonstrating the superior catalytic activity of materials. The degradation of methylene blue (MB) by various experimental groups was also studied as shown in Fig. 3(f). These results aligned with the findings from TMB experiments, providing further evidence of the efficient production of •OH radicals. Furthermore, a positive correlation between time and MB degradation by HA-CP@Fe₃O₄ was observed (Fig. S6, ESI†). In addition, the TMB method was used to investigate the formation of •OH radicals at various time intervals using different concentrations of HA-CP@Fe₃O₄ to gain deep insights into the catalytic performance of HA-CP@Fe₃O₄. The catalytic activity of HA-CP@Fe₃O₄ was found dependent on both

concentration and time (Fig. 3(b) and (c)) and the optimum activity was observed at pH 5.4, which was attributed to the fact that H₂O₂ is generated when CuO₂ decomposes upon acidic conditions. (Fig. S7, ESI†).

Cu(II) has the ability to generate cytotoxic singlet oxygen (¹O₂) via the Russell reaction. According to the Russell reaction mechanism (Fig. S8, ESI†), the carboxyl group present on the material is initially oxidized by H₂O₂ to form a peroxycarboxylic radical which is then catalytically converted into ¹O₂ by Cu²⁺.^{44–47} For investigating the Russell catalytic activity of HA-CP@Fe₃O₄, 1,3-diphenylisobenzofuran (DPBF) was used to determine the generation of ¹O₂. As shown in Fig. 3(d) and (e), the characteristic absorption peaks of CP@Fe₃O₄ and HA-CP@Fe₃O₄-treated DPBF were decreased with time. On contrary, the absorption peaks of Fe₃O₄ and the Fe₃O₄ + H₂O₂ group were nearly same as the blank control group, indicating that CuO₂ was responsible for the generation of ¹O₂. In addition, the HA-CP@Fe₃O₄-catalyzed generation of •OH and ¹O₂ was determined by electron spin resonance (ESR) spectroscopy using 5,5-dimethyl-1-pyrrole *n*-oxide (DMPO) and 2,2,6,6-tetramethylpiperidine (TEMP) as the trapping agents. As depicted in Fig. 3(g), the distinctive signals of DMPO••OH (1:2:2:1) and TEMP-¹O₂ (1:1:1) confirm the production of •OH and ¹O₂, respectively, while the six-line signal of superoxide anion was invisible. These observations firmly confirms the catalytic ability of HA-CP@Fe₃O₄ to generate •OH and ¹O₂. Furthermore, the result of ESR assay was verified by scavenging experiments, and the relative oxidation rate (%) decreased by 73.4 and 58.8% after treatment with isopropanol (IPA) and L-histidine (His), respectively. However, the relative oxidation rate was dropped by only 17.1% on addition of •O₂[−] scavengers, indicating that •OH and ¹O₂ are the major ROS species produced by HA-CP@Fe₃O₄ (Fig. 3(h)).

An essential intracellular regulatory molecule called glutathione (GSH) exhibits antioxidant properties and aids in maintaining the proper functioning of the immune system. Nevertheless, excessive glutathione produced by bacteria by anaerobic glycolysis in the diseased microenvironment inevitably scavenges ROS and limit antimicrobial actions. Hence, GSH depletion capacity of HA-CP@Fe₃O₄ was assessed by Ellman's reagent assay. In this assay, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) is usually react with the sulfhydryl group of GSH to generate yellow colored 5-thio-2-nitrobenzoic acid, with a maximum absorption peak at 412 nm. Hence, the consumption of HA-CP@Fe₃O₄ for GSH was determined by monitoring the absorbance at 412 nm. As shown in Fig. 3(i), the absorption peak at 412 nm gradually decreased, and the yellow color became lighter as the reaction time increased. This indicates that the residual amount of GSH gradually decreased after treatment with HA-CP@Fe₃O₄, whereas the absorption peak in the control group remained almost unchanged. The outcomes of this experiment proved that HA-CP@Fe₃O₄ exhibits a strong GSH scavenging capability.

