Supporting information

Folic-Acid-Functionalized Fe_3O_4@C@Pt Nanozyme for Synergistic Catalytic-Photothermal Tumor Therapy

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Synthesis of Fe_3O_4

Fe_3O_4 nanoparticles were prepared by a solvothermal method [1]. A mixture of 1.3 g of $FeCl_3 \cdot 6H_2O$, 0.5 g of PEG4000, and 3.6 g of sodium acetate was dissolved in 72 mL of ethylene glycol. The solution was transferred to a hydrothermal reactor and heated at 200 °C for 16 h. After cooling to room temperature, the product was washed with ultrapure water and ethanol three times. The product was then dried in a vacuum oven at 50 °C for 24 h to obtain Fe_3O_4 nanoparticles.

Synthesis of Fe_3O_4@C

Fe_3O_4@C were synthesized by the pyrolyzing of Fe_3O_4@PDA. Fe_3O_4@PDA nanoparticles were synthesized by a templating method[2]. Anhydrous ethanol and deionized water were used as dispersants, and ammonia was used as an alkaline catalyst. 100 mg of Fe_3O_4, 400 mg of F127, 150 mg of P123, and 500 mg of DA·HCl in 50 mL of ethanol/water (v/v=1:1) was mixed equably. Then, 2.0 mL of mesitylene was added to form an emulsion system. Afterwards, 2.45 mL of ammonia was added dropwise and the stirring was continued for 4 h. The Fe_3O_4@PDA were separated by external magnets. The P123/F127 template was removed by ultrasonication with anhydrous ethanol and acetone (*v*/*v*= 2:1) for 10 min. Place the materials prepared above in a tubular furnace with N_2 treatment for 30 min, then heat the samples to 800 °C with a heating rate of 5 °C/min and remained for 2 h. The black product obtained was Fe_3O_4@C.

Synthesis of Fe_3O_4@C@Pt

Commence by dispersing 100 mg of Fe_3O_4@FPDA into 20 mL of deionized water, followed by the addition of 20 mg of $(NH_4)_2PtCl_6$. Stir the mixture continuously for a duration of 2 h. Subsequently, introduce $NaBH_4$ into the solution and maintain stirring for an additional 2 h. After the reaction is complete, rinse the mixture twice with water and alcohol. Then, dry the product under vacuum at 60 °C to obtain a brownish powder, which is denoted as Fe_3O_4@C@Pt.

Synthesis of Fe_3O_4@C@Pt-FA

10 mg Fe_3O_4@C@Pt nanozyme was dispersed in 20 mL deionized water, and FA-PEG-NH_2 with the configured concentration of 10 mg/mL was added, mixed for 10 h, separated and precipitated to obtain Fe_3O_4@C@Pt-FA.

Characterization

The Transmission Electron Microscopy (TEM) images were obtained on JEOL JEM-2100 at 200 kV. Powder X-Ray Diffraction (XRD) patterns were recorded on Bruker D8 ADVANCE A25X X-ray diffractometer in the range of 10 ~ 80 with the scan rate of 10 degree/min. UV-visible spectrophotometry (UV-vis) absorption spectrum of the solution was obtained on Tecan Spark 20 M Multimode Microplate Reader. The confocal laser scanning microscope (Lecia, TCS SP8 STED, Germany) was used to obtain the fluorescence images of cells.

Determination of peroxidase (POD)-like activity

The POD-like activity was evaluated by using TMB as an enzyme catalytic substrate. In brief, the materials (2.5 μg/mL), H_2O_2 (0.048 mM) and TMB (0.208 mM) were mixed in 200 μL of NaAc-HAc buffer (0.1 mol/L, pH 4.5) at 37 °C, and the absorbance of the oxidized TMB at 652 nm was measured with a Tecan Spark 20 M Multimode Microplate Reader (Switzerland). The experiments were replicated three times each.

Cellular Experiments

CT26, 3T3 and L02 cells were used for the cellular experiment. The cells were incubated overnight in 6-well and 96-well plates before being used as adherent cells.

