Supporting Information

A Platelet-Mimicking Single-Atom Nanozyme for Mitochondrial Damage-Mediated Mild-Temperature Photothermal Therapy

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KEYWORDS: single-atom nanozyme, platelet membrane, mild-temperature photothermal therapy, peroxidase-like activity, mitochondria damage.

It is note that the hyperthermia damage induced by photothermal effect could induce the ROS production in cells, but the fluorescence intensity of ROS in PMS+NIR group didn’t differ significantly from that in PMS group, this might be the short measurement time after photothermal treatment and the difference could not be distinguished by solely looking at fluorescence image. However, by analyzing the mean fluorescence intensity (Figure S), the PMS+NIR group showed a slightly higher fluorescence than that in PMS.

**EXPERIMENTAL SECTION**

**Characterization:**

The morphology of synthesized materials was observed with field-emission TEM (JEM-F200), transmission electron microscopy (JEM-1400 plus, Japan) and scanning electron microscope (TESCAN MIRA4, Czech). The high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and corresponding energy-dispersive spectroscopy (EDS) mapping analyses were collected on a field-emission TEM (JEM-F200, Japan). And the Aberration-corrected HAADF-STEM (AC-HAADF-STEM) image was carried by TEM/STEM (JEM-ARM200F, Japan) with a spherical aberration corrector, observing the
atomically dispersed Fe in samples. Powder X-ray diffraction (XRD) patterns were conducted (Smartlab SE, Japan) with Ni-filtered Cu KR radiation (40 kV, 50 mA). The XPS experiment was carried out using a Vacuum Generators twin-crystal monochromatized Al Kα (hν = 1486.58 eV) source and a hemispherical electron analyzer (ESCALAB250Xi, USA) in a standard ultrahigh vacuum (UHV) chamber at a base pressure of ~3.0×10^-10 Torr. The total energy resolution is 0.5 eV, and the step size for all measured spectra is 0.02 eV. The powder samples were pressed on the carbon tape, and the binding energy (BE) values were referenced to the C 1s peak position at 284.8 eV BE. The Raman spectra were obtained on an InVia spectrometer (Thermo DXR, USA) at room temperature. The nitrogen adsorption-desorption isotherms and corresponding pore size distribution were tested at 77 K (Tristar II 3020, USA). The membrane-coated materials were observed on JEM-1400 plus TEM at the voltage of 100 kV.

**X-ray absorption fine structure (XAFS) data characterizations and analysis:**

The XAFS experiments at the Fe K-edge were performed at the BL14W1 beamline of Shanghai Synchrotron Radiation Facility operated at 3.5 GeV under “top-up” mode with a constant current of 240 mA \(^1\). Using Si (111) double-crystal monochromator, the data was collected under transmission mode using ionization chamber for Fe foil, FeO, Fe\(_2\)O\(_3\), and iron phthalocyanine (FePc) standards, and under fluorescence mode using a Lytle detector for the Fe-SAzyme sample, respectively. The photon energy (E0) was calibrated accordingly to the absorption edge of Fe foil (photoelectron energy origin E0 = 7112 eV).

The obtained XAFS data were processed according to the standard procedures using Athena and Artemis codes implemented in the IFEFFIT software packages \(^2\). For the X-ray near-edge structure (XANES) part, the experimental absorption coefficients as a function of X-ray energy \(\mu(E)\) were processed by background subtraction and standard normalization procedures, which were reported as “normalized absorption”. For the extended X-ray absorption fine structure (EXAFS) part, the Fourier
transformation (FT) data were modelled in R-space, and analyzed by applying first-shell approximate model for Fe-N path. The passive electron factor (S02) was determined by fitting the experimental data on Fe foil, and the coordination number of Fe-N (N) was variable for further analysis of the measured sample. The parameters describing the electronic properties (e.g., correction to E0, ΔE0) and local structure environment including bond distance (R), and Debye-Waller factor around the absorbing atoms (σ2) were determined during the optimization process. The modelling parameters were provided in Supplementary Table 1. Based on the scattering amplitude of the EXAFS signal alone, neighboring atoms on the periodic table cannot be distinguished. Thus, the atom types will need to be further assigned based on the local chemical environment through XPS measurements.

**Western blotting for the key proteins in Fe-SAzyme, PM and PMS:**

The total cellular protein in Fe-SAzyme, PM and PMS were extracted using a protein extraction kit (Dingguo, China). The extracted proteins were separated using SDS-PAGE electrophoresis. After electrophoresis, the gel was treated with Coomassie blue staining. Extraction of protein for western blot was performed as described above. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). This was followed by a blocking step for 1 h with 5% skim milk, and then the membrane was incubated with the primary antibody against P-selectin (Proteintech) overnight at 4 °C using Na, K-ATPase as the control. Finally, the membrane was incubated with the secondary antibody for 1 h at room temperature. The statistical analysis of expressions content performed using ImageJ software.