3.4 *In vitro* antibacterial activity of HA-CP@Fe₃O₄

Gram-negative *E. coli*, which does not produce HAase, was chosen as the control strain, while Gram-positive *S. aureus*,

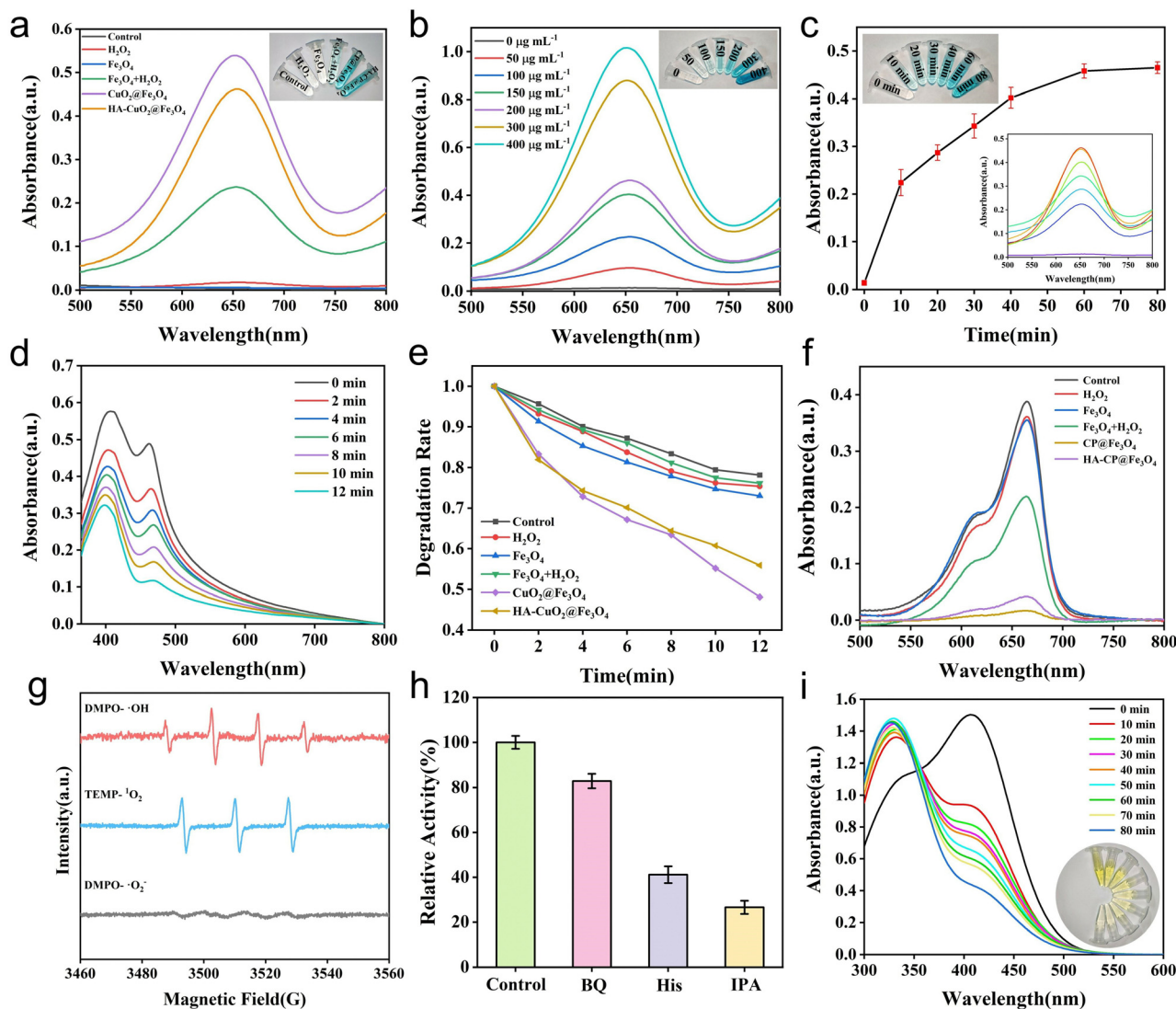


Fig. 3 (a) Confirmation of $\cdot\text{OH}$ radicals generated by Fenton-like reaction in different groups ($200\ \mu\text{g mL}^{-1}$) using TMB assay. Studying Fenton activity of HA-CP@Fe₃O₄ by varying (b) concentrations and (c) time ($200\ \mu\text{g mL}^{-1}$). (d) Absorption spectra of DPBF solution with HA-CP@Fe₃O₄ ($200\ \mu\text{g mL}^{-1}$) at increasing time intervals. (e) Comparison of the degradation rates of DPBF over time in different experimental groups ($200\ \mu\text{g mL}^{-1}$). (f) Absorption spectra of MB after treatment with different experimental groups ($200\ \mu\text{g mL}^{-1}$). (g) ESR spin-trapping spectra of HA-CP@Fe₃O₄ for the detection of DMPO- $\cdot\text{OH}$, DMPO- $\cdot\text{O}_2^-$, and TEMP- $\cdot\text{O}_2^-$. (h) Inhibition of TMB oxidation by IPA, BQ, and His in the presence of HA-CP@Fe₃O₄. (i) Time-dependent depletion of GSH by HA-CP@Fe₃O₄ ($200\ \mu\text{g mL}^{-1}$).

which does produce HAase, was selected as the model strain to investigate the antimicrobial properties of various nanomaterials (Fe₃O₄, CP@Fe₃O₄ and HA-CP@Fe₃O₄). *S. aureus* and *E. coli* were separately incubated with various nanomaterials, and the bacterial survival rate was subsequently