Cytotoxicity assay

Briefly, 3T3 cells and L02 cells were first seeded in 96-well plates at a density of 1×10^5 cells per well and cultured for 12 h in Dulbecco's modified eagle medium (DMEM). Then, the cells were

incubated at different concentrations nanozyme (37 °C 24 h), respectively. Finally, 10 μL CCK-8 solution was added to each well, and the plates were incubated for another 2 h. A microplate reader was used to measure the absorbance at 450 nm. The experiments were replicated three times each.

Cell Fluorescence Imaging

To perform fluorescence imaging to differentiate between live and dead cells upon exposure to Fe_3O_4@C@Pt, the procedure involved incubating the cells under four different conditions: in DMEM medium without exposure to a NIR laser, in DMEM medium with exposure to a NIR laser, with Fe_3O_4@C@Pt without exposure to a NIR laser, and with Fe_3O_4@C@Pt with exposure to a NIR laser. Following incubation, the cells were co-stained with Hoechst and propidium iodide (PI) for 15 minutes. This staining process labels live cells with a blue fluorescence and dead cells with a red fluorescence. Finally, the cells were observed and imaged using confocal fluorescence microscopy to analyze the effects of the different treatments on cell viability.

***In Vitro* Catalytic-Photothermal Therapy**

For the *in vitro* catalytic-photothermal therapy, CT26 cells were seeded in 96-well plates at a density of 1×10^5 cells per well and cultured for 24 h followed by treatment with the different concentrations of the nanomaterials. After incubation for 4 h, the cells were exposed to the 808 nm NIR laser (1 W/cm^2) for 2 min. Subsequently, irradiation was followed by a waiting period of either 20 h. Finally, 10 μL CCK-8 solution was added to each well, and the plates were incubated for another 2 h. A microplate reader was used to measure the absorbance at 450 nm. All experiments repeated three times.

Photothermal experiments

The photothermal performance of the as synthesized nanomaterials at specific concentration was evaluated by recording the temperature change upon their exposure under 808 nm laser irradiation

with various power density for 10 min [3]. The temperature was monitored by a thermocouple thermometer. The photothermal stability experiments were carried out as follows: Fe_3O_4@C@Pt were exposed to 808 nm laser for 10 min, followed with the natural cooling to room temperature, and the above process was repeated for 3 times.

The photothermal conversion efficiency of Fe_3O_4@C@Pt were calculated using the following equation:

$$\eta = \frac{hS(T_{max} - T_{am}) - Q_0}{I(1 - 10^{-A})}$$

Where h is the heat transfer coefficient, S is the surface area, T_{max} is the maximum equilibrium temperature, T_{am} is the ambient surrounding temperature, I is the laser power (1 W/ cm^2), A is the absorbance of the sample solution at 808 nm, and Q_0 represents the heat absorption of the quartz cell.

Cellular mitochondria potential assay

To evaluate mitochondrial membrane potential changes, CT26 cells were treated with 50 μg/mL nanomaterials for 24 h, then stained with the JC-1 for 20 min at 37 °C. Following two washes as per the protocol, cells were examined under a confocal laser scanning microscope. The red fluorescence indicates JC-1 aggregates in mitochondria, a sign of potential retention, whereas green fluorescence indicates JC-1 monomers in the cytosol, signifying mitochondrial membrane depolarization. Cells treated with 10 mM CCCP served as a positive control to validate the assay.

Blood Analysis

Healthy adult mice (Balb/c, 5 weeks old) were employed in this study. Blood samples were collected 7 days post-injection (20 mg/kg) for the analysis of hematological parameters and biochemical markers.