**Cell lines and animal model:**

4T1 mouse breast cancer cell lines was provided by the College of Life Science of Wuhan University. All the cells were cultured in the standard cell medium recommended by American Type Culture Collection. Female BALC/c mice aged 4-5
weeks were purchased from Vital River Company (Beijing, China). \(1 \times 10^6\) 4T1 cells suspended in 100 \(\mu\)L PBS were subcutaneously injected into each mouse to establish the tumor models. After the tumor volume reached around 200 mm\(^3\), the tumor bearing mice were used for further experiments. The animal experiments were carried out according to the protocol approved by the Ministry of Health in the People’s Republic of PR China and were approved by the Administrative Committee on Animal Research of the Wuhan University.

*In vitro* tumor-specific uptake:

At first, Dil labeled RMS and PMS (Containing 100 \(\mu\)g/mL Fe-SAzyme) were incubated with 4T1 cells for 2 h at 37 °C. The cells were then washed with PBS several times, fixed with PFA for 30 min at room temperature, stained with Lyso-Tracker Green and then imaged by using a fluorescence microscope (IX81, Japan). The fluorescence intensity was measured by ImageJ software. The RAW 264.7 cell uptake test method is similar to the above.

*JC-1 mitochondrial membrane potential assay:*

For the JC-1 assay, 4T1 cells were co-incubated with five different groups: 1) Control (PBS); 2) RMS; 3) PMS; 4) RMS + NIR; 5) PMS + NIR. The cells were washed twice with PBS and stained with JC-1 for 20 min at 37 °C in the dark. Subsequently, the cells were rinsed twice with ice-cold incubation buffer (1×), incubated in fresh culture medium again and detected using a fluorescence microscope. The mitochondrial damage/disruption was detected by fluorescence microscopy (IX81, Japan).

*In vitro* hydroxyl radical (·OH) and reactive oxygen species (ROS) generation

The ROS generation was detected in vitro on 4T1 cells by chemical method using dichlorodihydrofluorescein diacetate (DCFH-DA) as probe, and the ·OH generation was detected using hydroxyphenyl fluorescein (HPF) as probe. Specifically, 4T1 cells
were incubated for 24 h with five different groups: 1) Control (PBS); 2) RMS; 3) PMS; 4) RMS + NIR; 5) PMS + NIR. The Fe-SAzyme concentration was 200 μg/mL in group 2, 3, 4, and 5. Then, cells in group 4 and 5 were irradiated with the 1064 nm laser at a power density of 0.5 W/cm² for 5 min. Afterward, DCFH-DA or HPF was added into the treated cells and the fluorescence images were observed by confocal laser scanning microscope (CLSM).

**In vitro anti-cancer effect of PMS:**

The anti-tumor effect was measured by MTT assay. 4T1 cells were seeded in 96-well plates at a density of 5 × 10³ cells per well and incubated for 12 h. Afterwards, cells were incubated for 5 different groups: 1) Control (PBS); 2) RMS; 3) PMS; 4) RMS + NIR; 5) PMS + NIR. The Fe-SAzyme concentration was 200 μg/mL in group 2, 3, 4, and 5. Then, cells in group 4 and 5 were irradiated with the 1064 nm laser at a power density of 0.5 W/cm² for 5 min. At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 570 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%).

**Biocompatibility of PMS:**

The biocompatibility of PMS to a normal cell lines was also evaluated by MTT assay. Specifically, RAW 264.7 mouse monocyte macrophage cells were seeded in 96-well plates at a density of 5 × 10³ cells per well and incubated for 12 h. Afterwards, cells were incubated for 4 h with different concentration of PMS (0, 50, 100 and 200 mg/mL), with PBS as control. At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 6 h. The cytotoxicity was evaluated and calculated in the same way as former MTT assay.
**In vivo pharmacokinetics, and distribution study:**

For the bio-distribution study, BALB/c mice (n = 5) received an intravenous (i.v.) injection of 100 mL PBS containing RMS or PMS (with equivalent Cy5 labeled-Fe-SAzyme dose of 20 mg/kg). In vivo images were recorded using the IVIS system at a Cy5 channel at 6, 12 and 24 h post-injection. All of the mice were euthanized, and their major organs were them collected 24 h after injection to determine the biological distribution of the particles.

