Supplementary Material

A multi-enzyme-like activities mussel-inspired nanozyme hydrogel for bacteria-infected wounds healing

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1.1. Materials

Tannic acid (TA), and 3,3',5,5'-Tetramethylbenzidine (TMB, 99%), 1,2-diaminobenzene (OPD, 99%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 99%), horseradish peroxidase (HRP), H_2O_2 (30%), single-component substrate solution were obtained from Aladdin Chemistry Co., Ltd. $FeCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$ and silver nitrate, ammonium hydroxide, sodium hydroxide, polyvinyl alcohol (alcoholysis degree 99%, Mw 98000), dextran70 (Mw=70000, Dex), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethanol, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals were used as received without further purification.

1.2. Characterization

The transmission electron microscopy (TEM) was performed on a JEM-2100F microscope (JEOL, Japan) working at 200 kV. X-ray photoelectron spectroscopy (XPS) was conducted using an ESCALAB 250XI electron spectrometer (Thermo Fisher Scientific Inc., USA). The UV–vis absorption spectra and the fluorescence spectra were carried out on Cary Eclipse (G9800A, Agilent Technologies) fluorescence spectrophotometer and TU-1901 (Persee, Beijing) spectrophotometer, respectively. Fourier transform infrared spectroscopy (FT-IR) was performed on a Bruker Vertex 70 spectrometer (Bruker, Germany). X-ray diffractometer (XRD) patterns were recorded with a D8-advance (Bruker, Germany).

1.3 Synthesis of S. aureus-based CDs

The S. aureus-CDs were synthesized by one step hydrothermal strategy. Briefly, 2 mL S. aureus suspension was dispersed in 20 mL distilled water under ultrasonic vibration for 15 min. The suspension was transferred to a Teflon-lined autoclave and reacted at 200 °C for 24 h. After naturally cooling to room temperature, the resulting solution was centrifuged at 8,000 rpm for 20 min to remove large aggregated particles, and filtered through a 0.22 μm filter. The resulting solution was dialyzed against deionized water in a 3000 Da dialysis tube for 24 h to remove unreacted reactants.

1.4 Determination of ROS

1.4.1 AA as ROS probe

Ascorbic acid (AA) can be used as a ROS probe by measuring changes in UV absorption when it was mixed with nanozyme hydrogels. AA shows strong UV absorption at 279 nm, but it can be oxidized by ROS to form dehydroascorbic acid [1]. Specifically, 150 μL AA (5 mM) was mixed with nanozyme hydrogel (1 mg mL^{-1}, 100 μL) in phosphate-buffered saline (PBS, pH 7.4) and then incubated at 37 °C for 1 h, followed by centrifugation at 8000 rpm for 10 min to remove insoluble material. The absorbance values of the mixture were measured at 279 nm.

1.4.2 DCFH-DA as probe

2′,7′-dichlorofluorescein diacetate (DCFH-DA) dye was applied to determine the ROS levels [2]. Specifically, DCFH-DA solution (5 mg mL^{-1}, 10 μL) was mixed with nanozyme hydrogels (1 mg mL^{-1}, 100 μL) in phosphate buffer solution (PBS, pH 7.4) and then incubated for 30 min at 37 °C. Finally, the fluorescence intensity at Ex=488 nm was measured using a fluorescence spectrophotometer.

1.4.3 Detection of hydroxyl radical (·OH)

p-Phthalic acid (PTA), as a fluorescent probe, was used for the detection of ·OH when it was mixed with nanozyme gels and H_2O_2. Specifically, PTA solution (10 mM, 100 μL) was mixed with H_2O_2 (50 mM, 100 μL) and nanozyme gels (1 mg mL^{-1}, 100 μL) in phosphate buffer. After incubated at room temperature for 4 h, the fluorescence intensity at Ex=305 nm was recorded. The emission intensity was positively correlated with the content of ·OH. Meanwhile, ·OH was also detected by

electron spin resonance (ESR). Nanozyme gels, H_2O_2 and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (0.5 mM, 100 μL) were mixed with phosphate buffer (pH=3), and then ESR was performed to record the ·OH signal of the mixture.

1.4.4 Detection of $O_2^{\cdot-}$

The analysis of $O_2^{\cdot-}$ was performed by evaluating the color change of the nitro blue tetrazolium chloride monohydrate (NBT)-nanozyme gels reaction system using spectrophotometry [3]. In the catalytic system of nanozyme gel, $O_2^{\cdot-}$ can reduce NBT to a blue-purple methylhydrazone with maximum UV absorption at 580 nm. After the reaction of the nanozyme gel with NBT (4.5 mM, 100 μL) in phosphate buffer for 15 min, the absorbance change at 575 nm was measured to verify the presence of $O_2^{\cdot-}$.