***In vivo* antitumor experiments**

To study the combined impact of photothermal and catalysis on tumor suppression, CT26 tumor-bearing Balb/c mice were allocated into three groups, each comprising five mice: a control group receiving PBS, a group receiving an intravenous injection of Fe_3O_4@C@Pt, and a third group receiving an intravenous injection of Fe_3O_4@C@Pt combined with laser treatment (Fe_3O_4@C@Pt + L). This setup aimed to evaluate the therapeutic efficacy of Fe_3O_4@C@Pt under different conditions. Tumor volumes and body weights of the mice were measured after different treatments. On day 12, all the mice were euthanized. All mice were obtained from the Comparative Medicine Centre of Yangzhou University (China), and all animal experiments were approved by the Animal Ethics Committee of the Medical College of Yangzhou University. The tumors in all groups were weighed, and the main organs were harvested for H&E staining. Injection dose: 100 μL, 5 mg/kg. Laser irradiation condition: 808 nm, 1 W/cm^2, 10 min.

Reference:

1 Y. Liang, L. Jiang, S. Xu, W. Ju, Z. Tao, Y. Yang, X. Peng and G. Wei, *J. Mater. Eng. Perform.*, 2024, **33**, 6804–6815.

2 D. Wan, C. Yan and Q. Zhang, *Ind. Eng. Chem. Res.*, 2019, **58**, 16358–16369.

3 L. Fan, X. Xu, C. Zhu, J. Han, L. Gao, J. Xi and R. Guo, *ACS Appl. Mater. Interfaces*, 2018, **10**, 4502–4511.

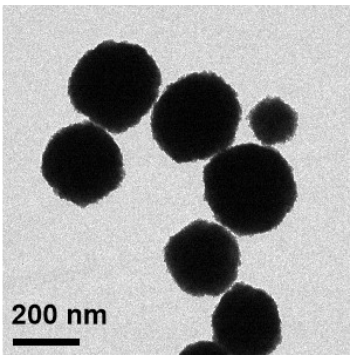

Figure S1. TEM image of Fe_3O_4.

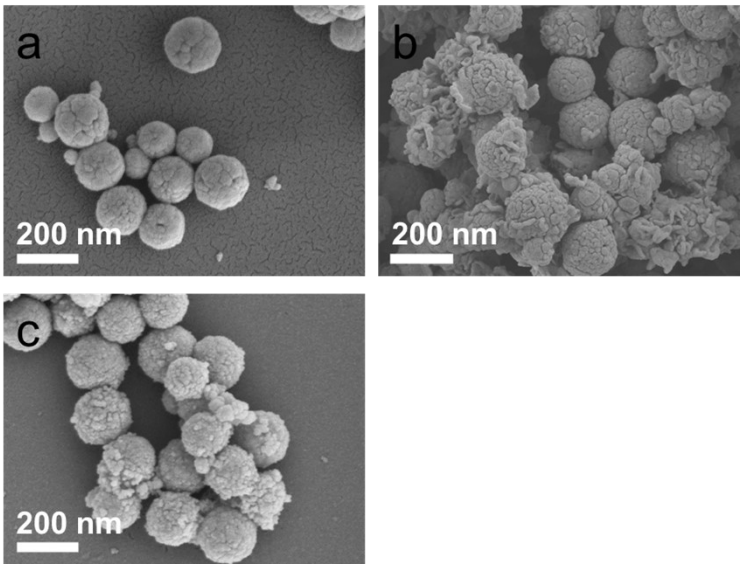

Figure S2. SEM images of (a) Fe_3O_4, (b) Fe_3O_4@C and (c) Fe_3O_4@C@Pt.