assessed using the plate counting method. As shown in Fig. 4(a)–(c), the results of the PBS group were similar to those of the bare H₂O₂ group and the Fe₃O₄ group, and neither *S. aureus* nor *E. coli* showed any significant harm. It turns out that the Fe₃O₄ + H₂O₂ showed some antibacterial effect which can be attributed to the generation of ROS by Fe₃O₄ via the Fenton effect. On the other hand, both the CP@Fe₃O₄ and HA-CP@Fe₃O₄ groups showed considerable antibacterial activity against *S. aureus* at a concentration of just $30\ \mu\text{g mL}^{-1}$, even without the use of H₂O₂.

Notably, the HA-CP@Fe₃O₄ group demonstrated superior anti-bacterial properties compared to CP@Fe₃O₄. As anticipated, the inhibitory effect on *E. coli* (which cannot release HAase) was significantly lower in the HA-CP@Fe₃O₄ group compared to the CP@Fe₃O₄ group, further confirming enzyme-responsive precise antibacterial property of HA-CP@Fe₃O₄. Additionally, it was found that HA-CP@Fe₃O₄ achieved a high bactericidal rate at a very low concentration ($30\ \mu\text{g mL}^{-1}$) without the need for external stimuli (light or heat) and could achieve specific killing of group streptococcus, which is superior to other comparable studies (Table 1). This is due to the fact that the HA coating prevents the decomposition of CuO₂ and minimizes wastage in non-infected areas, enabling precise sterilization while shielding healthy tissues from ROS.

