Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Fe-Based Single-Atom Nanozyme with Multi-enzyme Activity for Parallel Catalytic Therapy via Cascade Reaction

Rui Niu, Yang Liu, Yinghui Wang and Hongjie Zhang**

R. Niu, Y. Liu, Prof. Y. Wang, Prof. H. Zhang

State Key Laboratory of Rare Earth Resource Utilization, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin, 130022, P. R. China.

E-mail: yhwang@ciac.ac.cn, hongjie@ciac.ac.cn

R. Niu, Prof. Y. Wang, Prof. H. Zhang

University of Science and Technology of China, Hefei 230026, Anhui, China.

Prof. H. Zhang

Department of Chemistry, Tsinghua University, Beijing 100084, PR China.

Experimental Section

Materials

Dopamine hydrochloride (DA), Tetraethyl orthosilicate (TEOS), Iron (III) acetylacetonate ($Fe(acac)_3$), 1,4-dicarboxybenzene (TA), Sodium hydroxide (NaOH) and 3,3',5,5′-tetramethyl-benzidine (TMB) were purchased from Macklin Inc. (Macklin, Shanghai, China). Ammonium hydroxide (28%), Ethyl Alcohol (95%) and Dimethyl Sulfoxide (DMSO) were purchased from Tian in Fuyu Fine chemical Co.Ltd. Bovine albumin（BSA）was purchased from Gentihold Biotechnology (Beijing, China). 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and 5-tert-butoxycarbonyl-5- methyl-1-pyrroline-N-oxide (BMPO) were purchased from Dojindo Co. Ltd. (Shanghai, China). 1,3-diphenylisobenzofuran (DPBF) was obtained from Alfa Aesar. 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl-imidacarbocyanine iodide (JC-1), 2,7-dichlorofluorescein diacetate (DCFH-DA), Calcein AM/Propidium Iodide (PI) Assay kit and Cell Counting Kit 8 (WST-8 / CCK8) were purchased from Beyotime Biotechnology (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640, penicillin-streptomycin, 0.25% trypsin-EDTA, fetal bovine serum (FBS) and Phosphate-buffered saline (PBS) were purchased from Hyclone, Cytiva. All chemicals were used without any further purification.

Characterization

TEM and EDS images were characterized by FEI Tecnai G2 F20 transmission electron microscope at a working voltage of 100 kV. HAADF-STEM images were characterized by JEM-ARM200F with a spherical aberration corrector working at 200 kV. Powder X-ray diffraction patterns of all samples were performed by D8 Advance Powder X-ray Diffractometer. X-ray photoelectron spectroscopy (XPS) of all samples were performed by VG ESCALAB MK II electron spectrometer. Fourier-transform infrared spectra (FT-IR) were recorded by a Vertex Perkin-Elmer 580BIR spectrophotometer (Bruker). Inductively coupled plasma optical emission spectrometer (ICP-OES) was used to measure the amount of Fe element. XAFS spectra at the Fe K-edge was collected at BL14W1 station in Shanghai Synchrotron

Radiation Facility (SSRF). XAFS data are analyzed by Athena and Artemis software, according to the standard procedures.

Preparation of FeSA-HNCS

The fabrication of the single-atom Fe dispersed N-doped hollow carbon spheres (FeSA-HNCS) was based on a literature method[1]. SiO_2 nanospheres with a diameter of about 250 nm were first synthesized as sacrificial templates via modified stöber method. In detail, 90 mL ethanol, 30 mL deionized water and 5.6 mL ammonium hydroxide were mixed and stirred at room temperature for 20 min. Then, 5 mL TEOS was dropwise added to the above mixed solution and stirred evenly for one hour. It was observed that the reaction system gradually changed from colorless to milky white. Subsequently, 500 mg DA and 5 mg $Fe(acac)_3$ were dissolved in 10mL mixture of deionized water and ethanol ($V_{water}/V_{ethanol}$=6:4), and then dropwise added to the above silica reaction system. After overnight continuous stirring at room temperature, the core-shell SiO_2@Fe-PDA nanospheres were successfully prepared. The grayish black products were collected by high-speed centrifugation and washed alternately with deionized water and ethanol at least three times. The collected SiO_2@Fe-PDA nanospheres was dried at 40℃ overnight in a vacuum oven. Next, the powder was transferred to a quartz boat and calcined at 900 °C for 2 h under N_2 atmospheres with a heating rate of 5 °C min^{-1}. After natural annealing, the pure black product is ground into powder and then added to sodium hydroxide solution and stirred for 24 h to etch the SiO_2 template. Finally, the FeSA-HNCS were collected by centrifugation, washed with water, and dried under vacuum to further use.

Surface modification

10 mg FeSA-HNCS was dispersed in 10 mL deionized water. After sonication for 2 h, 1mL BSA aqueous solution (1mg/mL) was added dropwise to FeSA-HNCS solution under stirring condition. Then, the mixture was vigorous stirred for overnight at room temperature. Next, the mixture was centrifuged and washed three times to obtain the FeSA-HNCSB.

Measurement of catalase-like activity

We used a dissolved oxygen instrument to measure the increasing rate of oxygen content in the solution to evaluate the CAT-like activity of FeSA-HNCSB nanozyme. Specifically, the detection electrode was immersed in 8 mL PBS (pH 6, 0.01M) or

deionized water and 1ml H_2O_2 (1mM) mixed solution, then 1 mL 1.0 mg mL^{-1} FeSA-HNCSB was quickly added and stirred for a few seconds. The oxygen concentration was immediately detected and the data have been continuous recording for 10 minutes.

Degradation experiment of DPBF

The production of $\cdot O_2^-$ catalyzed by FeSA-HNCSB nanozyme was determined by DPBF (1,3-diphenyl isobenzofuran). 100 μg/mL FeSA-HNCSB and 25 μg/mL DPBF were incubated in PBS (0.01 M, pH 6.0) with or without 0.1 mM H_2O_2 at room temperature. The absorbance of the color reactions was recorded after 10min using a UV-vis spectrophotometer.

POD-like activity and kinetic assay

POD-like activity assays of FeSA-HNCSB were carried out with H_2O_2 as reaction substrate and 3,3',5,5'-tetramethylbenzidine (TMB) as chromogenic agent. In brief, FeSA-HNCSB (20 μg mL^{-1}), TMB (1 mM) and various H_2O_2 concentrations (0.1mM, 0.2 mM, 0.25 mM, 0.5 mM, 1mM) were added to 2 mL of PBS solution (pH 6) at room temperature. The absorbance at 650 nm was recorded at an interval of five seconds via UV-vis spectrophotometer. The steady-state kinetic parameters of FeSA-HNCSB were obtained by fitting the absorbance data to the Michaelis-Menten equation. The initial reaction rates (V_0) of various H_2O_2 concentration were calculated from the absorbance changes by Beer-Lambert Law. The Michaelis-Menten kinetic curve was obtained by fitting the mathematical equation of the relationship between reaction rates and H_2O_2 concentrations. a linear double-reciprocal plot (Lineweaver-Burk plot) was used for calculating the K_m and V_{max}

$$A = \varepsilon bc \quad \text{(Eq1)}$$

$$v_0 = \frac{v_{max}.[s]}{k_m + [s]} \quad \text{(Eq2)}$$

$$\frac{1}{v_0} = \frac{k_m}{v_{max}}.\frac{1}{[S]} + \frac{1}{v_{max}} \quad \text{(Eq3)}$$

In these equations, ε is the molar absorption coefficient of oxTMB (39000 m^{-1}), [S] is the substrate concentrations.

OXD-like activity and kinetic assay.

