Supporting Information

Synthesis of polyacrylic acid coated AuPd@Fe_xO_y nanoparticles for synergistic chemodynamic and photothermal therapy of osteosarcoma

Zeping Liu [a], Haoyu Sun [b], Yongjie Shan [c,d], Haodong Wang [e], Wenqi Luo [a], Rui Gu [a*], and Zhenxin Wang [c,d,f*]

a Department of Orthopaedic Surgery, China-Japan Union Hospital of Jilin University, Changchun, 130033, PR China

b Department of Hand and Foot Surgery, China-Japan Union Hospital of Jilin University, Changchun 130033, P. R. China

c State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, P. R. China.

d School of Applied Chemistry and Engineering, University of Science and Technology of China, Hefei, 230026, P. R. China.

e Department of Cardiovascular Medicine, The First Hospital of Jilin University, Changchun, 130021, P. R. China.

f National Analytical Research Center of Electrochemistry and Spectroscopy, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, P. R. China.

E-mail: wangzx@ciac.ac.cn

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1. Additional Experimental Section

1.1 Materials

$HAuCl_4 \cdot 4H_2O$, Na_2PdCl_4 and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Co. (MO, USA). $FeCl_2 \cdot 4H_2O$ and sodium polyacrylate (PAA, Mw = 4,500 g/mol, PDI ≤1.5) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Calcium green acetoxymethyl ester (Calcium Green AM) and propidium iodide (PI) were purchased from Beyotime Biotechnology Co., Ltd. (Jiangsu, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Jiangsu Keyuan Biotechnology Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (New York, USA). The trypsin cell dissociation kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The MG63 cell line was purchased from Shanghai Fuheng Biotechnology Co., Ltd. (Shanghai, China), which is affiliated with the Chinese Academy of Sciences (CAS). Other reagents were obtained from Beijing Chemical Reagent Co., Ltd. (Beijing, China). BALB/c male mice (6-8 weeks old), with an average weight of 20 g, were purchased from Vita River Laboratory Animal Technology Co., Ltd. (Beijing, China). All chemicals were of analytical grade and used as received without further purification. Milli-Q water (18.2 MΩ cm) was used in all experiments.

1.2 Characterization.

The morphology of the nanoparticles was recorded using a Hitachi H-600 electron microscope (Hitachi Ltd., Japan) at an accelerating voltage of 100 kV. Powder X-ray diffraction (XRD) analysis was performed using Cu-Kα radiation (0.15406 nm) on a D8 ADVANCE diffractometer (Bruker Co., Germany). X-ray photoelectron spectroscopy (XPS) measurements were carried out using a VG ESCALAB MKII spectrometer (VG Scientific Ltd., UK). Zeta potential and hydrodynamic size measurements were conducted on a Malvern Zetasizer (Malvern Instruments Ltd., UK). The MTT assay was performed using a Versamax microplate reader (Bio-Tek Instruments, Inc., USA). T_1-weighted MRI images were acquired using a Siemens 3T MRI scanner (Magnetom Avanto, Siemens, Erlangen, Germany). UV-Visible spectra were recorded using a Mini 1240 UV-Visible spectrophotometer (Shimadzu Co., Japan). Fluorescence images were obtained using a confocal laser scanning microscope (CLSM, Nikon Co., Japan).

1.3 Synthesis of AuPd@Fe_xO_y NPs

500 μL of $HAuCl_4 \cdot 4H_2O$ (0.024 mol/L) and 500 μL of Na_2PdCl_4 (0.024 mol/L) were added to 100 mL of deionized water. Under continuous stirring, 5 mL of ammonia solution (0.0075 %) was quickly added to the mixed solution at 25 °C. After stirring for 2 min, 3 mL of $FeCl_2$ aqueous solution (0.1 mol/L) were quickly added and the mixture was continuously stirred for another 20 min. The product was collected by centrifugation

(8000 rpm, 10 min), and washed three times with 100 mL of H_2O by centrifugation (8000 rpm, 10 min).[1]

1.4 Synthesis of PAA-AuPd@Fe_xO_y NPs

10 mL PAA solution (3 % wt/v) were added into 100 mL of AuPd@Fe_xO_y NPs (200 μg/mL). The mixture was continuously stirred at 25 °C for 2 h. The product was collected by centrifugation (8000 rpm, 10 min), and washed three times with 100 mL of H_2O by centrifugation (8000 rpm, 10 min).