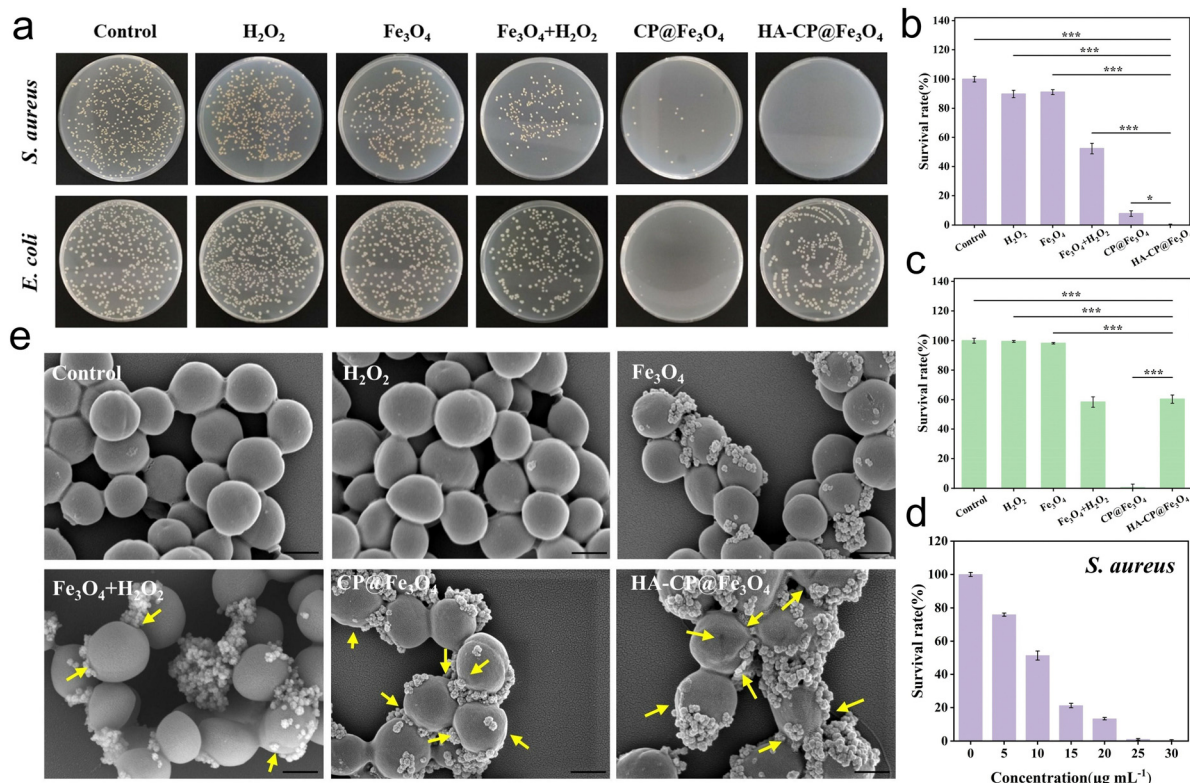


Fig. 4 Study on antibacterial activity of $HA-CP@Fe_3O_4$ *in vitro*. (a) Images of the LB agar plates of *S. aureus* and *E. coli* after treatment with different experimental groups. (materials concentration: $30 \mu g mL^{-1}$, H_2O_2 concentration $62.5 \mu M$) survival rate of (b) *S. aureus* and (c) *E. coli* after incubation with different groups. (d) Concentration dependent antibacterial activity of $HA-CP@Fe_3O_4$ against *S. aureus*. (e) SEM images of *S. aureus* after treatment with different experimental groups. Scale bars: 500 nm.

Table 1 Antibacterial model and activity of CuO_2 composite nanomaterials against *S. aureus* at different concentrations

Materials	Antibacterial model	Antibacterial activity (%)	Concentration ($\mu g mL^{-1}$)	Specificity	Ref.
PDA/CP/ICG	CDT/PDT ^a /PTT ^b	99.9	50	No	27
MoS ₂ /CuO ₂	CDT/PTT	99	50	No	26
CP@WS ₂	CDT/PTT	96.56	100	No	48
rGO/CuO ₂	PTT/TOT ^c	99	100	No	49
CuO ₂ @TiO ₂	SCDT ^d /STT ^e	99.9	30	No	25
CuO ₂ @SiO ₂	CDT	95	100	No	50
CuO ₂	CDT	97.4	37.5	No	51
HA-CP@Fe ₃ O ₄	CDT	99.9	30	Streptococcus	This work

^a PDT: photodynamic therapy. ^b PTT: photothermal therapy. ^c TOT: topical oxygen therapy. ^d SCDT: sono-chemodynamic therapy. ^e STT: sono-thermal therapy.