1.4.5 Detection of GSH

The oxidation degree of glutathione (GSH) was evaluated by Ellman's assay [4]. Ellman reagent (5,5′-dithiobis (2-nitrobenzoic acid), DTNB) could oxidized thiol groups (-SH) to -S-S- links in GSSH, producing a yellow product (2-nitro-5 thiobenzoate acid). Typically, 1 mL of bicarbonate buffer solution (50 mM, pH = 8.7) containing the nanozyme gels samples was mixed with 1 mL of 0.8 mM GSH bicarbonate buffer solution, and then the mixture was incubated at room temperature at a shaking speed of 150 rpm. Subsequently, 1 mM H_2O_2+GSH and GSH solutions were used as positive and negative control groups, respectively. After that, 3.5 mL of 0.05 M Tris-HCl (pH = 8) solution and 670 μL of bicarbonate buffer solution with 100 mM DTNB were added and then centrifuged at 3000 rpm for 15 min. Finally, the absorbance of the solution at 412 nm was measured on a microplate spectrophotometer. The following equation was used to calculate the loss of GSH: GSH loss (%)=OD (control)-OD (sample)/OD (control)×100 %.

1.5 Bacterial cultivation

Pseudomonas aeruginosa (*P. Aeruginosa*, ATCC 9027) and *Staphylococcus aureus* (*S. aureus*, ATCC 6538) were selected as representative strains of Gram-negative and Gram-positive bacteria, respectively. The colonies formed in solid Luria-Bertani (LB) medium and solid nutrient broth medium were inoculated in liquid medium (5 mL), and then cultured at 37 ℃ for 12 h. The final concentration of bacterial suspension was diluted to 1×10^8 CFU mL^{-1}.

1.6 Labeling bacteria with *S. aureus*-CDs

The preparation of *S. aureus*-CDs was shown in *Supplementary data*. *S. aureus* and *P. aeruginosa* suspension (1×10^5 CFU mL^{-1}) were divided into four groups: control, Cu,Fe-NC, CDs/AgNPs and CDs/AgNPs@Cu,Fe-NC. Then *S. aureus*-CDs were incubated at 37 ℃ for 1h. Before fluorescence imaging, all samples were centrifuged and washed 6 times with sterile PBS at 4000 rpm 5 min, and then 20 μL bacterial suspension was taken on the slide and covered with the slide. Finally, the bacteria were observed with a CLSM.

1.7 Morphological observation of bacterial cell membrane integrity

The suspension of *S. aureus* and *P. aeruginosa* (1×10^8 CFU mL^{-1}) was centrifuged (4000 rpm, 5 min) and washed with PBS for 6 times. After the bacterial suspension was dropped on the glass slide, the bacteria in the sample was fixed with 4 % glutaraldehyde solution for 4 h, and then gradually dehydrated with 30, 50, 70, and 100 % ethanol solution for 10 min. Bacteria samples were naturally dried at room temperature for 12 h. After sputter Au and carbon coating, the bacterial samples were observed by a SEM.

1.8 Biocompatibility of the nanozyme gel

The biocompatibility of nanozyme gel was evaluated by hematological analysis in rats. Specifically, female rats, aged 6-8 weeks with a body weight of 180-220 g, were divided into two groups (5 mice in each group). Rats were intragastrically administered with PBS (3 mL per rat) and nanozyme gels suspension (3 mL per rat, 10 mg kg^{-1}), respectively, once daily for 2 consecutive days. One day and the seventh day after intragastrically administered, respectively, blood was taken from the eyes of the rats and mixed with EDTA disodium anticoagulant. Hematology tests are performed using an automated blood analyzer.

At the same time, the cytotoxicity of nanozyme gel on mammalian cells (HT29 cells) was evaluated by MTT method. Specifically, the cells were first cultured to a density of 1×10^5 cells mL^{-1}. Then, the cells were added to a 96-well plate, cultured in a 37 °C incubator (5% CO_2) for 24 h, and then incubated with nanozyme gels of different qualities (0-600 μg) for 24 h. The liquid medium and nanozyme gels were sucked out and washed twice with sterile PBS. The MTT solution (50 μL) was added and incubated for 4 h (37 ℃). After the color turns dark purple, 200 μL of DMSO was added to each well and shaken for 15 minutes. Finally, record the absorbance at 570 nm was recorded on a microplate reader.

