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Electronic Supporting Information

Al centre-powered graphitic nanozyme with high catalytic efficiency for pH-independent chemodynamic therapy of cancer

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Experimental Section

1. Chemicals and Reagents

Aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$, ≥98.0%), aqueous hydrofluoric acid (HF, 40 wt%), iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥99.0%), nitric acid (HNO_3, 65.0%-68.0%), anhydrous *n*-hexane (≥97.0%), *N,N*-dimethylformamide (DMF, ≥99.5%), dimethyl sulfoxide (DMSO, ≥99.0%), hydrogen peroxide (H_2O_2, GR, 30 wt%), ethanol (C_2H_5OH, >99.8%), ethylenediaminetetraacetic acid (EDTA, ≥99.0%), acetonitrile (MeCN, ≥99.8%) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Benzene-1,3,5-tricarboxylic acid (BTC, ≥95.0%), 2-aminoterephthalic acid (≥99.0%), dicyandiamide (DCD, ≥99.0%) were obtained from J&K Scientific Ltd. (Beijing, China). Reduced iron powder, methylene blue (MB, ≥98.5%), MTT reagent (≥98.0%), propidium iodide (PI, ≥95.0%) were obtained from Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). Calcein-AM (≥96.0%) was purchased from Aladdin Biochemical Technology Co. Ltd (Shanghai, China). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, ≥99.7%) was obtained from MedChemExpress (New Jersey, USA). N-methylacridone (NMA, ≥98.0%) was obtained from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). Deionized water used in this study was prepared by Milli-Q ultrapure water system. 0.01 M phosphate buffers (PB) with different pH values were obtained by mixing 100 mM Na_2HPO_4 and 100 mM NaH_2PO_4 in the proper ratios. The pH of each buffer was then calibrated with a PHB-4 pH-meter (INESA Scientific Instrument Co. Ltd., China).

2. Cells and Animals

SMMC-7721 cell (Human liver cancer cell line) used in this study was purchased from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum albumin (FBS), 100 U/mL penicillin and streptomycin. Cells were maintained at 37 ℃ in humidified atmosphere with 5% CO_2.

Male athymic BALB/c mice (4-week-aged) were purchased from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). Tumor-bearing mice were prepared by

subcutaneously injecting 5×10^6 in vitro-propagated cancer cells into the right legs of nude mice. All animal operations were performed according to protocol No. SCXK (Xiang) 2018-0006 approved by the Laboratory Animal Center of Hunan Province.

3. Preparation of Nanozymes

3.1 Preparation of Fe@Al-MOF

Firstly, Al-MOF (MIL-101-NH_2) was prepared according to the reported method,[1] then DCD and iron chloride were co-encapsulated into the pores of Al-MOF to gain Fe@Al-MOF composite via double solvent method (DSM). Typically, dehydrated Al-MOF was added into anhydrous n-hexane (hydrophobic solvent) in mass-to-volume concentration of 0.5%, the resulting mixture was subsequently sonicated for ~ 30 min in order to obtain a homogenous system. After stirring for 10 min, equivalent DCD and iron chloride (100 mg) that suspended in 0.067 mL DMF solution (hydrophilic solvent) were slowly added with continuous and all-inclusive agitation. After another 3 h agitation, the synthesized product (Fe@Al-MOF) was precipitated from the mixed solution. The collected precipitates were firstly dried in ambient air, then dehydrated in vacuum at 120 ℃ for 12 h.

3.2 Preparation of Fe/Al-GNE

Fe@Al-MOF was loaded into a ceramic boat and then carbonized at 800 ℃ in a nitrogen atmosphere for 5 h with a heating rate of 10 ℃/min. After cooling to ambient temperature, the resulting solid was treated with HF (20 wt%) solution for 24 h to remove fluctuating and inert components, and subsequently collected by centrifuging with a speed of 8000 rpm. After thorough wash with deionized water, the black product (Fe/Al-GNE) was dried in vacuum at 120 ℃ for 12 h. Notably, the experiment with corrosive HF was done in fume cupboard, and the operator was equipped with gloves, goggle and gauze mask.

As comparative nanozymes, Fe-GNE as well as Al-GNE that were derived from Fe-MOF and Al-MOF, respectively, were prepared using the same procedure. Thereinto, Fe-MOF (MIL-100) was synthesized according to the reported method.[2]

4. Characterization

Powder X-ray diffraction (PXRD) patterns of the products were detected on a D8-ADVANCE powder diffractometer (Bruker, Germany). Transmission electron microscopic and energy-dispersive X-ray detector (EDX) images were acquired by Tecnai G2 F20 transmission electron microscope (FEI, USA). Size distribution experiments were performed by Zetasizer Nano ZS90 (Malvern, USA).