1.5 Synthesis of Au@Fe_xO_y NPs

500 μL of $HAuCl_4 \cdot 4H_2O$ (0.024 mol/L) was added to 100 mL of deionized water. Under continuous stirring, 5 mL of ammonia solution (0.0075 %) was quickly added to the mixed solution at 25 °C. After stirring for 2 min, 3 mL of $FeCl_2$ aqueous solution (0.1 mol/L) were quickly added and the mixture was continuously stirred for another 20 min. The product was collected by centrifugation (8000 rpm, 10 min), and washed three times with 100 mL of H_2O by centrifugation (8000 rpm, 10 min).

1.6 *In Vitro* Depletion of GSH by PAA-AuPd@Fe_xO_y NPs.

The 50 μL of PAA-AuPd@Fe_xO_y NPs at different concentrations (200, 100, and 50 μg/mL) were mixed with 950 μL of GSH solution (20 μmol/L in PBS (pH 7.4)). After incubating the mixture at 37 ° C for 1 h, the mixture was treated by centrifugation (10000 rpm, 10 min), the supernatant was collected, and tested by a commercial GSH assay kit according to the

manufacturer's manual.

To investigate the mechanism of GSH depletion induced by PAA-AuPd@Fe_xO_y NPs, we first evaluated the role of AuPd surface adsorption by blocking its active sites with non-thiol ligands (sodium citrate). PAA-AuPd@Fe_xO_y NPs were incubated with excess sodium citrate at room temperature for 2 hours, followed by centrifugation (7500 rpm, 10 min) and redispersion. GSH consumption was then measured in both uncoated and ligand-coated NPs under identical conditions (20 μmol/L GSH, 10 min incubation at 37°C). After centrifugation to remove NPs, residual GSH was quantified via UV-Vis absorbance at 412 nm.

1.7 Detection of Extracellular ·OH

The production of ·OH was detected using fluorescence spectroscopy with 2,5-dihydroxyterephthalic acid (DHTPA) as the fluorescent probe. To investigate the effect of pH on ·OH generation, PAA-AuPd@Fe_xO_y NPs (collected from the precipitate after reacting with GSH) were mixed with final H_2O_2 solutions (10 mmol/L) at different pH values (7.4, 6.5, and 5.4). Subsequently, 100 μL of Terephthalic acid (100 mmol/L) was added to the mixture. After incubation for 30 minutes, the mixtures were centrifuged, and the fluorescence spectrum of the supernatant was recorded using a fluorescence spectrophotometer at an excitation wavelength of 315 nm. To evaluate the temperature-dependent efficiency of ·OH production, the nanoparticles were incubated in PBS buffer (pH 5.4) containing 10 mmol/L

H_2O_2 at different temperatures (0, 15, 30, 45 and 60 °C) for 30 minutes. Following incubation, centrifugation was performed, and the fluorescence spectra of the supernatants were measured. The enhanced ·OH generation induced by photothermal effects was achieved by exposing the PAA-AuPd@Fe_xO_y NPs to 808 nm laser irradiation (2.0 W/cm²) for varying durations (0, 5, 10 and 15 minutes). The ·OH production was quantified by analyzing the fluorescence spectrum of the supernatants post-irradiation.

1.8 Photothermal Effect of PAA-AuPd@Fe_xO_y NPs.

Expose 1 mL of PAA-AuPd@Fe_xO_y NPs (200 μg/mL) to 808 nm laser (1.0, 1.5, and 2.0 W/cm²) for 480 seconds. Record the temperature every 30 seconds using an infrared camera. Place 1 mL of PAA-AuPd@Fe_xO_y NPs at different concentrations (0, 50, 100, 200 μg/mL) in quartz tubes and expose them to 808 nm laser (2 W/cm²) for 480 seconds. Record the temperature every 30 seconds using an infrared camera. Additionally, assess the photothermal stability by cycling the irradiation of PAA-AuPd@Fe_xO_y NPs. First, expose the PAA-AuPd@Fe_xO_y NPs (200 μg/mL) solution to an 808 nm laser with a power density of 2.0 W/cm² for 480 seconds. Then turn off the laser and allow the solution to cool naturally. Repeat this cycle 5 times, and record the temperature using an infrared camera. The photothermal conversion efficiency (η) of PAA-AuPd@Fe_xO_y NPs can be calculated by the following equations:

$$\theta = (T - T_{surr})/ (T_{max} - T_{surr})$$

$\eta = [hS (T_{max} - T_{surr}) - QDis] / [I (1 - 10^{-A808})] \times 100\ \%$

$hS = (\Sigma miCp,i)/\tau s$

$t = \tau s \times (-\ln\theta)$

Where h is the heat transfer coefficient, S is the irradiated area, and T_{max} is the equilibrium temperature, T_{surr} is the ambient temperature of the surroundings. The QD expresses the heat from light absorbed by the quartz sample cell itself, I is the laser power density, A is the absorbance of PAA-AuPd@Fe_xO_y NPs at 808 nm, m is the mass of the sample, Cp is the thermal capacity of the sample, t is cooling time after irradiation, and τs is the sample system time constant.[2]

1.9 Cell Culture and Cytotoxicity Assay of PAA-AuPd@Fe_xO_y NPs.

Add 100 μL of MG63 cells cultured in fresh medium (fresh DMEM supplemented with 10 % FBS and 100 U/mL penicillin-streptomycin) to a 96-well microtiter plate (1×10^4 cells per well), and incubate them at 37 °C in a humidified atmosphere containing 5 % CO_2 for 24 hours. Wash the cells three times with 100 μL of PBS, and then add different concentrations of PAA-AuPd@Fe_xO_y NPs (0, 50, 100, 200 μg/mL) to the wells. Incubate the cells under the same conditions for another 24 hours. Subsequently, the cells were washed three times with 100 μL PBS and replaced with DMEM medium containing MTT solution (0.5 mg/mL 100 μL) for another 4 hours of incubation. Then carefully remove the supernatant and add 100 μL of DMSO to each well. Measure the absorbance at 490 nm using a microtiter

plate reader. Use untreated MG63 cells as a control. Calculate the relative cell viability (%) by comparing the absorbance values to the control.

1.10 The intracellular uptake capacity of PAA-AuPd@Fe_xO_y NPs.

MG63 cells were seeded into 6-well plates at a density of 1×10^6 cells per well and incubated for 24 hours. Then, cells were divided into two groups: (1) Control Group (without treatment) and (2) PAA-AuPd@FexOy NPs Group (treated with 200 μg/mL PAA-AuPd@FexOy NPs). Both groups were incubated for 24 hours. After incubation, cells were washed twice with PBS and centrifuged (1000rpm, 5 min). Cells were then digested overnight at room temperature with aqua regia. Following digestion, cell debris was removed by centrifugation (1000rpm, 5 min), and the supernatant was analyzed via ICP-MS to quantify intracellular concentrations of Au and Fe.

1.11 Detection of Intracellular ROS Production.

Seed MG63 cells into a 96-well plate and incubate them for 24 hours. Then, culture the cells with DCFH-DA (1 μmol/L in DMEM) for 20 minutes. Afterward, replace the medium with acidified medium (pH 6.5) containing 100 μmol/L H_2O_2, normal medium containing 200 μg/mL PAA-AuPd@Fe_xO_y NPs, and acidified medium (pH 6.5) containing 200 μg/mL PAA-AuPd@Fe_xO_y NPs and 100 μmol/L H_2O_2. Culture the cells in normal medium containing 200 μg/mL PAA-AuPd@Fe_xO_y NPs and in acidified medium (pH 6.5) containing 200 μg/mL PAA-AuPd@Fe_xO_y NPs and 100

μmol/L H_2O_2, and then irradiate them with an 808 nm laser for 8 minutes. Use normally cultured cells as the control sample. After 2 hours, wash the cells with PBS (100 μL, three times). Evaluate intracellular ROS by detecting the fluorescence of DCF (λex = 488 nm, λem = 525 nm) using a confocal laser scanning microscope.

1.12 *In vitro* PTT and PTT-enhanced CDT.

Seed MG63 cells in a 96-well plate and incubate them for 24 hours. Then, culture the cells in a normal medium containing different concentrations of PAA-AuPd@Fe_xO_y NPs (0, 50, 100, 200 μg/mL) and in an acidified medium (pH 6.5) containing 100 μmol/L H_2O_2. After incubating for another 24 hours, wash the cells three times with 100 μL of PBS, and then irradiate them with an 808 nm near-infrared laser (2.0 W/cm²) for 10 minutes. Replace the medium with fresh medium and incubate for another 24 hours. Then, assess cell viability by MTT assay and by imaging after co-staining with Calcein AM/ PI for 20 minutes. Use normally cultured cells as the control sample.