Finally, the hemolytic properties of the nanozyme gel were further evaluated. Specifically, nanozyme gels aqueous (50-300 mg mL^{-1}), PBS (as a negative control) and 0.1% Triton X-100 (as a positive control) were mixed thoroughly with well-isolated rat red blood cells (RBCs) and incubated at 37°C for 2 h. After centrifugation,

the supernatant was analyzed by full wavelength scanning and the absorbance of the mixture was tested at 570 nm.

1.9 Adhesion strength

Bulk adhesion property of the nanozyme gels was studied by lap shear tests[5]. The lap-shear tests with fresh pigskin tissue skin as adherent were conducted to study the adhesive ability of the nanozyme gels to the host tissue. 200 μL of the nanozyme gels was applied to the surface of the fresh tissue (3 cm × 1 cm), and another tissue was placed on top of the polymer mixture. The contact area of the two skin tissues was 1 cm × 1 cm. The tissues were then incubated at room temperature for 2 h for complete gelation before the lap shear test. The samples were tested with a universal testing machine (MTS Criterion 43; MTS Criterion, USA). The sensor was 50 N, and the speed was 5 mm/min. Each measurement was repeated at least 3 times.

Figures:

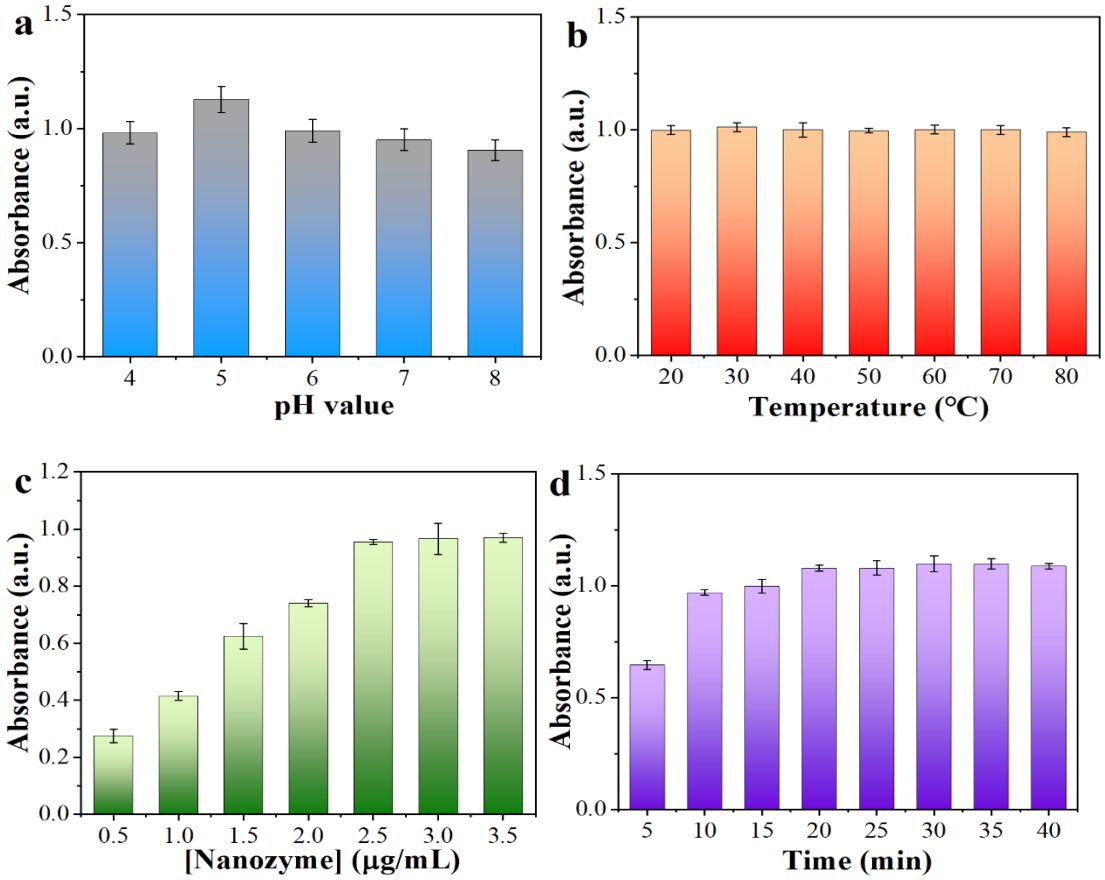


Fig. S1 Different conditions effect on OXD-like activity (TMB as substrate), (a) the nanozyme concentration, (b) pH, (c) temperature and (d) the reaction time.