5. Study of •OH Generation at Different pH Value

Firstly, to confirm the generation of •OH, ESR spectroscopy was measured by using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as •OH spin trap agent. In brief, Fe/Al-GNE (2 μg) was incubated with DMPO (100 mM) in the presence of H_2O_2 (6 mM) for 3 min under different pH conditions, then X-band ESR spectra of the mixture was immediately recorded by an EMX-8/2.7 spectrometer (Bruker, Germany) at room temperature. The settings as follow: microwave frequency = 9.872 GHz, microwave power = 6.375 mW, modulation frequency = 100.00 kHz and modulation amplitude = 1.00 G.

To investigate the catalytic activity of different nanozymes in a wide range of pH, classical colorimetric assays were performed based on •OH-triggered degradation of methylene blue (MB). Generally, MB (5 mg/L) was incubated with nanozymes (20 μg/mL) in PBS with or without H_2O_2 (13 mM) for 1 h at different pH values. After centrifugation, the supernatant was measured by a UV-2700 spectrometer (Shimadzu, Japan).

6. Fluorescence Analysis of Catalytic Efficiency at Weakly Acidic pH_e

Generally, DCFH-DA (5 μM) was incubated with different nanozymes (20 μg/mL) in PBS with or without H_2O_2 (13 mM, unless otherwise noted) for 10 min at pH 6.9, respectively. Then, the fluorescence responses of DCFH-DA were recorded by both PTI QM8000 fluorescence spectrometer (Photo Technology International, USA; Ex = 488 nm, Em = 503-640 nm) and IVIS Lumina Series III in vivo imaging system (Caliper Life Sicence, USA).

To investigate the catalytic kinetics, time-lapse fluorescence responses of DCFH-DA that treated with Al-MOF, Fe-MOF, Fe@ Al-MOF and Fe/Al-GNE, respectively, were monitored in PBS containing 13 mM H_2O_2 (Ex = 488 nm, Em =525 nm).

7. Fluorescence Analysis of Lewis Acidity by N-methylacridone

To determine the Lewis acidity of Fe/Al-GNE, fluorescent N-methylacridone (NMA) was used as indicator. In an N_2-filled glovebox, nanozymes were added to NMA solution (10 μM in MeCN), and then the resulting mixture was sonicated for 2~3 min until well blended. After 30 min incubation at room temperature, fluorescence spectra of the supernatant were recorded by PTI QM8000 fluorescence spectrometer (Ex = 396 nm, Em = 406-540 nm).

8. Laser Scanning Confocal Microscopy Imaging

Generally, SMMC-7721 cells were seeded in the CLSM culture plates one day in advance, and then imaged after different treatments. The confocal fluorescent images were obtained by a FV3000 confocal microscope (Olympus, Japan) with a 40× objective. Excitation wavelength and emission filters were described as follows. DCFH: Ex=488 nm, Em=500–600 nm bandpass; Calcein-AM: Ex=488 nm, Em=500-540 nm bandpass; PI: Ex=561nm, Em=570-670 nm bandpass.

pH-independent Fenton reaction was assessed by incubating SMMC-7721 cells (pre-stained by DCFH-DA) with nanozymes (20 μg/mL), H_2O_2 (50 μM) in PBS with different pH value for 20 min, then visualized by confocal microscope immediately.

To investigate the Fenton catalytic efficiency at pH_e 6.9, SMMC-7721 cells pre-treated with DCFH-DA (5 μM) were incubated with nanozymes (20 μg/mL) in the presence of H_2O_2 (50 μM) for different times at pH = 6.9, then visualized by confocal microscope immediately.

For tissue slice imaging, tumor tissues and normal muscle tissues were all acquired from tumor-bearing mice. After embedded in OCT, the tissues were rapidly frozen to -28 ℃ and cut into 10 μm-sections using CM1950 (Leica, Germany). The frozen slices were quickly adsorbed on glass slides, and subsequently treated with nanozymes (20 μg/mL) for 1 h at room temperature before imaging.

9. *In Vitro* Cytotoxicity Assays

9.1 MTT assay

pH-independent chemodynamic cytotoxicity of Fe/Al-GNE was evaluated using MTT colorimetric assay. In order to mimic the slightly acidic extracellular

microenvironment of solid tumor, RPMI 1640 medium (FBS-free) was acidified to different pH values before used. SMMC-7721 cells (2×10^3 cells per well) were seeded in 96-well microplates and allowed to adhere overnight. Then, the culture medium of each well was replaced by fresh medium (FBS-free) with different pH values (pH 7.4, 6.9, 6.5 and 5.4), respectively. After incubating cells with nanozymes (20 μg/mL) and H_2O_2 (100 μM) for 6 h at 37 ℃, supernatant was replaced with fresh medium (10% FBS) for further cell growth (24 h). Subsequently, MTT reagent (0.35 mg/mL) was added to each well and incubated for another 4 h at 37 ℃. After removing the MTT solution, 100 μL of DMSO was added for formazan dissolution. Cell viability was determined by recording the absorbance at λ =490 nm with a versa max microplate reader (Molecular devices, USA).