OXD-like activity assays of FeSA-HNCSB were performed with TMB as reaction substrate. Concisely, FeSA-HNCSB (20 μg mL^{-1}) and various TMB concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mM) were added to 2 mL of PBS solution (pH 6) at room temperature. The absorbance at 650 nm was recorded at an interval of five seconds via UV-vis spectrophotometer. The steady-state kinetic parameters of FeSA-HNCSB were obtained by fitting the absorbance data to the Michaelis-Menten equation. The initial reaction rates (V_0) of various TMB concentration were calculated from the absorbance changes by Beer-Lambert Law. The Michaelis-Menten kinetic curve was obtained by fitting the mathematical equation of the relationship between reaction rates and TMB concentrations. a linear double-reciprocal plot (Lineweaver-Burk plot) was used for calculating the K_M and $V_{max.}$

Electron paramagnetic resonance (ESR) detect the generation of ROS

ESR assay was used to verify the production of different kinds of ROS. 100 μg mL^{-1} FeSA-HNCSB and 100 μM DMPO (as the ·OH trapping agent) was added into PBS (0.01 M, pH 6) containing 0.1 mM H_2O_2. 100 μg mL^{-1} FeSA-HNCSB and 100 μM BMPO (as the $\cdot O_2^-$ trapping agent) was added into PBS (0.01 M, pH 6). The mixture was quickly and evenly shaken and transferred to quartz tube for ESR assay.

Evaluation of Photothermal Properties

FeSA-HNCSB aqueous solution (1mL) with different concentrations (0, 25, 50, 100, 200 μg mL^{-1}) were irradiated by 808m laser (1.0 W cm^{-2}) for 10 minutes, and the temperature variation were detected by the thermocouple microprobe. After that, The temperature changes of aqueous FeSA-HNCSB aqueous solution (200 μg mL^{-1}) under various laser power density irradiation (0.25, 0.5,0.75 and 1.0 W cm^{-2} of 1064 nm laser) was monitored in the same way. The photothermal cycle stability and photothermal conversion effect of FeSA-HNCSB were further studied. The FeSA-HNCSB aqueous solution (200 μg mL^{-1}, 1 mL) was irradiated for 10 min via an 808 nm laser (1.0 W cm^{-2}), followed by shutting off the NIR laser until the solution cooled to room temperature. The heating and cooling experiment were repeated for five times. In the meantime, the temperature rise and fall data are recorded by thermocouple microprobe. The photothermal conversion efficiency (η) of FeSA-HNCSB was

calculated through the following equation:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{dis}}{I\left(1 - 10^{-A_{808}}\right)} \quad (Eq4)$$

$$hS = \frac{m_D C_D}{\tau} \quad (Eq5)$$

where h is the heat transfer coefficient, S is the surface area of container, T_{max} is the highest equilibrium temperature, Tsurr is environment temperature, Q_{dis} is the heat dissipated from light absorbed by the centrifuge tube containing PBS, I means the laser power density, and A_{808} is the absorbance of samples at 808 nm. m_D refers to the solution mass, C_D means the heat capacity of the solution, and τ is the sample system time constant. which is the time ratio to -lnθ ($\theta = \frac{\Delta T}{T_{max} - T_{surr}}$) in the cooling process.

Cell culture

Breast cancer 4T1 cells were seeded in RPMI 1640 medium with addition of 10% FBS, 1% penicillin-streptomycin, and culture at 37 °C in a thermostatic incubator (ThermoFisher) with 5% CO_2.

Intracellular ROS detection

DCFH-DA was used as a fluorescent probe to detect the generation of intracellular ROS. 4T1 cells were seeded in 6-well plates and cultured for 24 h. After that, the cells were treated with fresh acidified medium (pH 6.0) containing (a) Control; (b) 100 μM H_2O_2; (c) 200 μg/mL FeSA-HNCSB; (d) FeSA-HNCSB + H_2O_2 and sequentially incubated for another 12 h. Afterwards, the culture medium was removed and washed twice with PBS, and then RPMI 1640 containing DCFH-DA (10 μM) was added. After a further 15 minutes of incubation, the medium was removed and the cells were washed twice times with PBS. Fluorescence microscopy was applied to analyze the fluorescence imaging of cells.

Cytotoxicity Assay

The CCK8 assay was performed to evaluate cell viability. 4T1 cells were seeded in

96-well plates at the cell density of 1×10^4 cells/well and cultured for 24 h. After that, the neutral medium was removed. Four groups of different medium were divided into (a) neutral medium; (b) neutral medium+100 μM H_2O_2; (c) acidified medium (pH 6.0); (d) acidified medium (pH 6.0) + 100 μM H_2O_2. Culture medium of each group containing FeSA-HNCSB at serial concentrations (0, 12.5, 25, 50, 100 and 200 μg mL^{-1}) were severally added into each well and incubated with the cells for another 24 h. For PTT, acidified medium containing H_2O_2 and serial concentrations of FeSA-HNCSB were severally added into each well and incubated with the cells for 4 h. After that, each well was irradiated under 808 nm laser (1.0 W/cm^2) for 5 min and sequentially incubated for another 20 h. Afterwards, the medium of each well was removed and washed twice with PBS, then 100 μL RPMI 1640 containing 10 μL CCK8 was added to each well. After further incubation for 2 h, The cell viability was assessed by measuring the absorbance of each well at 450 nm via microplate reader.

live/dead cell staining assay

Calcein-AM and PI agents were used to stain the live cells and dead cells. 4T1 cells were seeded in 6-well plates and cultured for 24 h. After that, the cells were treated with fresh acidified medium (pH 6.0) containing (a) Control; (b) 100 μM H_2O_2; (c) 200 μg mL^{-1} FeSA-HNCSB; (d) FeSA-HNCSB + H_2O_2 and sequentially incubated for another 24 h. For PTT, acidified medium containing H_2O_2 and 200 μg mL^{-1} FeSA-HNCSB were incubated with the cells for 4 h. Then, the cells were irradiated under 808 nm laser for 5 min (1.0 W/cm^2) and sequentially incubated for another 20 h. Afterwards, the medium of each well was removed and washed twice with PBS, followed by staining with Calcein-AM (5 μM) and PI (2.5 μM). After further incubation for 15 min, the medium was removed and the cells were washed twice times with PBS. Fluorescence microscopy was applied to analyze the fluorescence imaging of cells.

JC-1 staining assay

JC-1 is a fluorescence probe for mitochondrial membrane potential measurement. 4T1 cells were seeded in 6-well plates and cultured for 24 h. After that, the cells were treated with fresh acidified medium (pH 6.0) containing (a) Control; (b) 100 μM H_2O_2;

(c) 200 μg mL^{-1} FeSA-HNCSB; (d) FeSA-HNCSB + H_2O_2 and sequentially incubated for another 24h. For PTT, acidified medium containing H_2O_2 and 200 μg mL^{-1} FeSA-HNCSB were incubated with the cells for 4 h. Then, the cells were irradiated under 808 nm laser for 5 min (1.0 W/cm^2) and sequentially incubated for another 20h. Afterwards, the medium of each well was removed and washed twice with PBS, followed by staining with JC-1 (2 μM). After further incubation for 20 min, the medium was removed and the cells were washed twice times with PBS. Fluorescence microscopy was applied to analyze the fluorescence imaging of cells.

Animal model

Female BALB/c mice (~ 20 g) were purchased from the Center for Experimental Animals, Jilin University (Changchun, China). 4T1 cells were injected subcutaneously into the left axilla of BALB / c mice to establish 4T1 tumor model. When the tumor size reached about 100 mm^3, the in vivo experiments were carried out.

In vivo anti-tumor assay

4T1 tumor-bearing BALB/c mice were randomly divided into four groups (five mice each): (a) PBS (100 μL); (b) 808 nm (1.0 W cm^{-2}, 10 min); (c) FeSA-HNCSB (10 mg kg^{-1}, 100 μL); (d) FeSA-HNCSB + 808 nm. After vein injection of PBS or FeSA-HNCSB solution for 24 h, the tumor region was irradiated by 808 nm laser. Measurements of tumor sizes and weights were taken every two days. On the 14th day after treatment, each group of mice were sacrificed to obtain the major organs and tumors for histological examination.