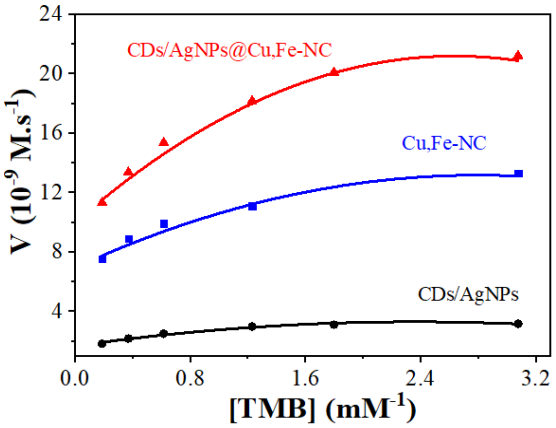


Fig. S2 Michaelis–Menten curves for the OXD-like activity of the nanozymes with varied TMB concentrations without the presence of H_2O_2.

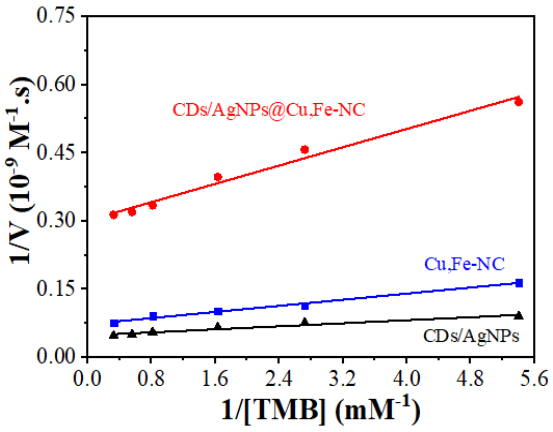


Fig. S3 The steady-state kinetics of nanozymes toward various concentrations of TMB.

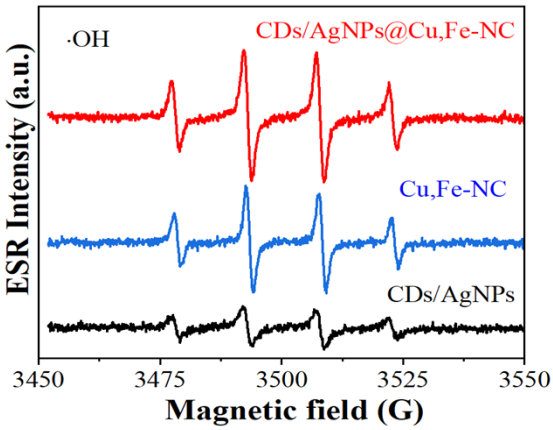


Fig. S4 Electron spin resonance (ESR) spectra of nanozyme in the presence of APS and DMPO.

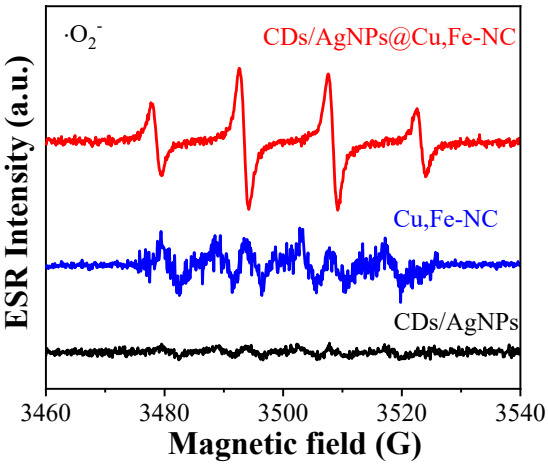


Fig. S5 Electron spin resonance (ESR) spectra of nanozyme in the presence of APS

and DMPO.