1.13 *In vitro* and *in vivo* MRI Contrast Performance.

Disperse PAA-AuPd@Fe_xO_y NPs with different Fe concentrations (0.05, 0.1, 0.2, 0.4, and 0.60 mmol/L) in PBS at different pH values (7.4 and 5.4) and measure the relaxation time. For *in vivo* MRI, intravenously inject 100 μL of PBS containing PAA-AuPd@Fe_xO_y NPs (20 mg/kg body weight) into tumour-bearing mice. Record MRI images at 0, 1, 2, 4, 6, 8 and 24

hours post-injection.

1.14 *In vivo* Photothermal Imaging.

Divide the tumour-bearing mice into two groups, and intravenously inject them with either 100 μL of PBS or 100 μL of a PBS solution containing PAA-AuPd@Fe_xO_y NPs (20 mg/kg body weight). Six hours after injection, irradiate the tumours with an 808 nm near-infrared laser. Record the temperature of the tumours every 2 minutes using an infrared thermal imager.

1.15 *In vivo* therapy studies

Select male BALB/c nude mice (6-8 weeks old, average weight 20 g) to establish an MG63 tumour model. Subcutaneously inject MG63 cells (3×10^6 cells in 100 μL) into the flank of the mice. When the tumours have grown to a diameter of 4-5 mm, randomly divide the mice into four groups (n=3): Group I: Control group; Group II: Intravenously receive 100 μL of PBS and are only irradiated with an 808 nm laser; Group III: Intravenously receive a PBS solution of PAA-AuPd@Fe_xO_y NPs (100 μL, 20 mg/kg body weight); Group IV: Intravenously receive a PBS solution of PAA-AuPd@Fe_xO_y NPs (100 μL, 20 mg/kg body weight) and are irradiated with an 808 nm near-infrared laser (1.3 W/cm², 10 minutes) 6 hours after injection. The tumour volume is calculated using the following formula: Volume (V) = (Tumour Length) × (Tumour Width)² / 2, where V_0 represents the initial tumour volume.

1.16 *In vivo* Toxicology Analysis

The mice in the four different treatment groups were euthanized and dissected 30 days after treatment, and the main organs, including heart, spleen, liver, lungs, kidneys, and tumours, were collected for hematoxylin and eosin (H&E) and TUNEL staining. For blood analysis, healthy mice were intravenously injected with a single dose of PAA-AuPd@Fe_xO_y NPs (100 μL, 20 mg/kg body weight). Thirty days after injection, extract blood and analyze it through hematological parameter determination. Use untreated healthy mice as a control.

2 Additional Figures S1-S10

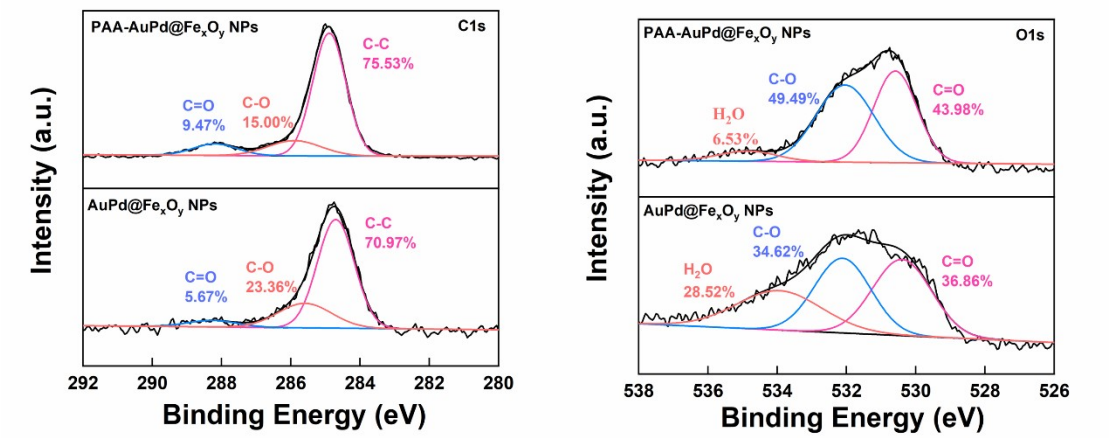

Fig. S1 The XPS spectra of PAA-AuPd@Fe_xO_y NPs and AuPd@Fe_xO_y NPs at the C1s and O1s level.