Results and Discussion

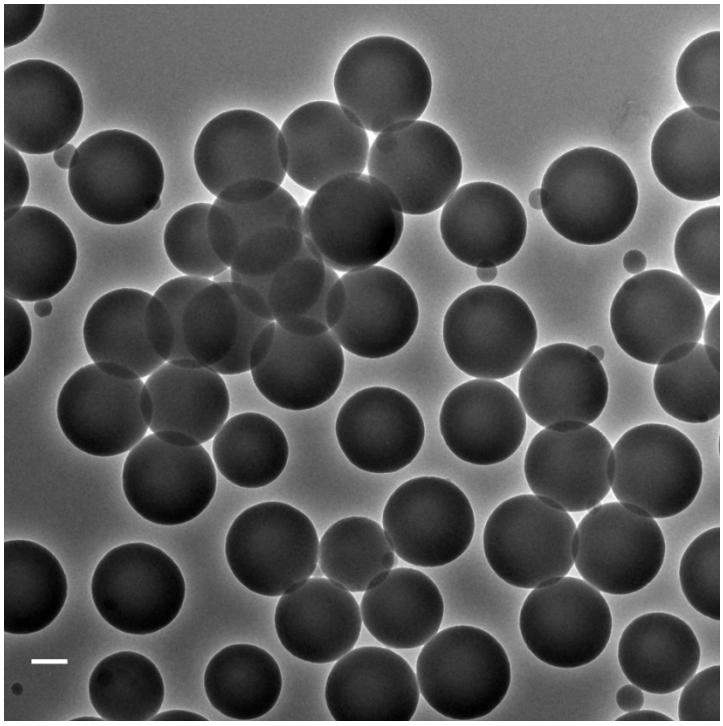

Figure S1. TEM image of SiO_2, which exhibits uniform spherical with a diameter of about 250 nm. Scale bar=50 nm.

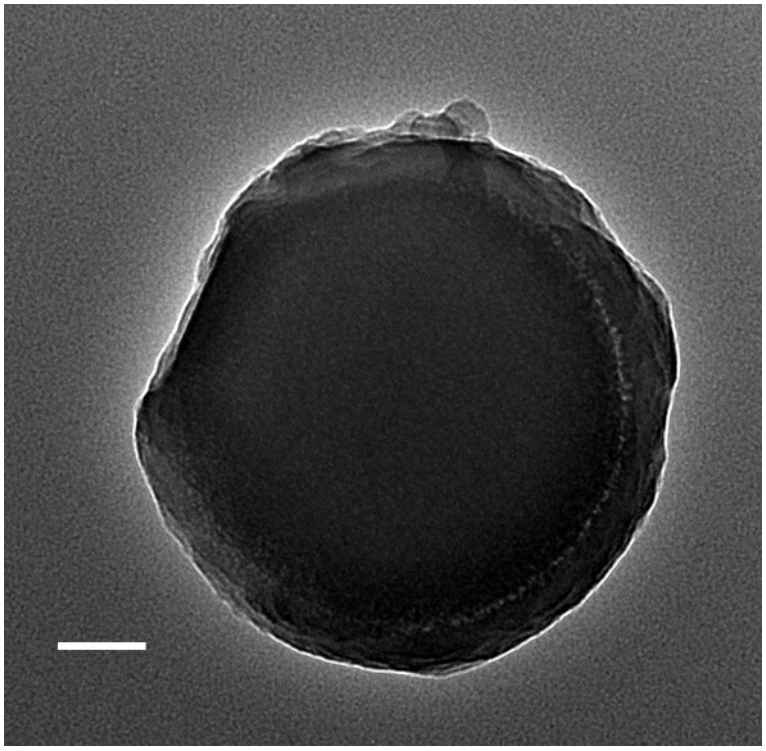

Figure S2. TEM image of SiO_2@Fe-PDA，which exhibits uniform core-shell structure. Scale bar=50 nm.

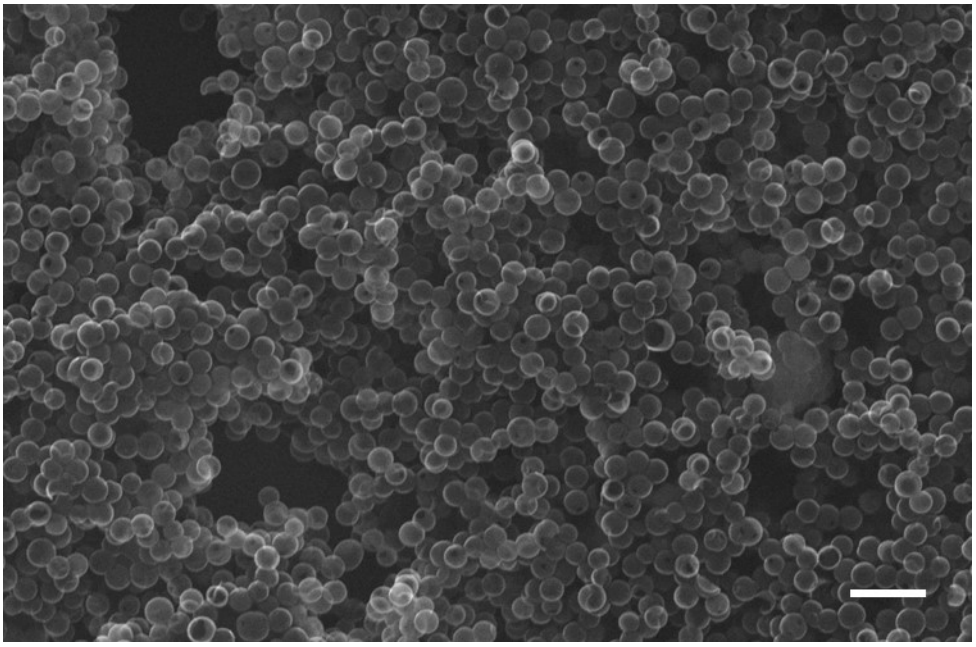

Figure S3. SEM image of FeSA-HNCS nanozyme. Scale bar=1 μm.

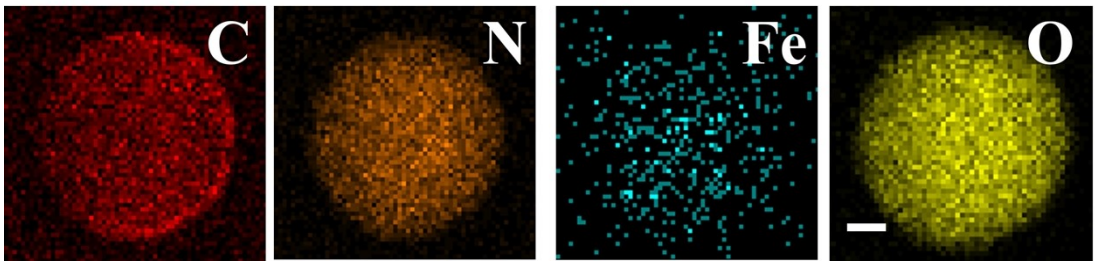

Figure S4. The EDS maps of C, N, Fe and O (scale bar 50 nm).