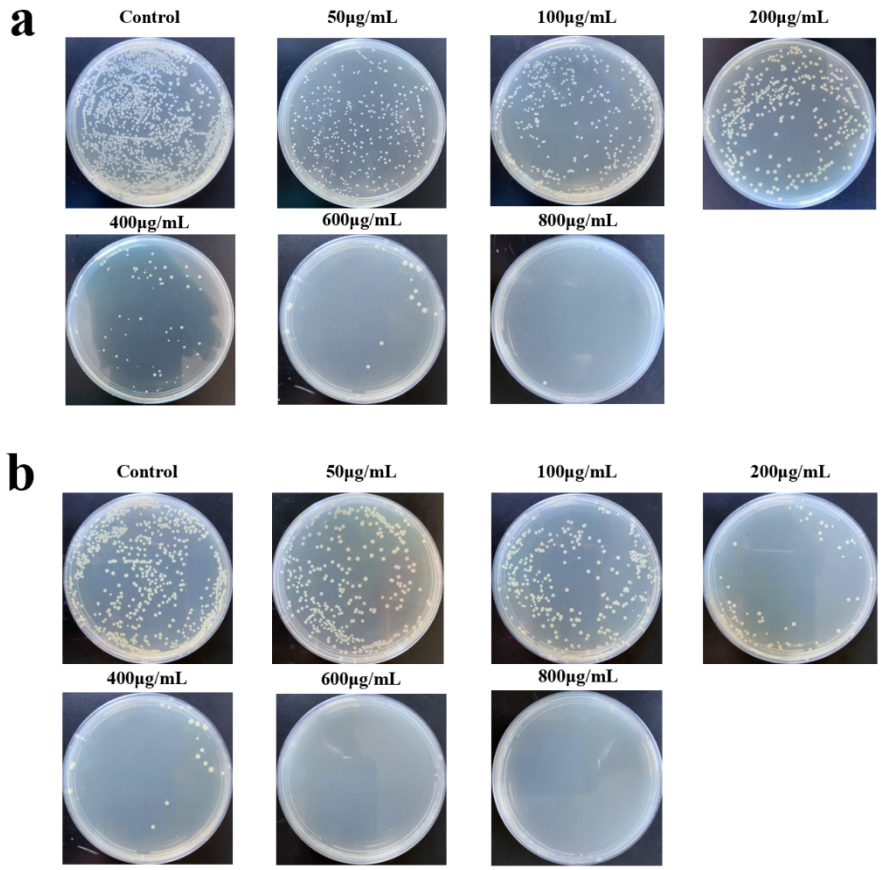
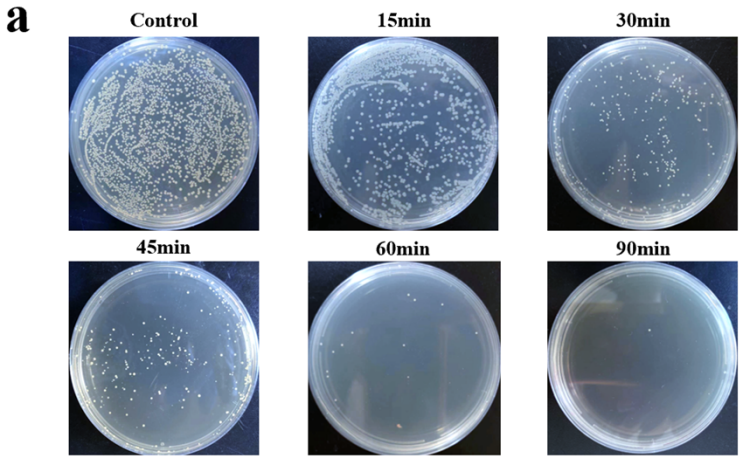


Fig. S6 Photographs of bacterial colonies of (a) *P. aeruginosa* and (b) *S. aureus* treated by Cu,Fe-NC (0, 50, 100, 200, 400, 600, 800 μg/mL).