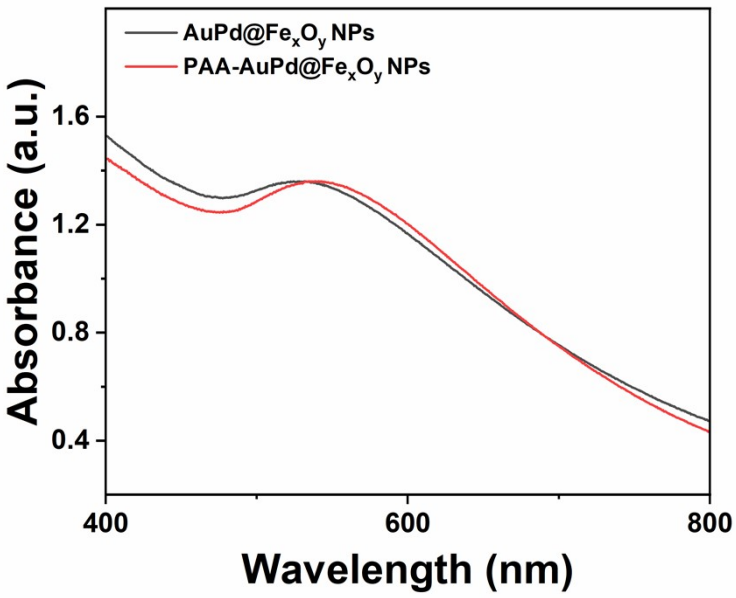

Fig. S2 UV-Visible absorption spectra of AuPd@Fe_xO_y NPs and PAA-AuPd@Fe_xO_y NPs.

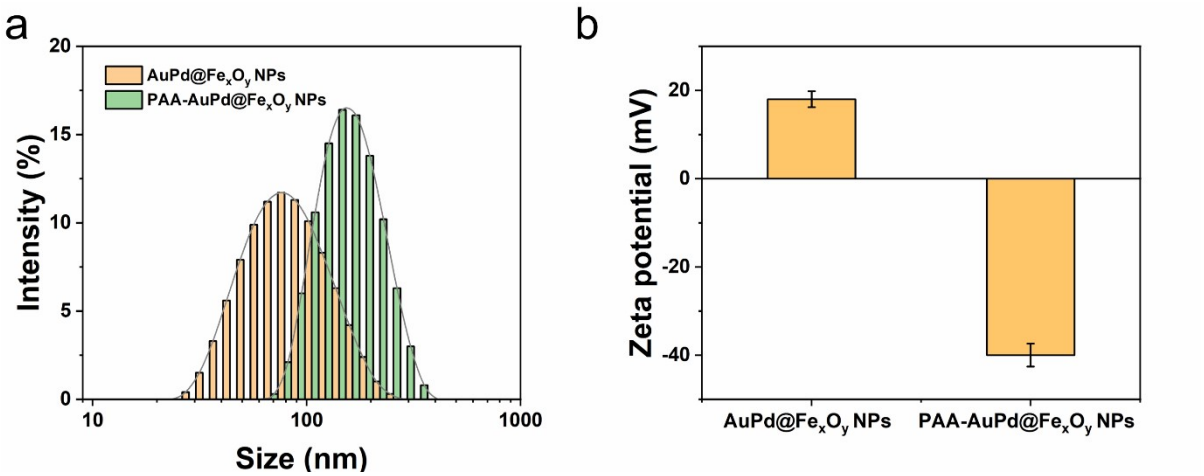

Fig. S3 (a) The hydrodynamic sizes and (b) Zeta potentials of AuPd@Fe_xO_y NPs and PAA-AuPd@Fe_xO_y NPs.