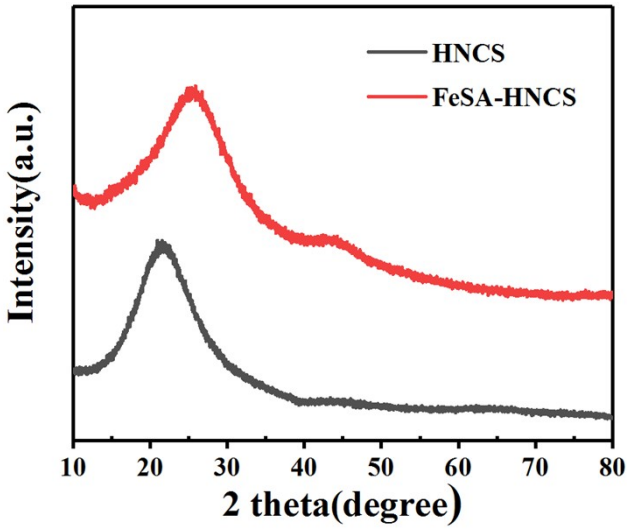

Figure S5. XRD patterns of FeSA-HNCS nanozymes and HNCS.

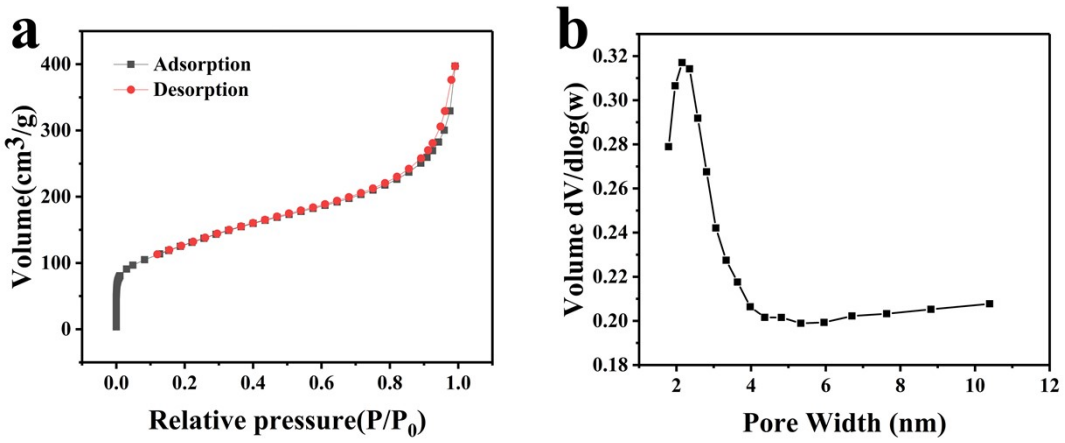

Figure S6. (a) N_2 sorption isotherms and (b) pore size distributions of FeSA-HNCS nanozyme.

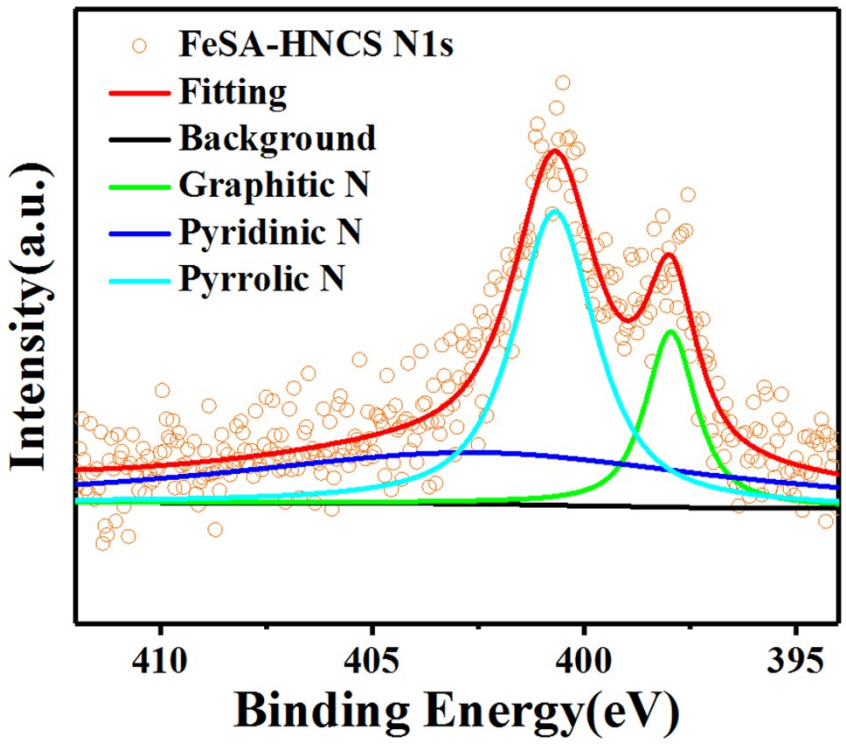

Figure S7. The N_{1s} XPS high resolution spectra of FeSA-HNCS.

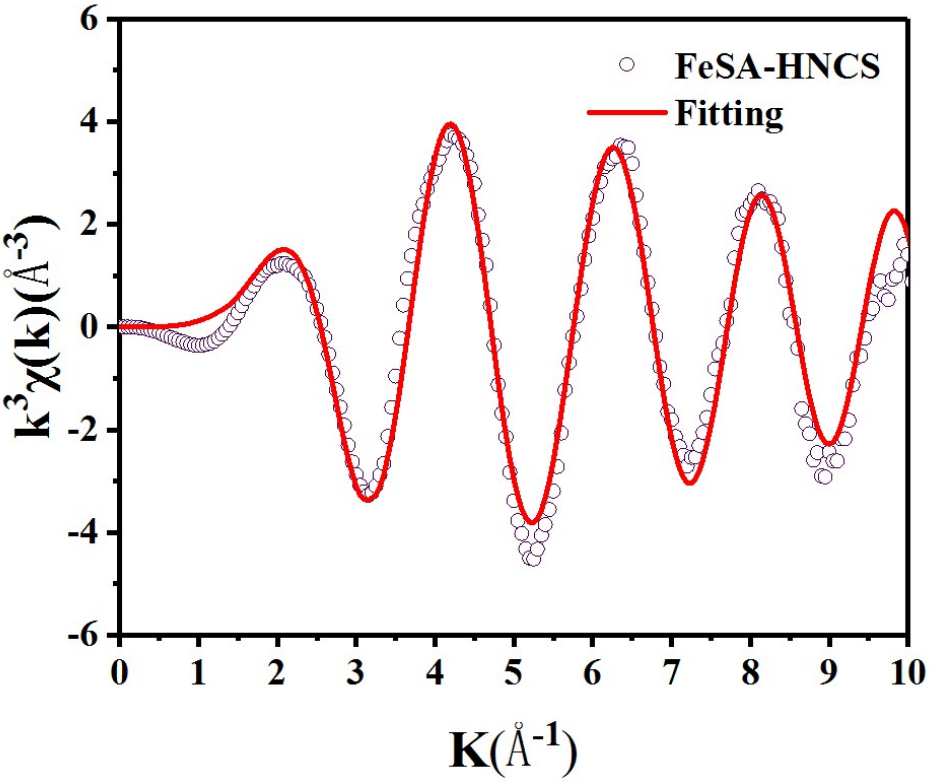

Figure S8. EXAFS fitting result of FeSA-HNCS at k space.