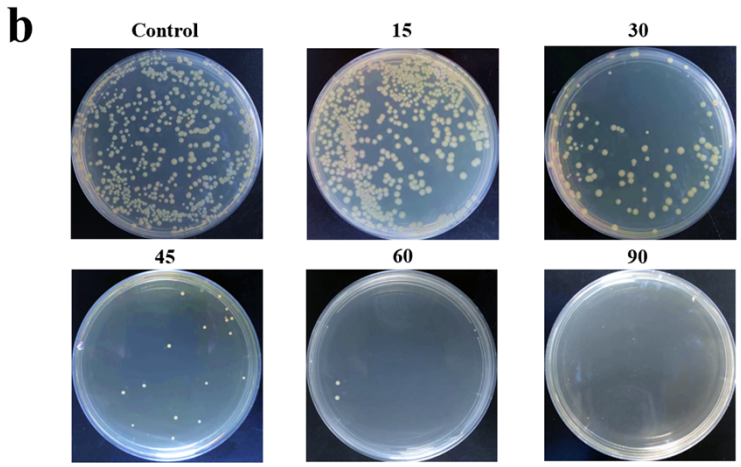
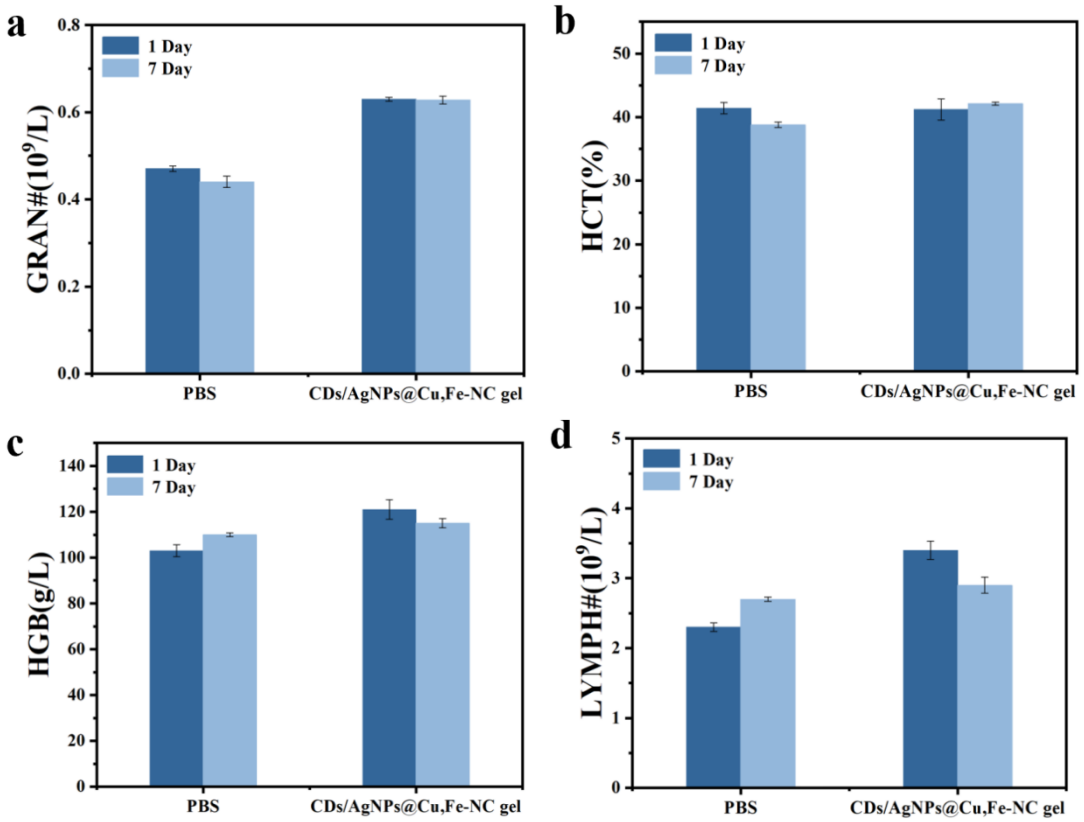
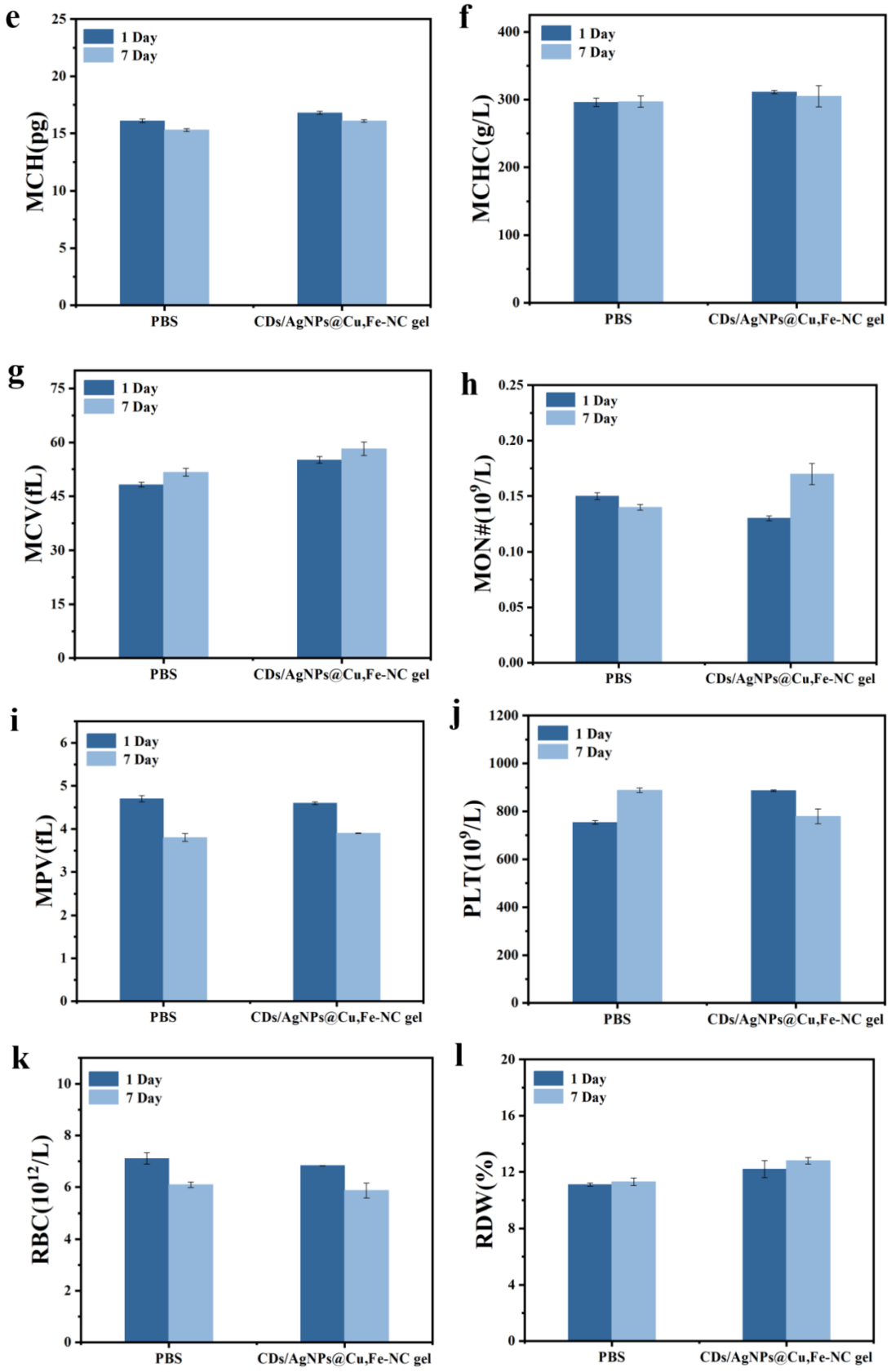