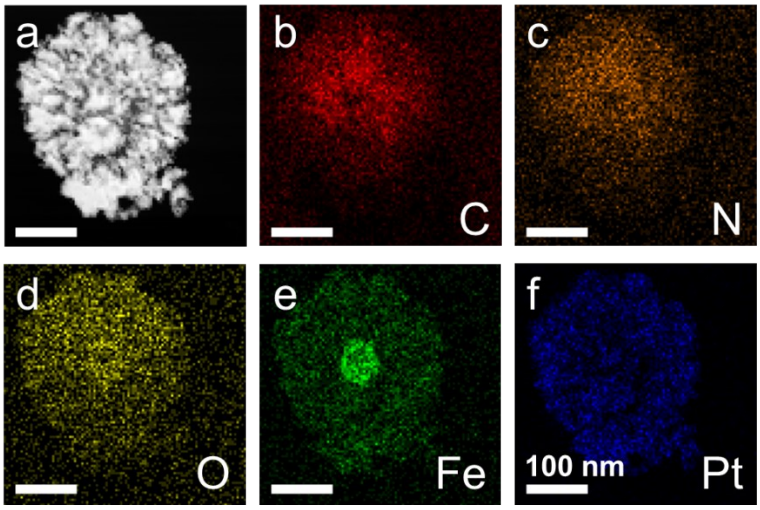

Figure S3. Dark-field STEM and corresponding elemental mapping of Fe_3O_4@C@Pt.

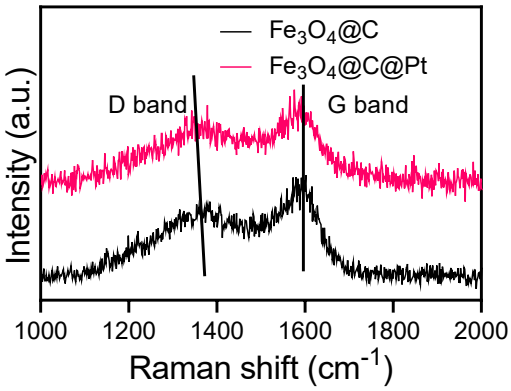

Figure S4. Raman patten of Fe_3O_4@C and Fe_3O_4@C@Pt.

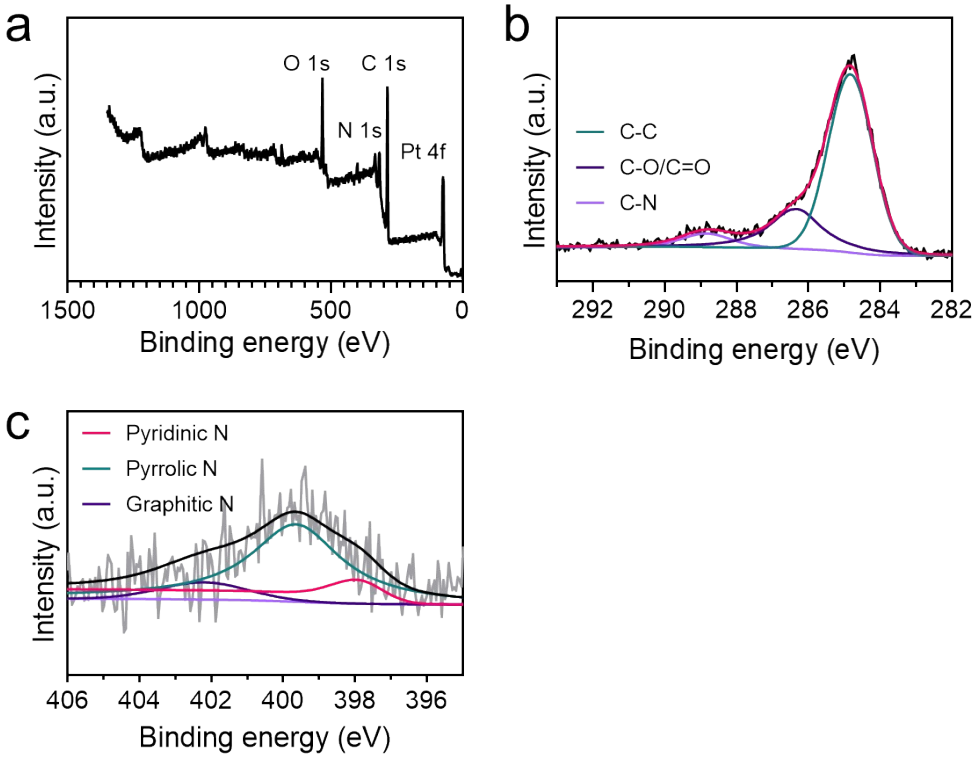

Figure S5. (a) XPS survey spectrum of Fe_3O_4@C@Pt, high-resolution XPS spectra of (b) C 1s and (c) N 1s in the Fe_3O_4@C@Pt.