The antibacterial activity of $CP@Fe_3O_4/HA-CP@Fe_3O_4$ against *S. aureus* exhibited a concentration-dependent trend, with an increase in concentration resulting in a steady reduction in the bacterial survival rate. (Fig. 4(d) and Fig. S9a–S11a, ESI†). The death rate of *S. aureus* induced by $CP@Fe_3O_4/HA-CP@Fe_3O_4$ was close to 99.9% at a concentration of $40/30 \mu g mL^{-1}$. In case of *E. coli*, it was necessary to increase the concentration of $CP@Fe_3O_4$ to $100 \mu g mL^{-1}$ to achieve a similar bactericidal rate (Fig. S9b and S11c, ESI†), which was attributed to the robust outer membrane structure of Gram-negative bacteria. However, the antibacterial effect of $HA-CP@Fe_3O_4$ against *E. coli* did not show a concentration dependence, and when the concentration reached $200 \mu g mL^{-1}$,

the antibacterial effect was only 56.3%. It is evident from these results that $HA-CP@Fe_3O_4$, which incorporates hyaluronic acid as an end-sealing material, can efficiently and selectively kill *S. aureus* and possibly other hyaluronidase-secreting bacteria. Hence, $HA-CP@Fe_3O_4$ could be a promising chemodynamic antibacterial material for the treatment of infections particularly associated with hyaluronidase secreting bacteria. Although the use of HA sealing material can achieve accurate/selective sterilization, it also has certain disadvantages, such as difficulty in achieving broad spectrum antibacterial effects.

To gain a better understanding of the antimicrobial effect of the materials on *S. aureus*, the morphological alterations of the bacteria after treatment with different materials were

investigated using field emission scanning electron microscopy (FE-SEM). As shown in Fig. 4(e), the bacterial morphology remained smooth and free of defects after treatment with H_2O_2 group, Fe_3O_4 group and PBS group alone, exhibiting minimal morphological damage. The presence of minor depression and distortion on the bacterial surface following treatment with $\text{Fe}_3\text{O}_4 + \text{H}_2\text{O}_2$ group indicates some level of damage. In contrast, the bacteria treated with the HA-CP@ Fe_3O_4 group and the CP@ Fe_3O_4 group exhibited clear signs of cell rupture and collapse (yellow arrows), indicating severe structural damage and injury to the bacteria. The results obtained from both plate antibacterial testing and FE-SEM were largely consistent, providing evidence that the materials possessed excellent and precise *in vitro* antibacterial activity.

3.5 *In vivo* antibacterial activity of HA-CP@ Fe_3O_4

Motivated by the remarkable antibacterial action of HA-CP@ Fe_3O_4 observed *in vitro*, we investigated the potential of this

system for healing and disinfecting animal wounds. The *in vivo* antimicrobial performance of HA-CP@ Fe_3O_4 was evaluated by establishing a mouse wound model through injection of *S. aureus* suspension ($50 \mu\text{L}$, $1 \times 10^8 \text{ CFU mL}^{-1}$) into freshly created circular wounds on the back of Balb/c mice, as depicted in Fig. 5(a)–(c). The mice were then randomly divided into six treatment groups as follows: PBS, Fe_3O_4 , $\text{Fe}_3\text{O}_4 + \text{H}_2\text{O}_2$, CP@ Fe_3O_4 , HA-CP@ Fe_3O_4 , and HA-CP@ $\text{Fe}_3\text{O}_4 + \text{M}$ (M refers to magnetic accumulation). The treated wounds have been photographed using a digital camera on a regular basis to assess wound size and healing process in each group as shown in Fig. 5(b). Notably, the HA-CP@ $\text{Fe}_3\text{O}_4 + \text{M}$ group showed the best healing outcomes, demonstrating superior effectiveness compared to other groups. To accurately compare the wound healing process among different groups, the relative wound area of each group was quantified (Fig. 5(c) and (e)). After 7 days of treatment, the relative wound areas for the PBS, Fe_3O_4 , $\text{Fe}_3\text{O}_4 + \text{H}_2\text{O}_2$, CP@ Fe_3O_4 , HA-CP@ Fe_3O_4 , and HA-CP@ $\text{Fe}_3\text{O}_4 + \text{M}$