Fig. S7 Photographs of bacterial colonies of (a) *P. aeruginosa* and (b) *S. aureus* different treated time by Cu,Fe-NC (0, 15, 30, 45, 60, 90 min).




e
MCH(pg)
1 Day
7 Day
PBS
CDs/AgNPs@Cu,Fe-NC gel
f
MCHC(g/L)
g
MCV(fL)
h
MON#(10^9/L)
i
MPV(fL)
j
PLT(10^9/L)
k
RBC(10^12/L)
l
RDW(%)

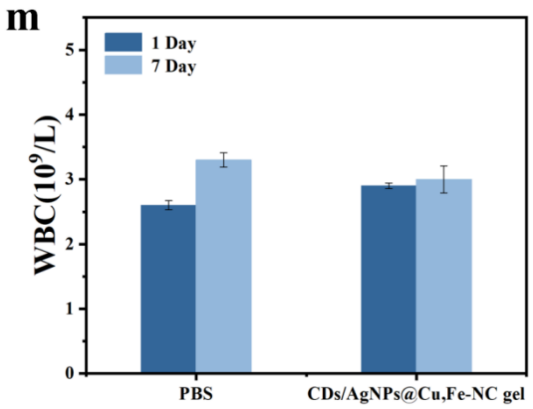


Fig. S8 Hematological indicators of (a) GRAN#, (b) HCT, (c) HGB, (d) LYMPH, (e) MCH, (f) MCHC, (g) MCV, (h) MON#, (i) MPV, (j) PLT, (k) RBC, (l) RDW and (m) WBC of mice on the 1st and 7th day after treatment. Data are presented as mean ± SD (n = 5).