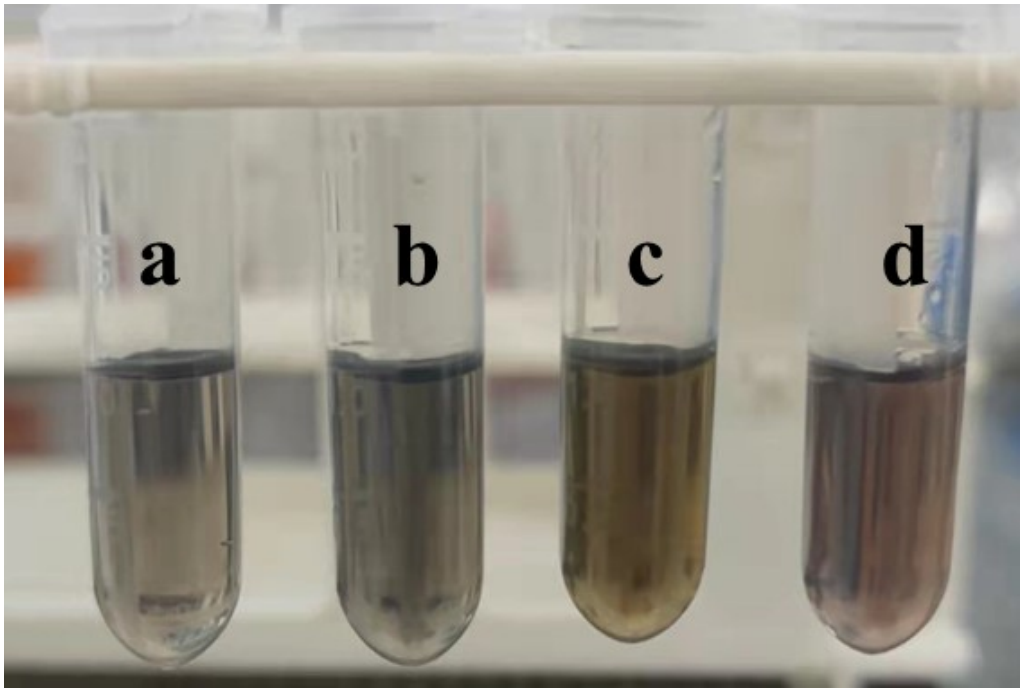

Figure S9. Thephotographs of FeSA-HNCSB dispersed in different solution systems (a:Water, b:PBS, c:FBS, d:RPMI 1640).

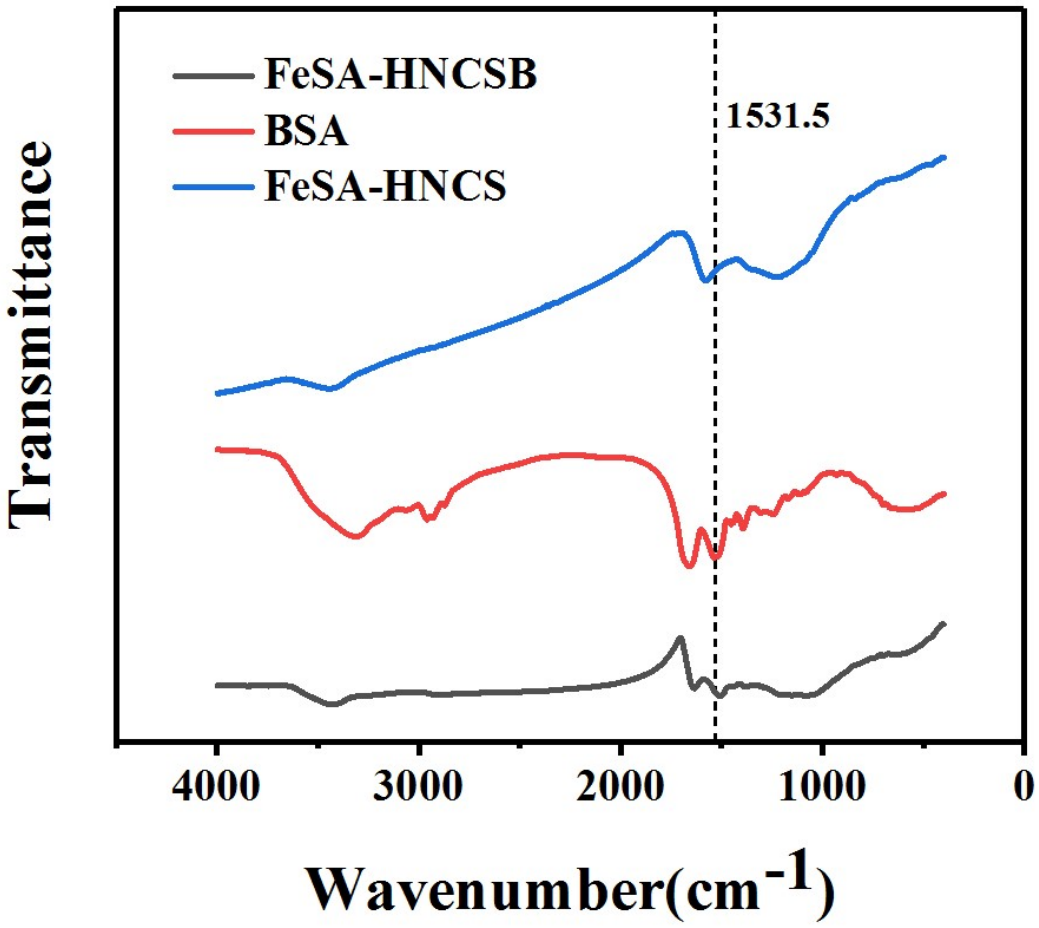

Figure S10. The Fourier transform infrared (FTIR) spectra of BSA, FeSA-HNCS, and FeSA-HNCSB.

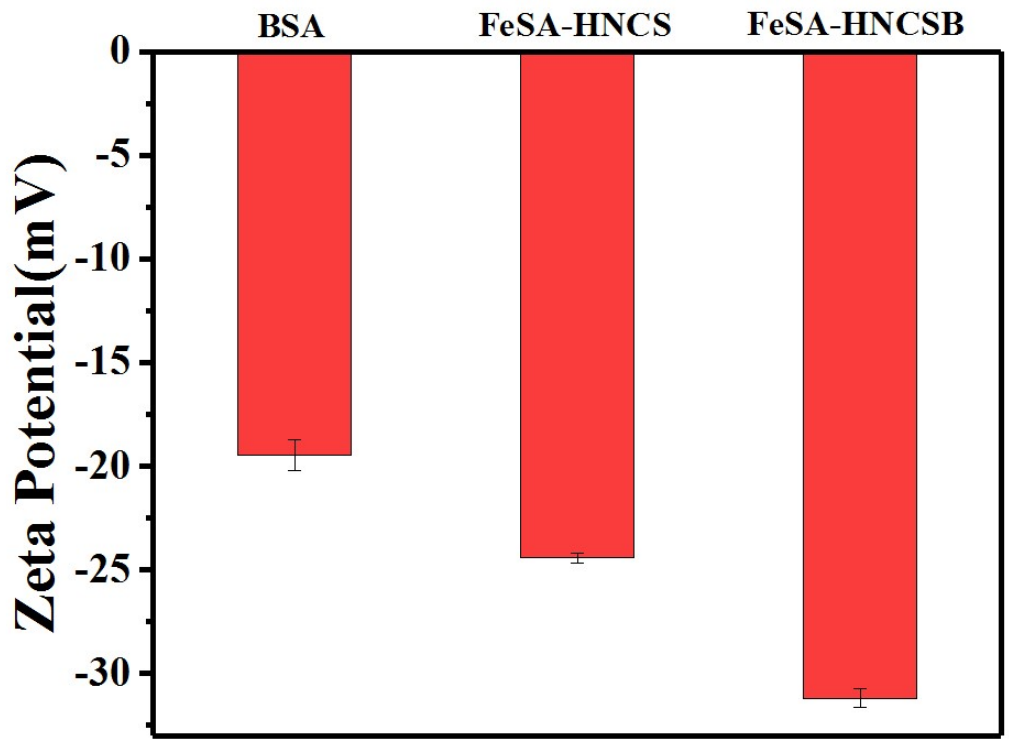

Figure S11. The zeta potential of BSA, FeSA-HNCS and FeSA-HNCSB.