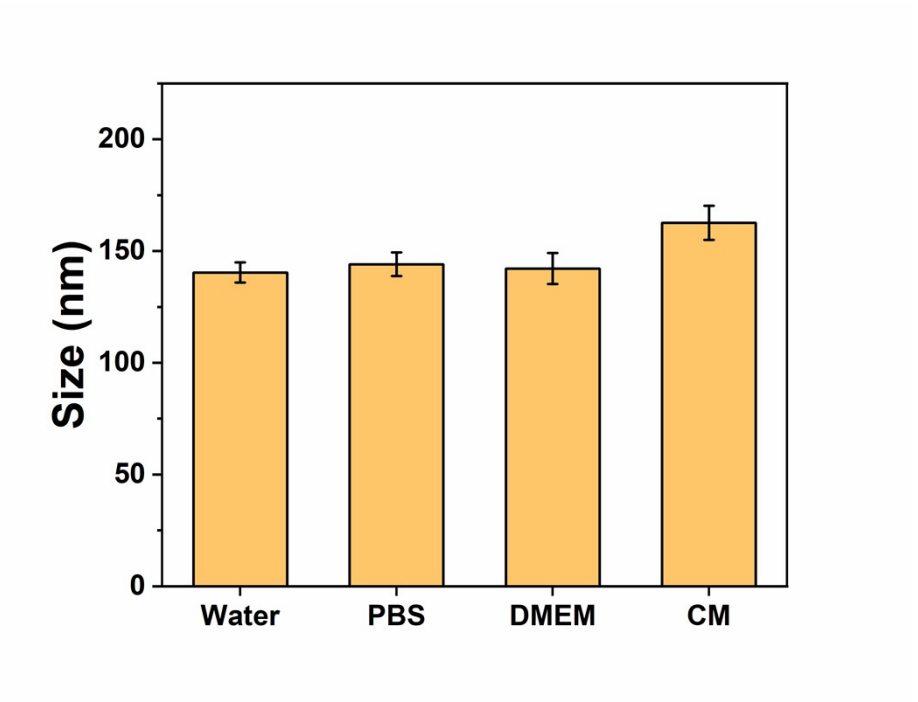

Fig. S4 The hydrodynamic sizes of PAA-AuPd@Fe_xO_y NPs in different dispersing media.

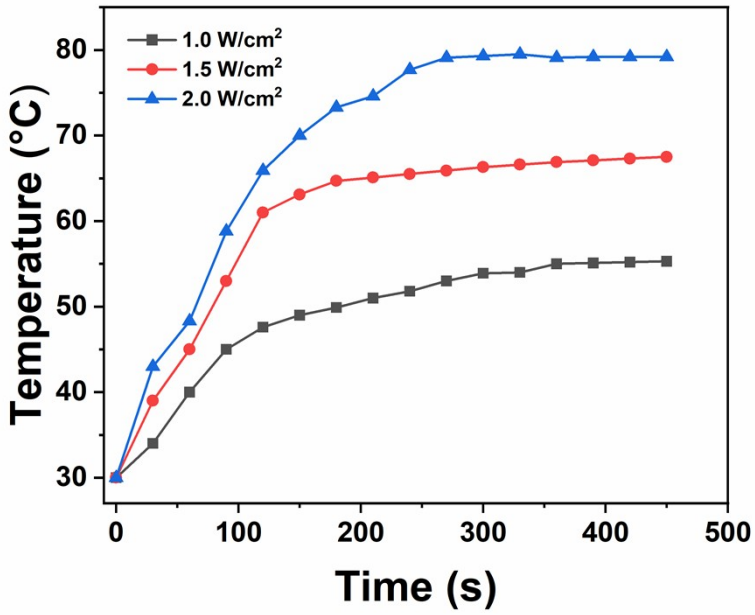

Fig. S5 The temperature profiles of 200 μg/mL PAA-AuPd@Fe_xO_y NPs solutions under 808 nm NIR laser irradiation at different powers.