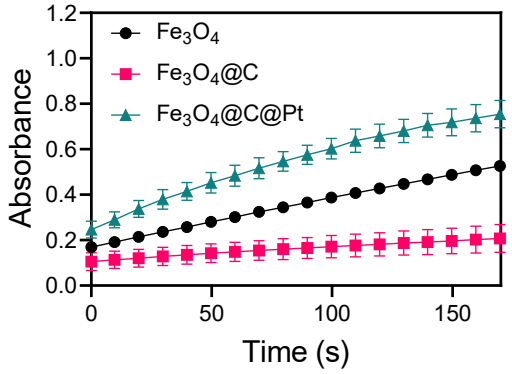

Figure S6. Steady-state kinetic assay and the catalytic mechanism study for the Fe_3O_4, Fe_3O_4@C and Fe_3O_4@C@Pt nanozymes.

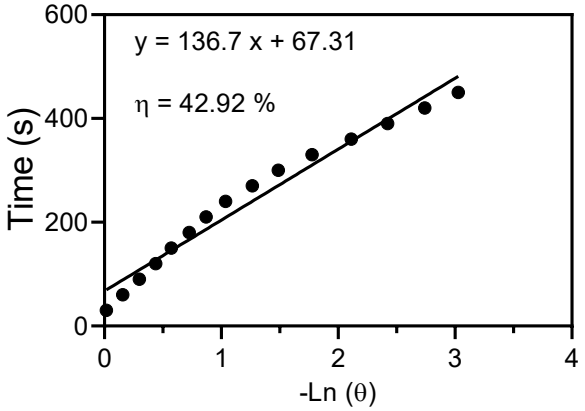

Figure S7. Linear relationship between the time data and -Ln(θ) obtained from the cooling period.

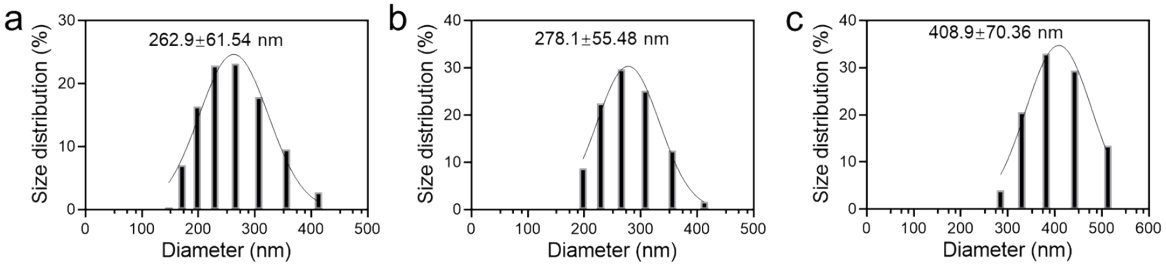

Figure S8. DLS analysis of (a) Fe_3O_4@C, (b) Fe_3O_4@C@Pt and (c) Fe_3O_4@C@Pt-FA.

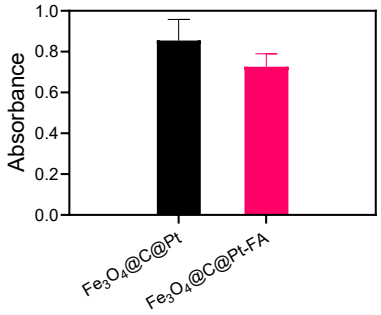

Figure S9. POD-like activity of Fe_3O_4@C@Pt and Fe_3O_4@C@Pt-FA.