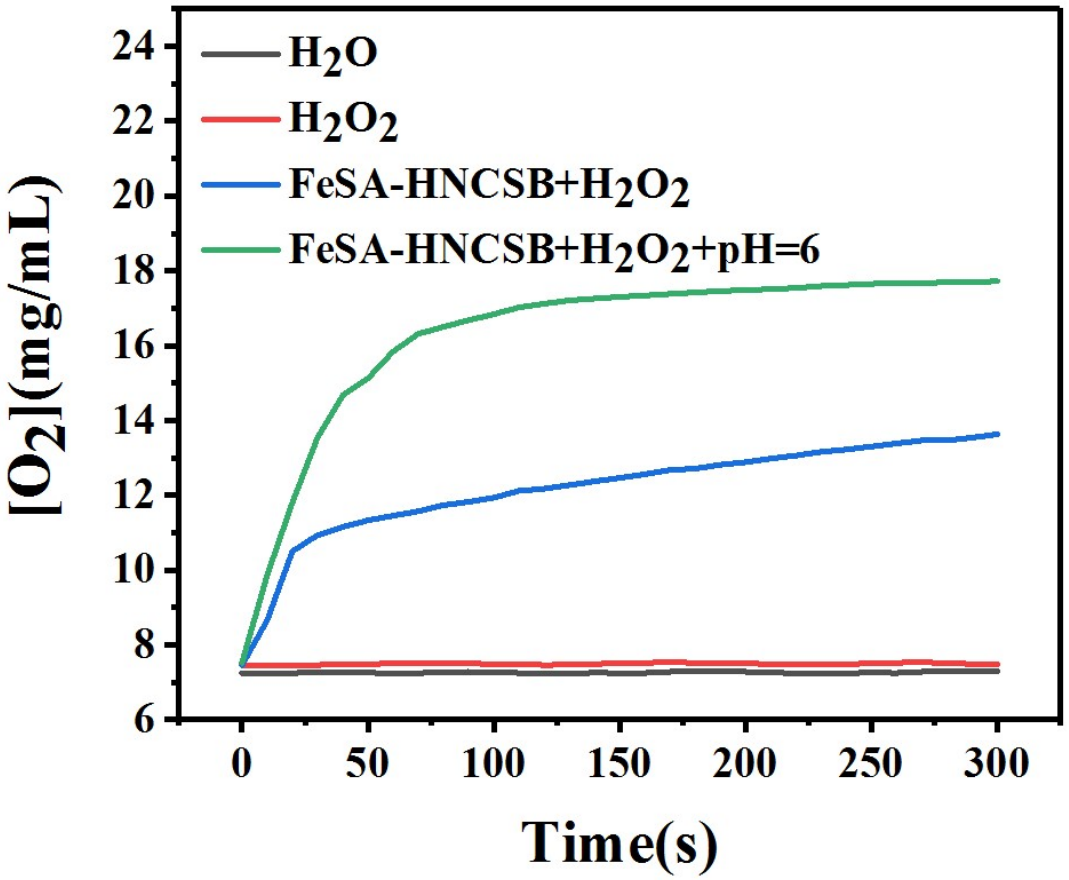

Figure S12. Concentration curves of dissolved oxygen in different experimental groups. 100 μg mL^{-1} FeSA-HNCSB, 0.1 mM H_2O_2.

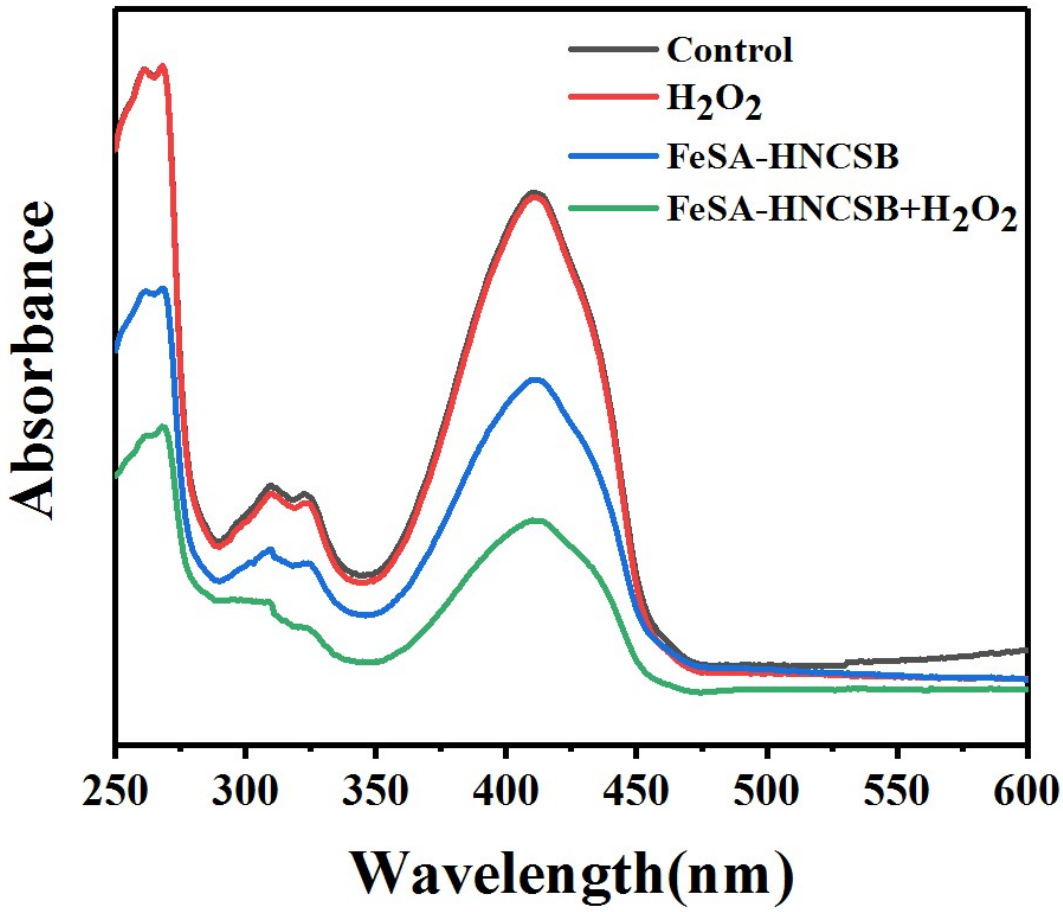

Figure S13. Absorption changes of DPBF in different experimental groups after 10 min.