**In vivo antitumor study:**

$1 \times 10^6$ 4T1 cells suspended in 100 μL PBS were subcutaneously injected into each mouse to establish the tumor models. When tumor size reached approximately 200 mm$^3$, the mice were divided randomly into 5 groups (each group included 5 mice): 1) Control (PBS); 2) RMS; 3) PMS; 4) RMS + NIR; 5) PMS + NIR. The Fe-SAzyme concentration was 20 mg/kg in group 2, 3, 4, and 5. Then, cells in group 4 and 5 were irradiated with the 1064 nm laser at a power density of 0.5 W/cm$^2$ for 3 min and then maintain current temperature for 7 minutes. Mice body weight and tumor volume in all groups were monitored every 2 days. A caliper was employed to measure the tumor length and tumor width and the tumor volume was calculated according to the following formula. Tumor volume = tumor length $\times$ tumor width $^2$ / 2. After 16 days of treatment, all the mice were sacrificed. Their blood samples and major organs (i.e., hearts, livers, spleens, lungs, and kidneys) were collected. Three important hepatic indicators (i.e., ALT: alanine aminotransferase, AST: aspartate aminotransferase, and ALP: alkaline phosphatase) and two indicators for kidney functions (i.e., BUN: blood urea nitrogen and CRE: creatinine) were measured by using a blood biochemical autoanalyzer (7080, HITACHI, Japan). And the tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the sections were stained with Ki-67 and TUNEL staining and finally examined by an optical microscope (BX51, Japan). Moreover, sections were also
stained with HPF to detect the ·OH generation level and the resultant fluorescence image were examined by optical microscope. Part of their organs were stained with H&E and examined as described above.

**Statistical analysis:**

Error bars represent the standard deviations for different parallel experiments. Significance between every two groups was calculated by the Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.

**Figure S1.** SEM images of Fe-SAzyme.
**Figure S2.** Raman spectra of Fe-SAzyme.

**Figure S3.** N\textsubscript{2} absorption and desorption curves and pore size distributions for Fe-SAzyme.
Figure S4. Fe K-edge EXAFS spectra (red dots) and the corresponding curve fit (black line) for Fe-SAzyme sample, shown in $k^3$-weighted $k$ space.

Figure S5. Fe K-edge EXAFS spectra (color dots) and the corresponding curve fits (solid lines) for Fe-SAzyme sample, shown in R space (including FT magnitude and imaginary component). The data are $k^3$-weighted without phase correction.
Figure S6. (A) The survey and (B) Fe 2p XPS spectra of Fe-SAzyme collected with Al Kα excitation (hv = 1486.6 eV).

Figure S7. (A) Hydrodynamic diameter and (B) zeta potential of Fe-SAzyme and PMS (n=3, mean ± SD).
Figure S8. P-Selectin content of PM and PMS in Figure 2B. The expressions content was calculated using ImageJ software (n=3, mean ± SD).

Figure S9. TEM images of PMS collected after incubation in various solution at 37 °C for two days.
**Figure S10.** The relative absorption spectra of H$_2$O$_2$-Ti(SO$_4$)$_2$ solution in the presence of Fe-SAzyme. pH = 6.0, 4.0 mM H$_2$O$_2$, 1%Ti(SO$_4$)$_2$ (n=3, mean ± SD).

**Figure S11.** (A) Michaelis–Menten kinetic analysis and (B) Lineweaver–Burk plot for Fe-SAzyme (80 μg/ml) with H$_2$O$_2$ as substrate. (C) Michaelis–Menten kinetic analysis and (D) Lineweaver–Burk plot for Fe-SAzyme (80 μg/ml) with TMB as substrate. The error bars represent the standard deviations detected for three parallel experiments (n =3, mean ± SD). The reaction was conducted at 25 °C and the pH is 4.
Figure S12. Cell viability of RAW 264.7 cell after the indicated treatments (n = 3, mean ± SD).

Figure S13. HSP90 content of different indicated groups in Figure 3B. The expression content was calculated using ImageJ software (n=3, mean ± SD).

Figure S14. Fluorescence images of 4T1 cells stained with HPF under different treatments.
**Figure S15.** Mean fluorescence intensity (MFI) of DCFH under different treatments. The MFI was analyzed by ImageJ.

**Figure S16.** Fluorescence images of tumor sections stained with HPF under different treatments.

**Figure S17.** Histopathological analysis results (H&E stained images) of the major organs, heart, lung, liver, kidneys, and spleen, of mice that were exposed to different treatments 16 days post-injection under laser irradiation (scale bars: 100 μm).

**Figure S18.** Bloods biochemistry data including kidney function markers: (A) ALT, ALP, and AST, (B) liver function markers: BUN and (C) CRE after various treatments (n=5, mean ± SD).