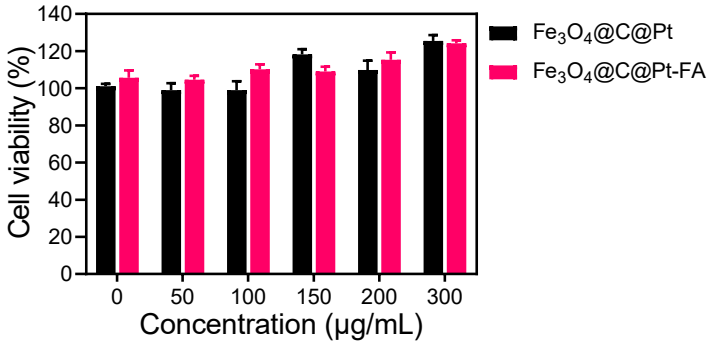

Figure S10. Cell viability of 3T3 cells treated with Fe_3O_4@C@Pt and Fe_3O_4@C@Pt-FA at different concentrations for 24 h without laser irradiation (n = 3).

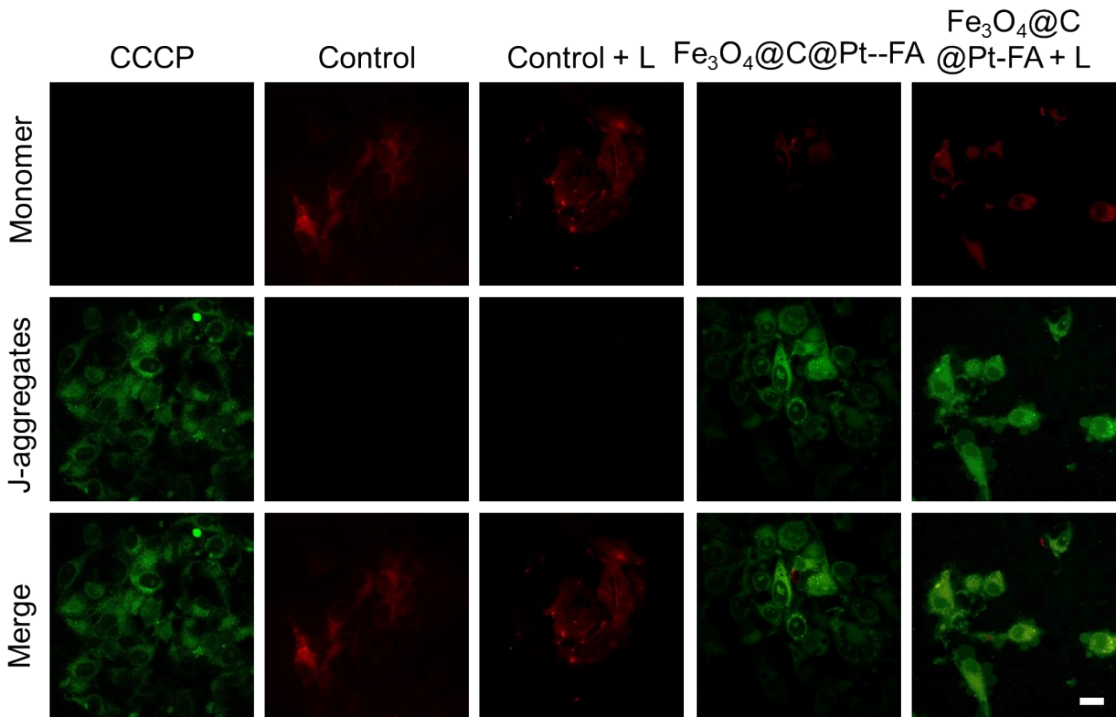

Figure S11. Changes of mitochondrial membrane potential in CT26 cells with different treatments