Inhibitory effect of Fe/Al-GNE at pH 6.9 was evaluated by taking Al-MOF, and Fe@Al-MOF as comparison. First, SMMC-7721 cells were incubated with nanozymes (20 μg/mL) in the presence of H_2O_2 (100 μM) for 6 h at 37 ℃. Then, supernatant was removed and replaced with fresh medium (10% FBS) for further cell growth (24 h). Cell viability was measured as described above.

9.2 Calcein-AM/PI stain assay

Firstly, SMMC-7721 cells were incubated with different nanozymes (20 μg/mL) in RPMI 1640 medium containing H_2O_2 (100 μM) for 6 h at 37 ℃, then stained by Calcein-AM (2 μM) and PI (4 μM) for 20 min. After twice washing by PBS, cells were imaged by confocal microscope. Similarly, the influence of pH_e to chemodynamic effect was investigated by incubating SMMC-7721 cells with Fe/Al-GNE in RPMI 1640 medium with different pH value.

10. *In Vivo* CDT Efficacy and Biosafety Evaluation

The CDT efficacy of Fe/Al-GNE was performed on SMMC-7721 tumor-bearing BALB/c nude mice. Once the tumors volume reached ~130 mm^3, mice were randomly divided into four groups and intratumorally injected with Al-MOF, Fe@Al-MOF and Fe/Al-GNE at a dose of 2 μg (resuspended in 50 μL APBS), respectively. The control group received an equal amount of PBS injection. Intratumor injection and volume

measurement of tumor were administrated every other day until day 16th. The tumor volume was calculated using the following equation: $V = length \times width^2 / 2$.

To evaluate the toxicity of Fe/Al-GNE, BALB/c nude mice were randomly divided into two groups, one group was intravenously injected with PEG-coated Fe/Al-GNE at a dose of 100 μg/kg. The control group received an equal amount of PBS injection. Intravenous injection and weight measurement of mice were administrated every other day until 14th day. Then, mice were sacrificed, and their major organs, including heart, liver, spleen, lung, and kidney were analyzed by hematoxylin and eosin (H&E) staining.

Hemolysis test was performed by using mice blood, which was obtained from the mice eye socket vEin. Subsequently, the red blood cells were collected by thrice centrifugation and washing, then treated with different concentration of Fe/Al-GNE (0, 10, 20, 50, 100, 200, 500, 1000 μg/mL) at 37 ℃. After 3 h incubation, all samples were centrifuged to observe the hemolysis. Ultrapure water and PBS were used as positive control and negative control, respectively.

Supporting Figures

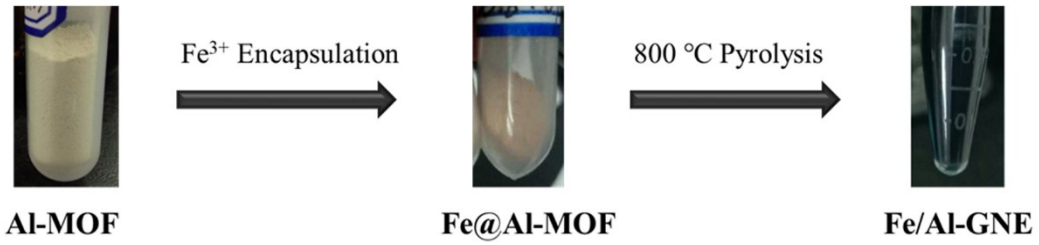

Fig. S1 Photographic images of Al-MOF, Fe@ Al-MOF and Fe/Al-GNE.

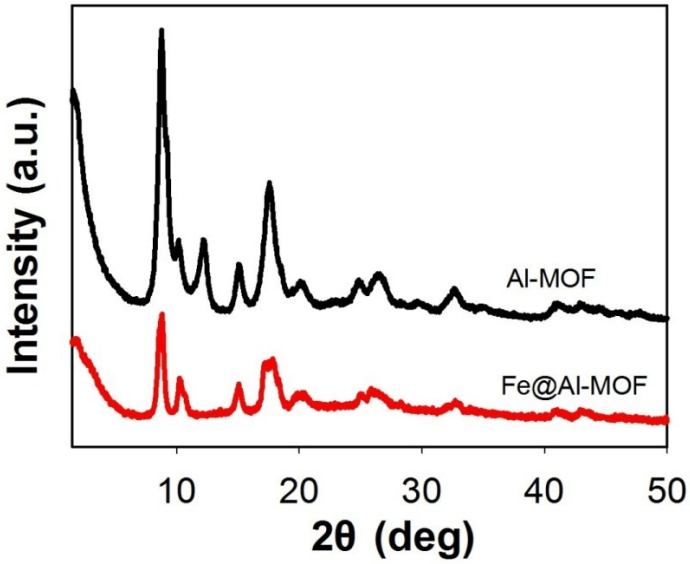

Fig. S2 XRD pattern of Al-MOF and Fe@Al-MOF.