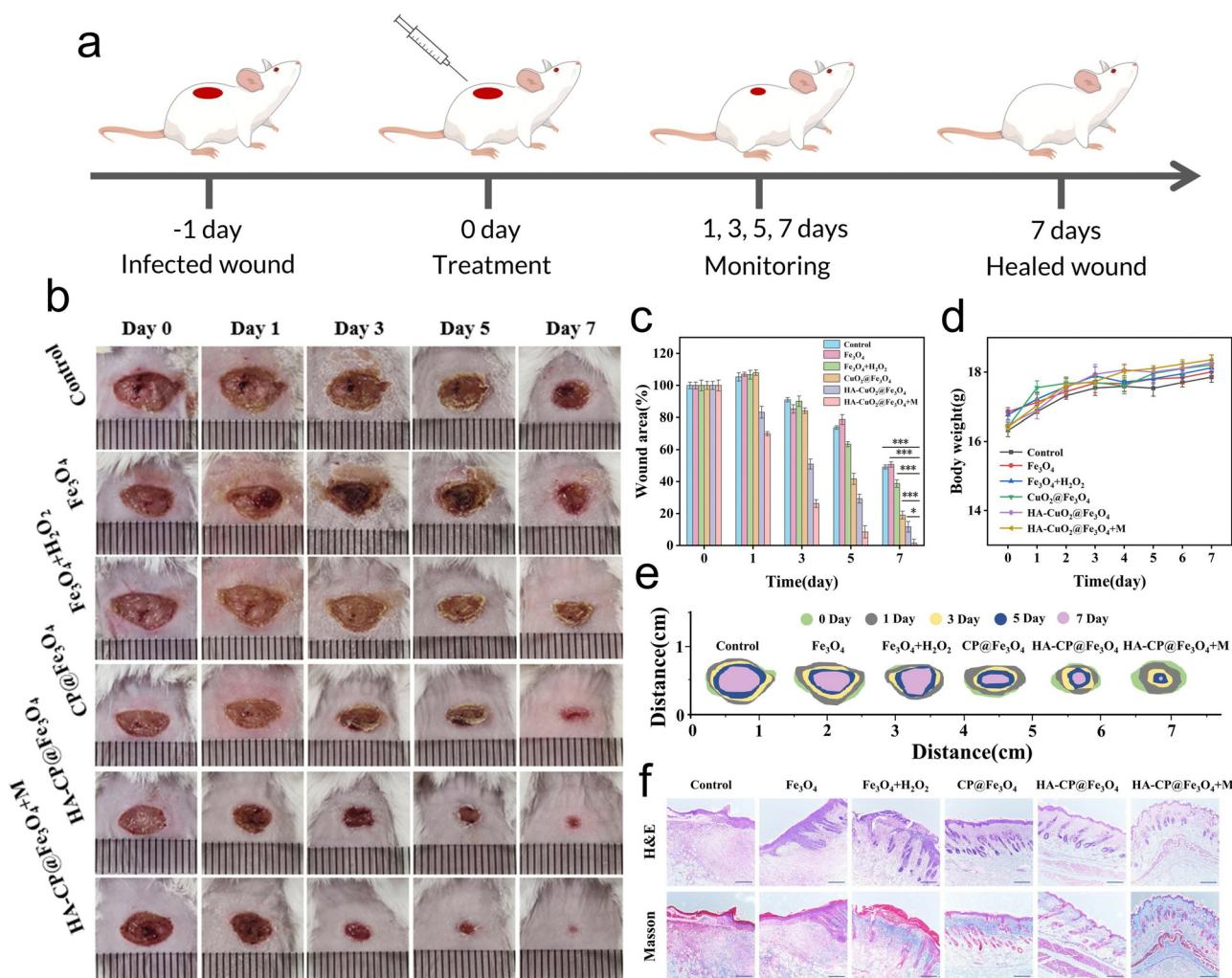


Fig. 5 Study on antibacterial activity of HA-CP@ Fe_3O_4 *in vivo*. (a) Schematic of the wound healing process. (b) Representative images of skin wounds after treatment with different groups ($30 \mu\text{g mL}^{-1}$). (c) Quantitative analysis of wound area for each group at different time points. (d) Change in body weight of mice in different treatment groups during the wound-healing process. (e) Dynamic process diagram illustrating the wound healing progression for each treatment group. (f) H&E staining and Masson staining of wounded tissues. Scale bars: 200 μm .

treatment groups were determined to be 49.2, 50.7, 38.7, 19.1, 11.7, and 1.5%, respectively. As anticipated, the HA-CP@Fe₃O₄ + M treatment group exhibited the most favorable wound healing and scabbing outcomes. The results imply that HA-CP@Fe₃O₄ aggregated at the infection site under the influence of a magnetic field can produce ROS to achieve adequate antimicrobial effects *in vivo*. Importantly, the body weight of mice did not show significant changes across the different treatments, suggesting that none of the treatment groups impeded the normal growth of mice (Fig. 5(d)).