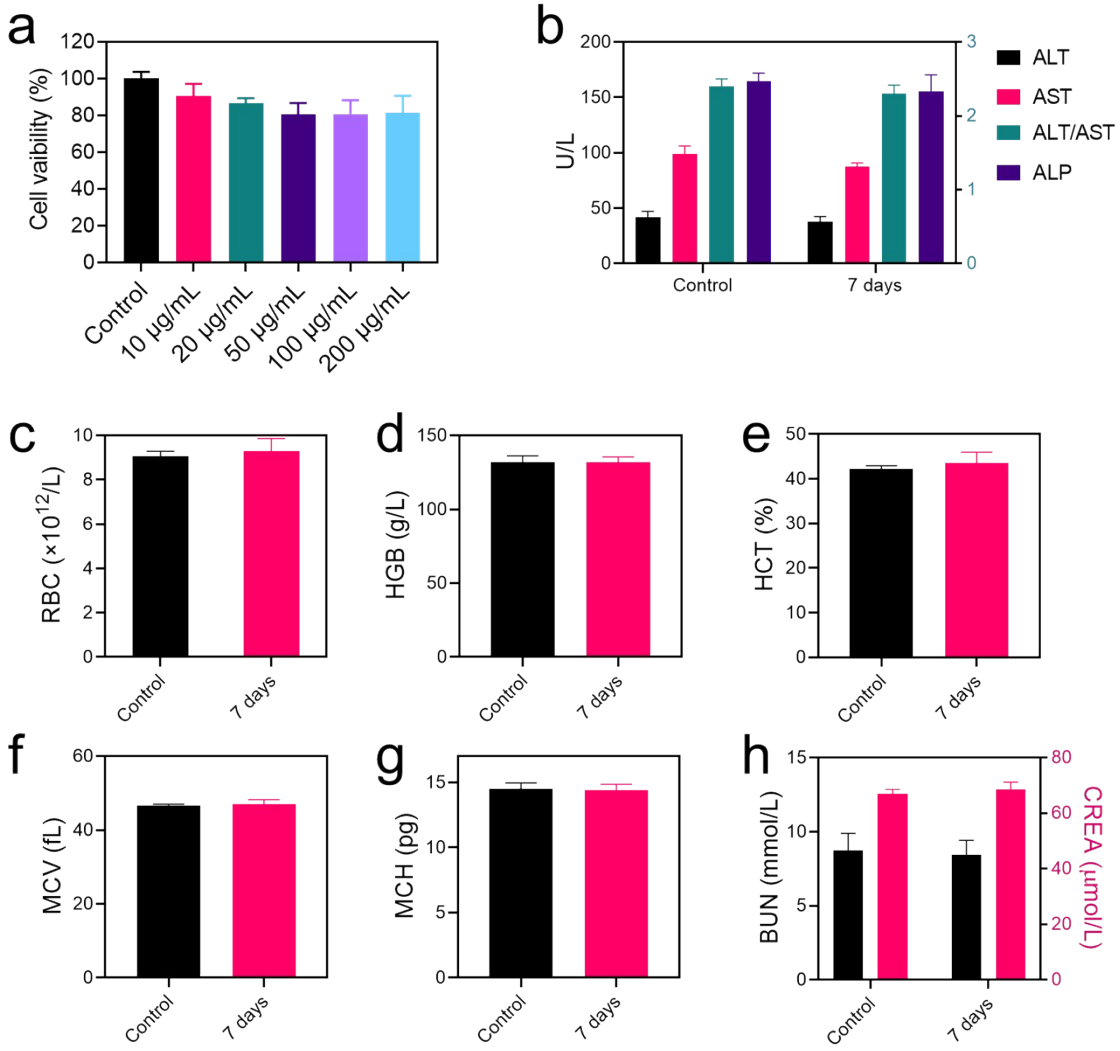

Figure S12. (a) The viability of L02 cells treated with a range of Fe_3O_4@C@Pt-FA concentrations for 24 h, (b-h) Blood biochemical analysis of mice 1 week after injection of Fe_3O_4@C@Pt-FA.

Table S1 The kinetic parameters of nanozyme as POD-like enzyme

Catalyst	[E] (μg·mL^{-1})	Substrate	K_m (mM)	V_{max} (10^{-8} M·s^{-1})
Fe_3O_4	5	TMB	0.19	3.54
Fe_3O_4		H_2O_2	87.42	4.96
Fe_3O_4@C		TMB	0.04	1.26
Fe_3O_4@C		H_2O_2	1.17	2.38
Fe_3O_4@C@Pt		TMB	0.04	8.38
Fe_3O_4@C@Pt		H_2O_2	2.91	16.27