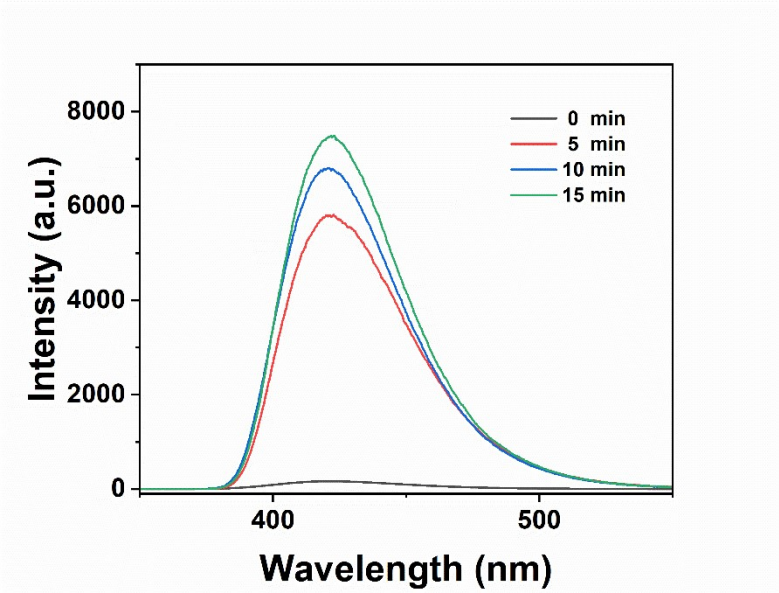

Fig. S6 The fluorescence intensity of DHTPA generated after reaction with PAA-AuPd@Fe_xO_y NPs under varying 808 nm NIR laser irradiation times (0,5,10 and 15 min).

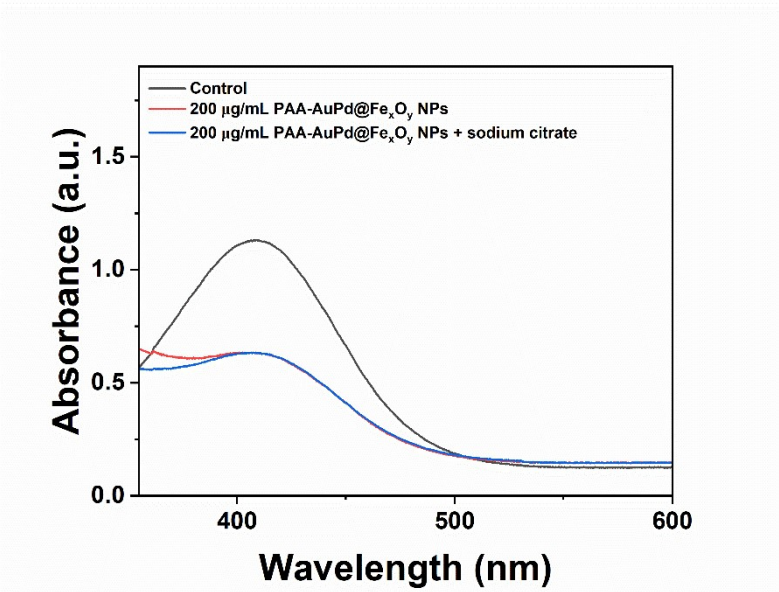

Fig. S7 GSH Depletion at 412 nm under reaction with PAA-AuPd@Fe_xO_y NPs and PAA-AuPd@Fe_xO_y NPs after blocking the active sites of AuPd with non-thiol ligands.

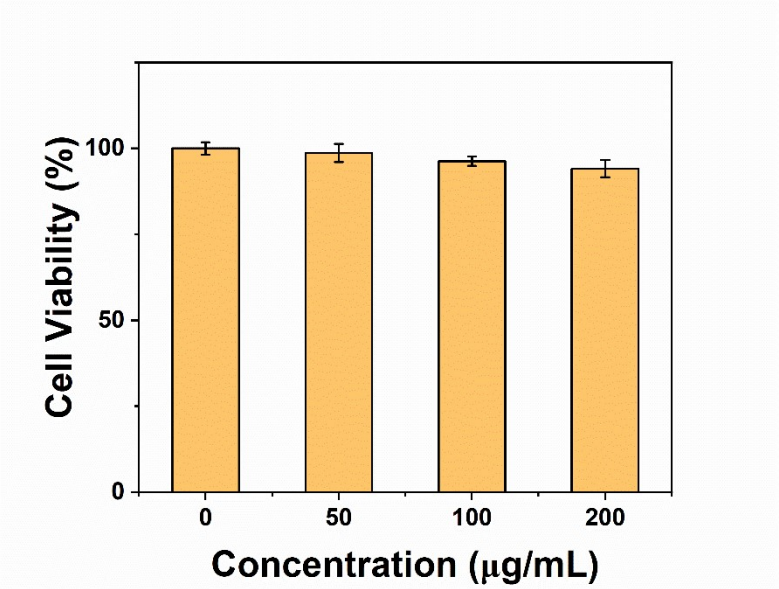

Fig. S8 The cell viability of LP929 cells under different concentration of PAA-AuPd@Fe_xO_y NPs.