The wound healing capacity was determined by collecting wound tissues from each group of mice on day 7 following treatment and staining with hematoxylin and eosin (H&E) (Fig. 5(f)). Severe inflammatory cell infiltration was observed in the PBS and Fe₃O₄ group. In contrast, the HA-CP@Fe₃O₄ + M group showed obvious and intact epidermal-dermal connections, along with significant reduction in inflammatory infiltration, suggesting remarkable wound healing capacity of HA-CP@Fe₃O₄. Masson staining was employed to closely examine the growth of collagen fibers in skin tissues. Remarkably, the HA-CP@Fe₃O₄ + M group exhibited the highest concentration of collagen fibers compared to the PBS and Fe₃O₄ groups, which showed the lowest amount of collagen fibers. Additionally, the HA-CP@Fe₃O₄ + M group showed a notable increase in hair follicle regeneration, demonstrating successful tissue regeneration. After 7 days of treatment, the infected tissue was removed for plate counting to conduct further *in vivo* antimicrobial assessment. As depicted in Fig. S12 and S13 (ESI[†]), the HA-CP@Fe₃O₄ + M group displayed the lowest

number of bacterial colonies among all the groups. In conclusion, the HA-CP@Fe₃O₄ nanomaterials exhibit outstanding *in vivo* wound healing and antimicrobial activities.

3.6 Cytotoxicity of HA-CP@Fe₃O₄

Biocompatibility has always been the primary safety criterion for *in vivo* application of antimicrobial nanoplateforms. In order to assess the overall biocompatibility of HA-CP@Fe₃O₄, we conducted assessments of its cytocompatibility and hemocompatibility. By using the MTT assay, the toxicity of HA-CP@Fe₃O₄ for L929 cells was initially evaluated. As depicted in Fig. 6(b), even at a concentration of 200 µg mL⁻¹, more than 85% of the cells remained viable, proving that HA-CP@Fe₃O₄ exhibited minimal cytotoxicity. In addition, we further evaluated the cytocompatibility of CP@Fe₃O₄, the results indicate that the biocompatibility of CP@Fe₃O₄ is slightly lower than that of HA-CP@Fe₃O₄ (Fig. S14, ESI[†]). However, at a concentration of 100 µg mL⁻¹ (determined as the *in vitro* antibacterial concentration against *E. coli*) of CP@Fe₃O₄, the viability of L929 cells remains above 80%. Therefore, CP@Fe₃O₄ may be utilized for the treatment of certain infections caused by multiple bacterial strains, but there is a potential risk of compromising the biocompatibility of CP@Fe₃O₄.

The hemolytic activity test was conducted to evaluate the potential hemolytic performance of HA-CP@Fe₃O₄ by co-incubating various concentrations of HA-CP@Fe₃O₄ with mouse erythrocytes. The results demonstrated that the supernatants of various concentrations of HA-CP@Fe₃O₄-treated groups resembled those of the negative control (PBS) group in terms