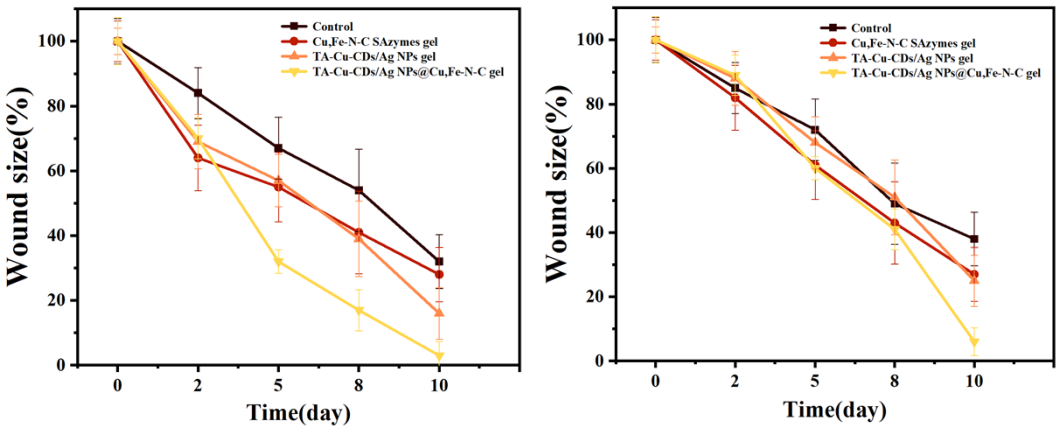


Fig. S9 The wound area values of (a) *P. aeruginosa* and (b) *S. aureus* treated by CDs/AgNPs@Cu,Fe-NC gel.

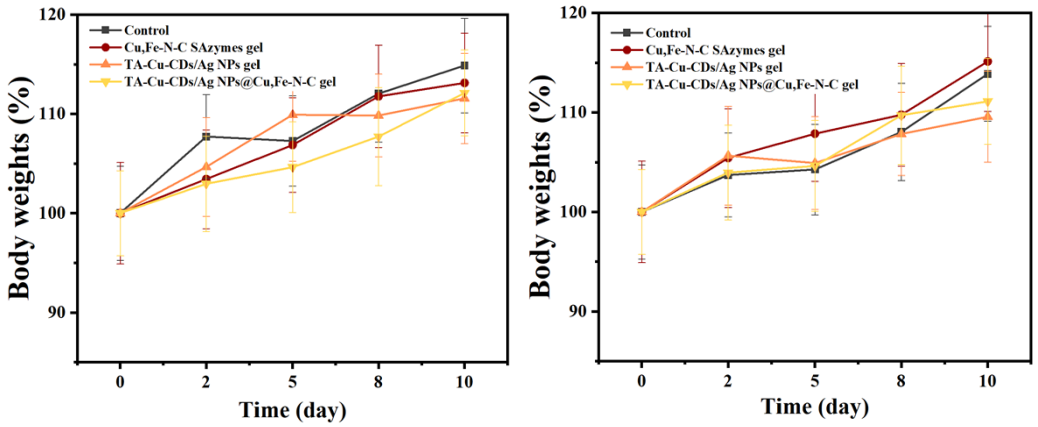


Fig. S10 The body weight of mice of treated by CDs/AgNPs@Cu,Fe-NC gel (a) *P. aeruginosa* and (b) *S. aureus*.

Tables:

Table S1 The Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) of the nanozymes for OXD-like catalysis.

Nanozymes	**TMB**	
	K_m （mM^{-1}）	V_{max}(×10^{-9} M·s^{-1})
CDs/AgNPs	0.60	1.19
Cu,Fe-NC	0.58	1.99
CDs/AgNPs@Cu,Fe-NC	0.44	5.95

References

[1] G. Fang, W. Li, X. Shen, J.M. Perez-Aguilar, T. Chong, X. Gao, Z. Chai, C. Chen, C. Ge, R. Zhou. *Nat. Commun.* 9 (2018) 129. https://doi.org/10.1038/s41467-017-02502-3.

[2] Y. Ma, W. Gao, Y. Zhang, M. Yang, X. Yan, Y. Zhang, G. Li, C. Liu, C. Xu, M. Zhang. *ACS Appl. Mater. Interfaces* 14 (2022) 6358−6369. https://doi.org/10.1021/acsami.1c21700.

[3] X. Zhou, W. Zeng, S. Rong, H. Lv, Y. Chen, Y. Mao, W. Tan, H. Li. *ACS Appl. Mater. Interfaces* 13 (2021) 47394−47406. https://doi.org/10.1021/acsami.1c15358.

[4] Y. Li, R. Fu, Z. Duan, C. Zhu, D. Fan. *Adv. Healthcare Mater.* 11 (2022) 2101849. https://doi.org/10.1002/adhm.202101849.

[5] R. Dong, X. Zhao, B. Guo, P.X. Ma. *ACS Appl. Mater. Interfaces*, 8 (2016) 17138. https://doi.org/10.1021/acsami.6b04911.