**Table S1.** Raman data for Fe-SAzyme.

<table>
<thead>
<tr>
<th>D band (cm⁻¹)</th>
<th>G bond (cm⁻¹)</th>
<th>l_D/l_G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1352.6</td>
<td>1582.3</td>
<td>1.02</td>
</tr>
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</table>

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Table S2. ICP-OES data for Fe-SAzyme.

<table>
<thead>
<tr>
<th>Element</th>
<th>Element content in solution (mg/kg)</th>
<th>Element content in sample (wt%)</th>
</tr>
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<tbody>
<tr>
<td>Fe</td>
<td>11197.4</td>
<td>1.12</td>
</tr>
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</table>

Table S3. List of the fitting structural parameters obtained from the analysis of the Fe K-edge EXAFS data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Path</th>
<th>k-range (Å⁻¹)</th>
<th>R-range (Å)</th>
<th>S₀²</th>
<th>N</th>
<th>R (Å)</th>
<th>σ² (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe foil</td>
<td>Fe-Fe</td>
<td>2.9-11.7</td>
<td>1.0-3.0</td>
<td>1.0</td>
<td>8</td>
<td>2.46</td>
<td>0.005(8)</td>
</tr>
<tr>
<td>Fe-SAzyme</td>
<td>Fe-N</td>
<td>3.1-9.15</td>
<td>1.0-3.0</td>
<td>1.0</td>
<td>4.4</td>
<td>1.83(2)</td>
<td>0.009(9)</td>
</tr>
</tbody>
</table>

*a The fitted ranges for Fe-NC sample were selected to be $3.1 \leq k \leq 9.15$ Å⁻¹ (k²-weighted) and $1.0 \leq R \leq 3.0$ Å, yielding the number of variable parameters being 4, out of a total of 7.5 independent data points.

*b $S₀²$ was fixed as 1.0, which was determined by fitting the experimental data on Fe foil with fixed coordination number for Fe-Fe path based on the crystal structure.

*c The coordination number N were varied.

*d The distance for Fe-N is from the crystal structure of FeN. The distance of Fe-N path for Fe-NC samples is 1.99(7) Å. $ΔE₀$ was refined as a global
fit parameter, giving a value of -0.19(1) eV. The percent miss fit between
the data and the model (R factor) for this fit is 1.7%.

Table S4. The Michaelis-Menton constant ($K_m$), maximum reaction rate ($V_{\text{max}}$) and
catalytic constant ($K_{\text{cat}}$) of as prepared Fe-SAzyme, natural HRP, and other single
atom nanozymes for POD-like catalysis.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>$V_{\text{max}}$ (M/s)</th>
<th>$K_m$ (mM)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work</td>
<td>$H_2O_2$</td>
<td>$4.64 \times 10^{-7}$</td>
<td>3.77</td>
<td>This Work</td>
</tr>
<tr>
<td>Fe–N–C</td>
<td>$H_2O_2$</td>
<td>$4.29 \times 10^{-7}$</td>
<td>28.3</td>
<td>3</td>
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<tr>
<td>SAN</td>
<td>$H_2O_2$</td>
<td>$8.25 \times 10^{-8}$</td>
<td>0.24</td>
<td>4</td>
</tr>
<tr>
<td>Fe SAEs</td>
<td>$H_2O_2$</td>
<td>$3.56 \times 10^{-7}$</td>
<td>12.2</td>
<td>5</td>
</tr>
<tr>
<td>Fe–N–C</td>
<td>$H_2O_2$</td>
<td>$5.98 \times 10^{-7}$</td>
<td>0.94</td>
<td>6</td>
</tr>
<tr>
<td>Fe-N/C</td>
<td>$H_2O_2$</td>
<td>$1.21 \times 10^{-7}$</td>
<td>40.16</td>
<td>7</td>
</tr>
<tr>
<td>PMCS</td>
<td>$H_2O_2$</td>
<td>$1.18 \times 10^{-7}$</td>
<td>4.84</td>
<td>8</td>
</tr>
<tr>
<td>Fe-N-C</td>
<td>$H_2O_2$</td>
<td>$6.2 \times 10^{-7}$</td>
<td>4.31</td>
<td>9</td>
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<tr>
<td>Co-N-C</td>
<td>$H_2O_2$</td>
<td>$1.65 \times 10^{-7}$</td>
<td>16.26</td>
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<td>Zn-N-C</td>
<td>$H_2O_2$</td>
<td>$0.48 \times 10^{-7}$</td>
<td>6.27</td>
<td>9</td>
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</table>

REFERENCES

2. Ravel, B.; Newville, M. ATHENA, ARTEMIS, HEPHAESTUS: Data Analysis