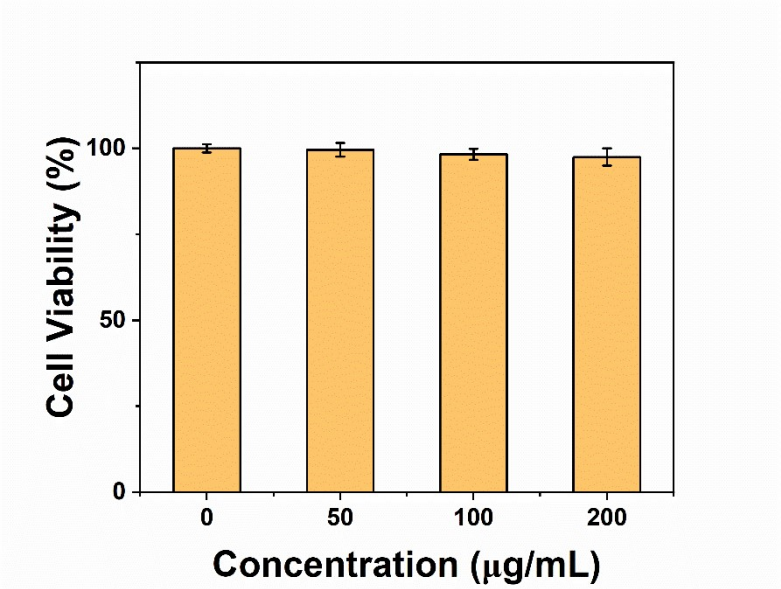

Fig. S9 The cell viability of MG63 cells as a function of concentration of PAA-AuPd@Fe_xO_y NPs.

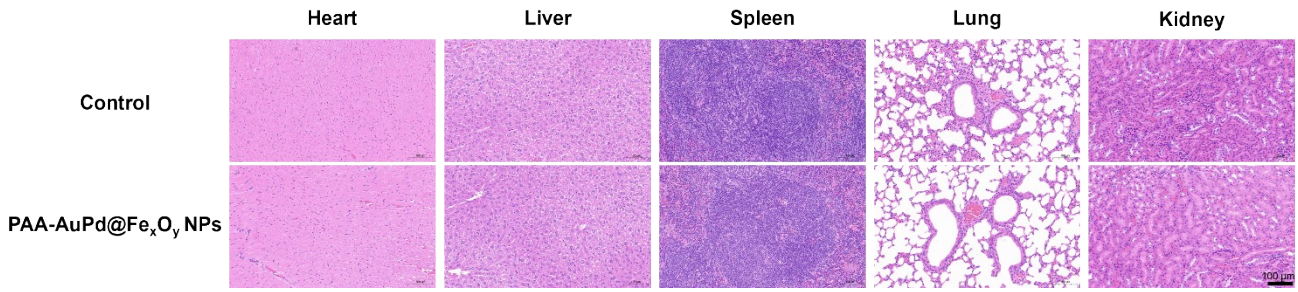

Fig. S10 H&E staining of heart, liver, spleen, lung, and kidney after 30 days treatment.

3 Additional Tables S1

Table S1 Hematology analysis of normal reared mice (control) or healthy mice treated with PAA-AuPd@Fe_xO_y NPs.

Hematological	Units	Control	PAA-AuPd@ Fe_xO_y NPs
WBS	$x10^9/L$	6.95	7.11
RBC	$x10^{12}/L$	7.43	7.43
HGB	g/L	152.55	153.85
MCV	fL	50.54	50.62
MCH	pg	20.53	20.71
PLT	$x10^9/L$	527.15	490.98
PDW	fL	30.85	30.56
MCHC	g/L	406.13	409.09

4. Additional References

1. Y. Sun, H. Chen, Y. Huang, F. Xu, G. Liu, L. Ma and Z. Wang, *Biomaterials*, 2021, **274**, 120821.
2. W. Y. Zhen, Y. Liu, L. Lin, J. Bai, X. D. Jia, H. Y. Tian and X. Jiang, *Angewandte Chemie-International Edition*, 2018, **57**, 10309-10313.