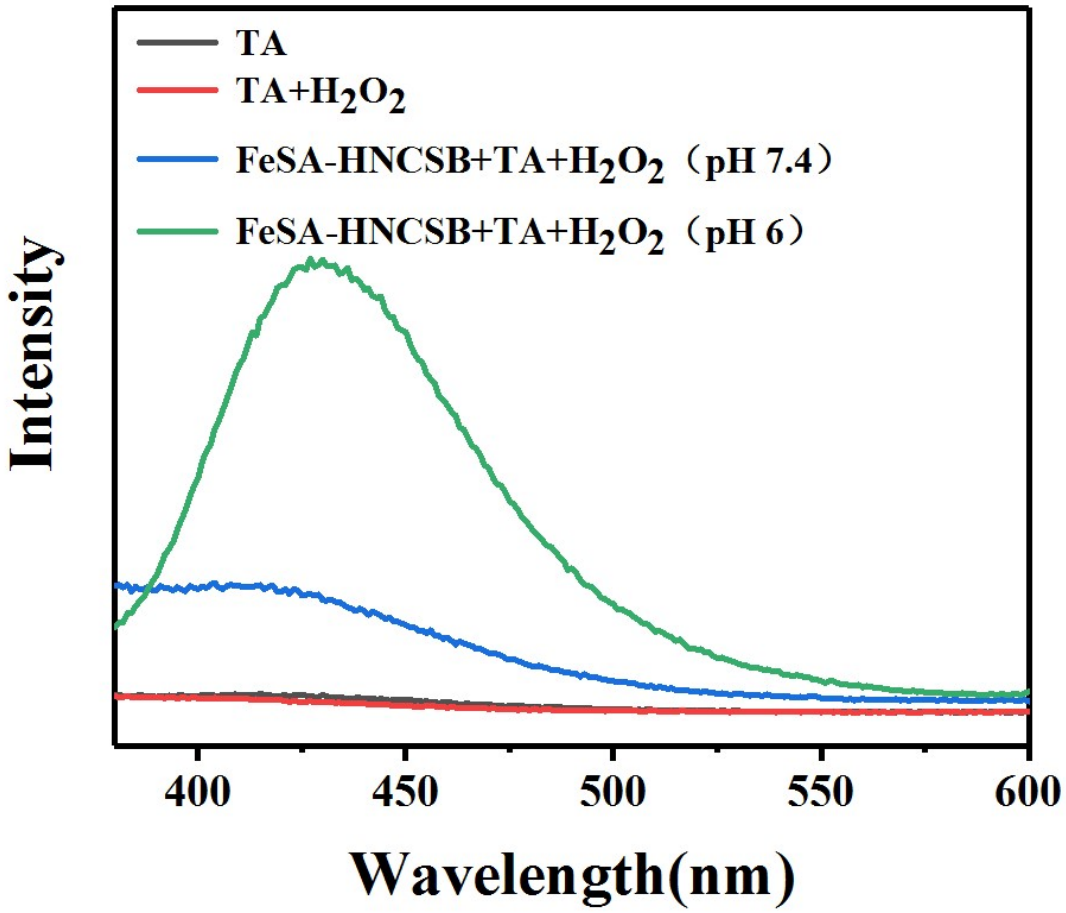

Figure S14. Fluorescence spectra of terephthalic acid representing the content of hydroxyl radical in different experimental groups.

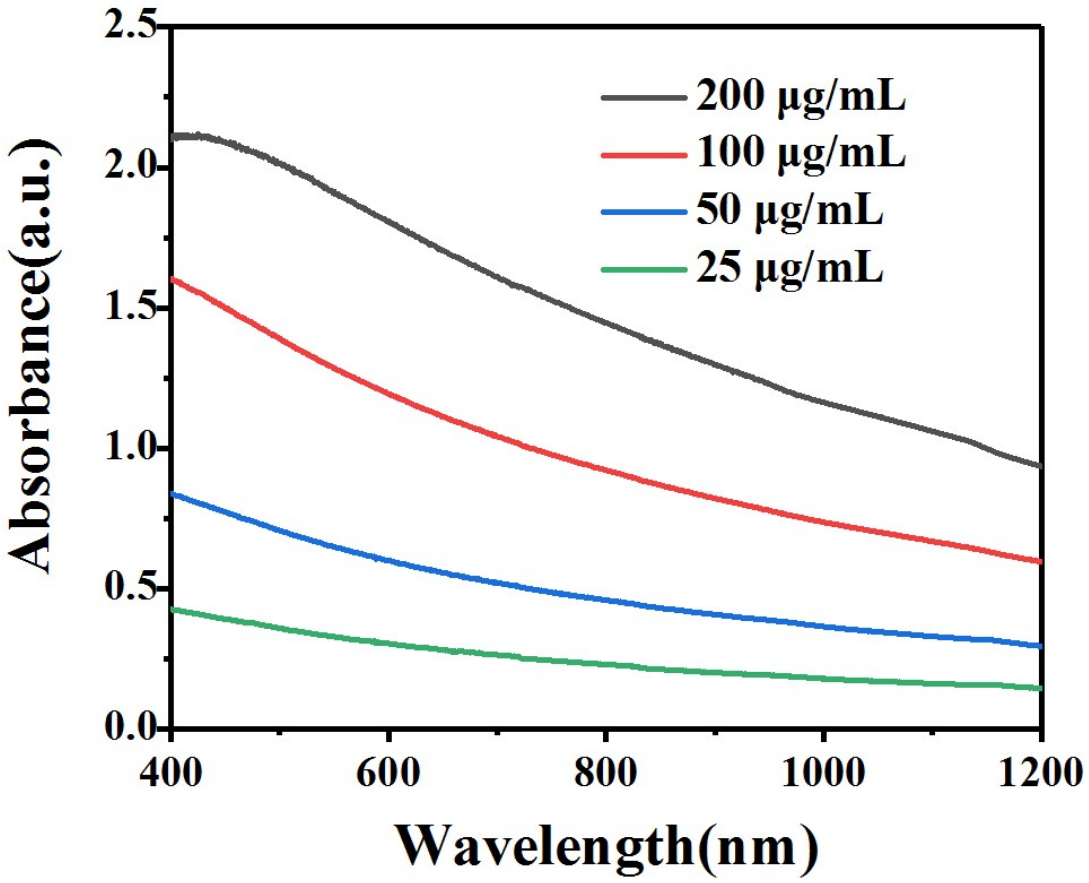

Figure S15. UV-vis-NIR absorption of the FeSA-HNCSB nanozyme with different concentrations.

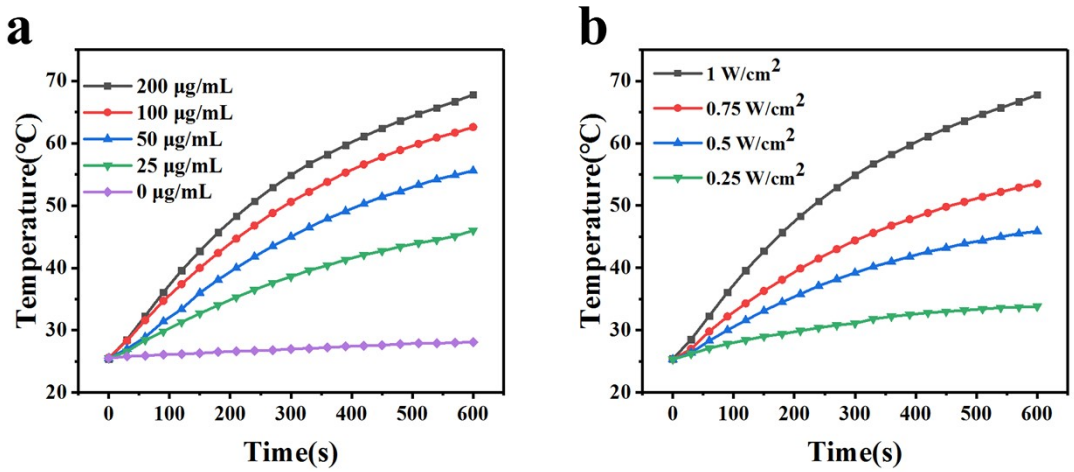

Figure S16. (a) Photothermal heating curves of FeSA-HNCSB nanozyme under a NIR laser (808 nm, 1.0 W cm^{-2}) at different concentrations for 600 s and (b) photothermal heating curves of FeSA-HNCSB nanozyme (200 μg mL^{-1}, 1 mL) with different power density of 808 nm laser irradiation for 600 s.