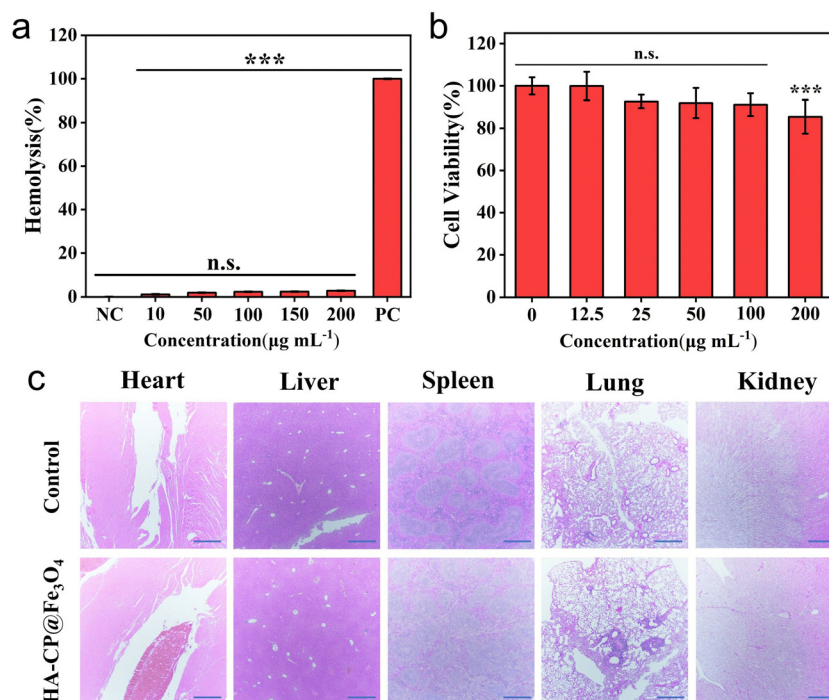


Fig. 6 (a) Hemolysis assay using HA-CP@Fe₃O₄ dispersions at different concentrations. Water is the positive control (PC), and PBS is the negative control (NC). (b) The survival rate of L929 cells after treatment with different concentrations of HA-CP@Fe₃O₄ for 24 h. (c) H&E staining images of the major organs (heart, liver, spleen, lungs, and kidneys) of mice from two different groups after 7 days. Scale bar = 500 µm.

of being translucent and colorless (Fig. 6(a)). Even at a concentration of $200\ \mu\text{g mL}^{-1}$, the HA-CP@Fe₃O₄ did not cause significant hemolysis of erythrocytes, with a hemolysis rate of only 2.85%. These findings indicate that the material poses excellent blood compatibility and further support its safety for potential biomedical applications. Finally, H&E staining was conducted on vital organs, including the heart, liver, spleen, lungs, and kidneys, to assess the *in vivo* biosafety of HA-CP@Fe₃O₄ (Fig. 6(c)). The lack of any visible histologic abnormalities or organ damage in mice treated with HA-CP@Fe₃O₄ demonstrated that the drug has favorable *in vivo* biosafety.

4. Conclusions

In summary, we have developed an on-demand chemodynamic antimicrobial nanoplatform (HA-CP@Fe₃O₄) with self-supply of H₂O₂ by successfully coating enzyme-responsive HA onto CuO₂-loaded Fe₃O₄ nanoparticles. Under weakly acidic conditions, HA-CP@Fe₃O₄ not only generates •OH radicals to facilitate H₂O₂ self-supplying controlled-release CDT *via* the Fenton effect but also produces ¹O₂ for synergistic sterilization through the Russell effect, enabling synergistic antimicrobial therapy. Furthermore, the encapsulation of HA provides (1) a targeting affect against HAase-secreting microorganisms, (2) protection against cytotoxicity, and (3) prevents resource waste due to improper copper ion release. Additionally, HA has the beneficial effect of accelerating the wound healing. *In vivo* studies confirm that the magnetic aggregation of HA-CP@Fe₃O₄ on the wound can accelerate the healing process while maintaining the excellent hemocompatibility and cytocompatibility. Overall, this study presents a novel and effective on-demand antimicrobial platform with self-supplied H₂O₂, showing great potential for the clinical treatment of bacteria-infected wounds.

Author contributions

Sijie Zhang: validation, methodology, writing original draft. Sameer Hussain: conceptualization, data curation. Yuhai Tang: conceptualization, supervision. Kaili Wang: validation, data curation. Xingyan Wang: investigation, methodology. Long Zhang: validation. Yuheng Liao: data curation. Chen Wang: data curation. Yi Hao: conceptualization, investigation, data curation. Ruixia Gao: project administration, supervision, funding acquisition. All persons who have made substantial contributions to the work reported in the manuscript.

Conflicts of interest

There are no conflicts to declare.

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