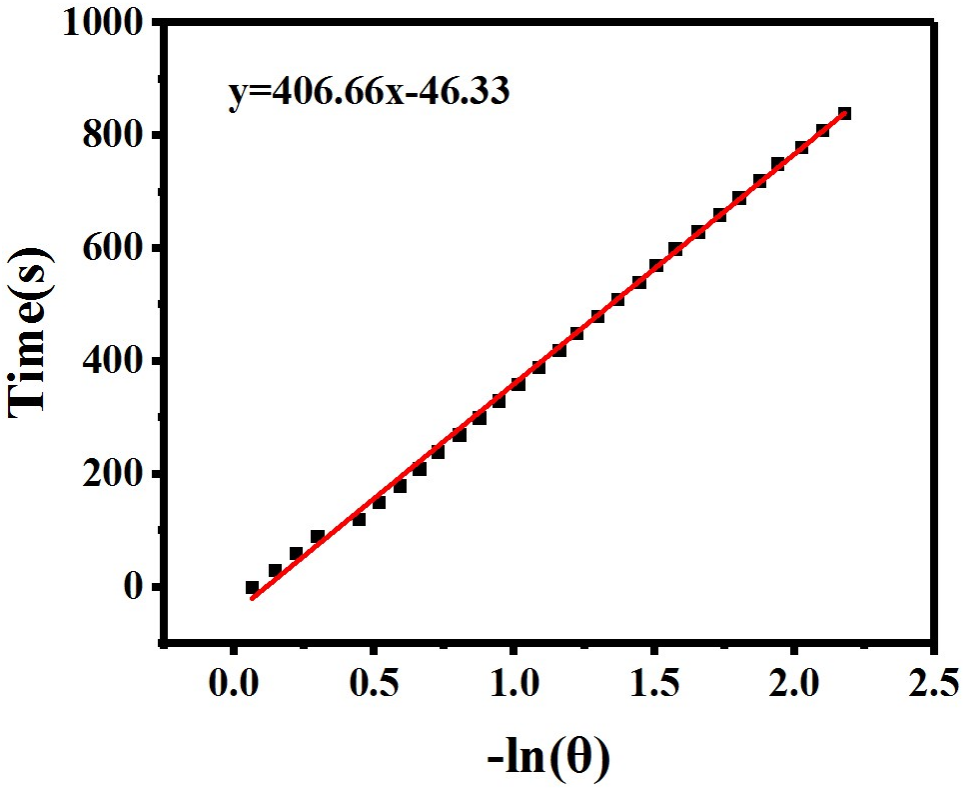

Figure S17. The linearity curves about time and -lnθ fitted from the temperature cooling process of FeSA-HNCSB nanozyme.

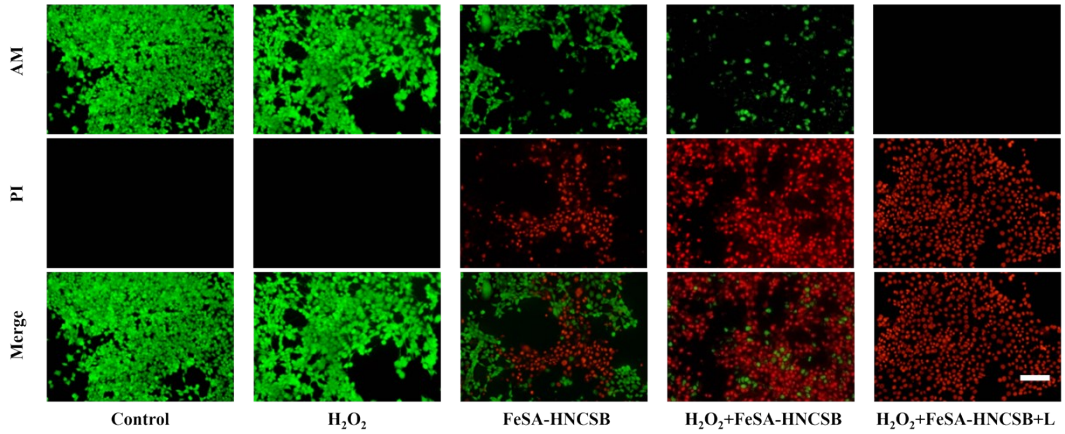

Figure S18. Fluorescence images of Calcein-AM and PI-stained 4T1 cells. Scale bar=400 μm.

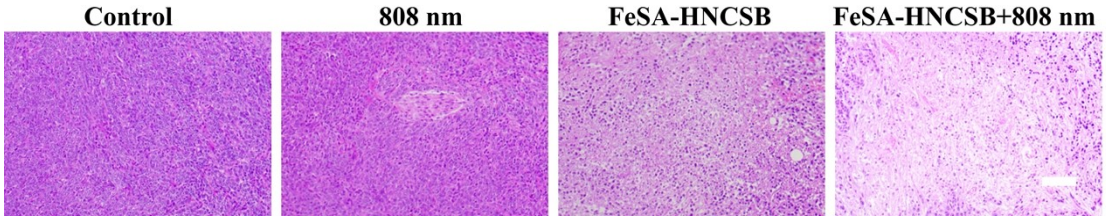

Figure S19. H&E staining in tumor slices after treatment. Scale bar=400 μm.

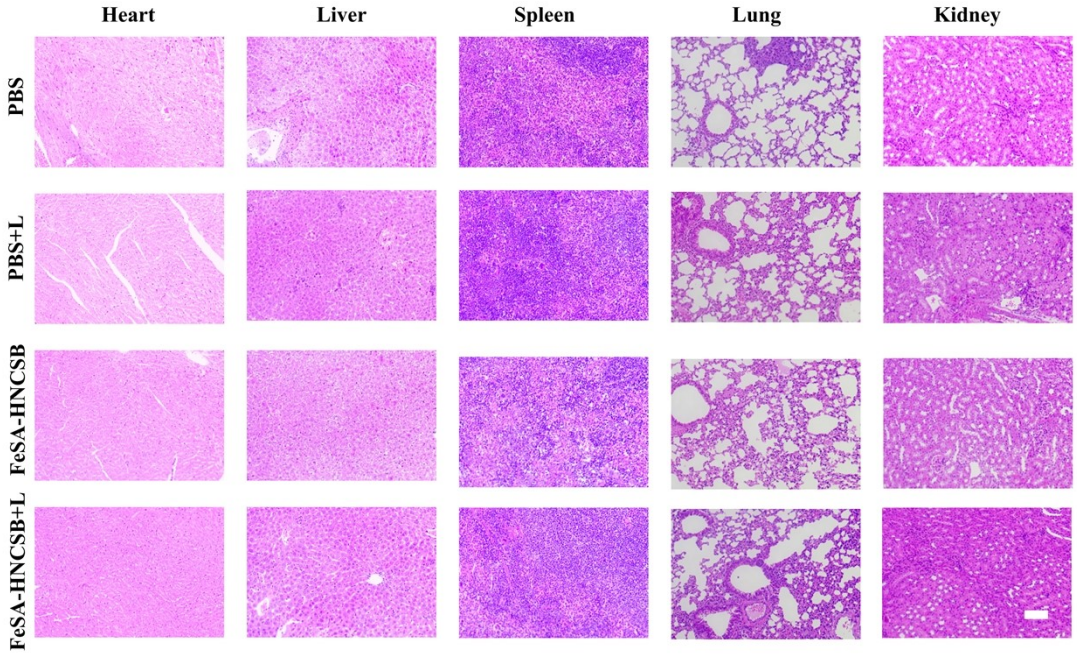

Figure S20. Hematoxylin eosin (H&E) stained images of major organs (heart, liver, spleen, lung, kidney). Scale bar=400 μm.

Table1. EXAFS fitting parameters at the Fe K-edge（S_0^2=0.69）

Sample	Path	C.N.	R (Å)	$\sigma^2\times10^3$ (Å^2)	ΔE (eV)	R factor
Fe foil	Fe-Fe	8*	2.47±0.01	4.5±1.0	6.4±1.4	0.002
	Fe-Fe	6*	2.85±0.01	5.5±1.8	4.8±2.7	
FeSA-HNCS	Fe-N	4	1.98±0.02	6.0±2.0	5.5±1.5	0.003
	Fe-O	1	2.09±0.01	4.2±1.1	7.1±3.8	

C.N.: coordination numbers; R: bond distance; σ^2: Debye-Waller factors; ΔE: the inner potential correction. R factor: goodness of fit. * fitting with fixed parameter.