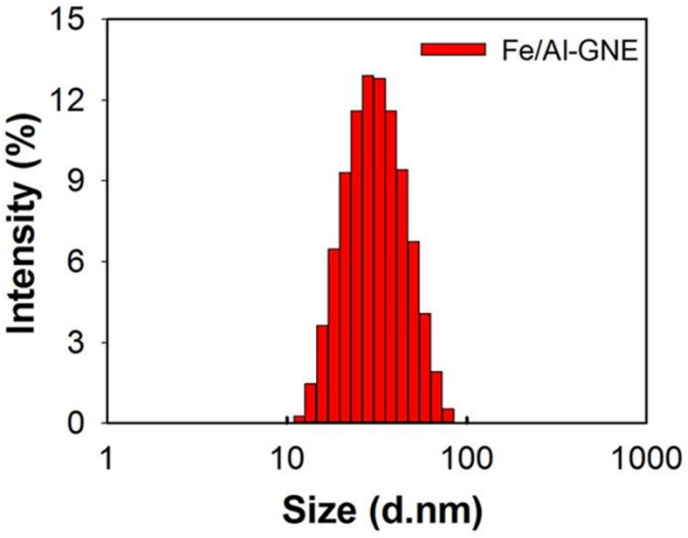

Fig. S3 Size distribution pattern of Fe/Al-GNE.

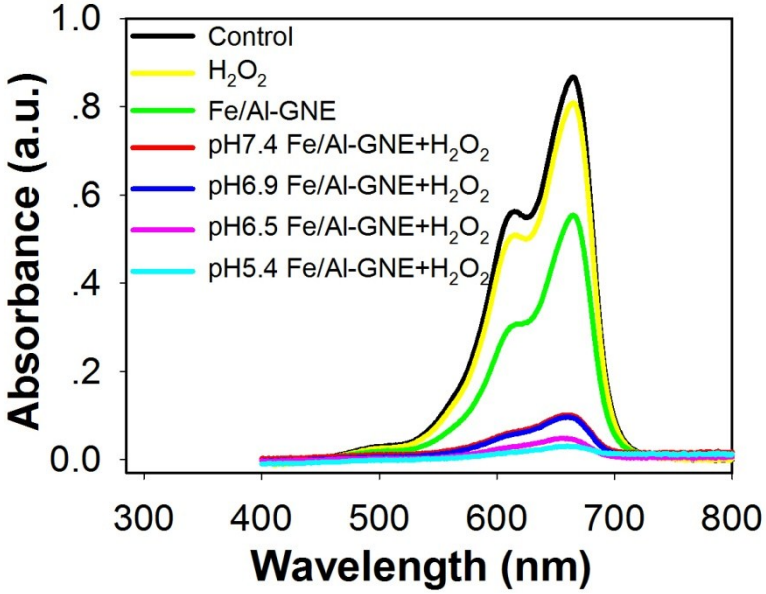

Fig. S4 UV-vis spectra of MB treated with Fe/Al-GNE at different pH condition. (H_2O_2: 13 mM)

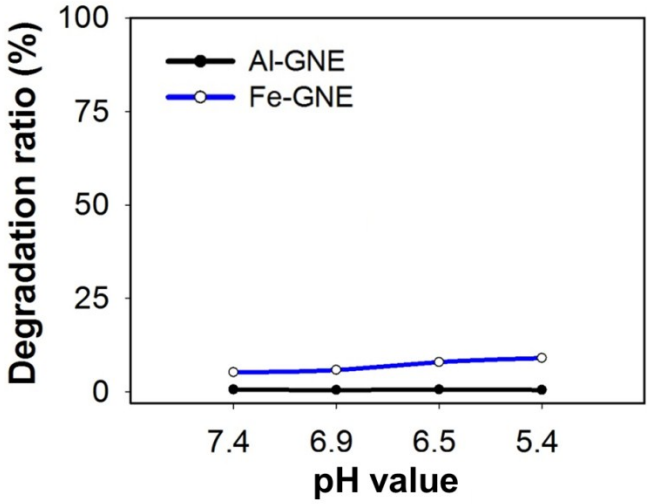

Fig. S5 Degradation curves of MB after incubation with Al-GNE and Fe-GNE in presence of H_2O_2 (13 mM), respectively.

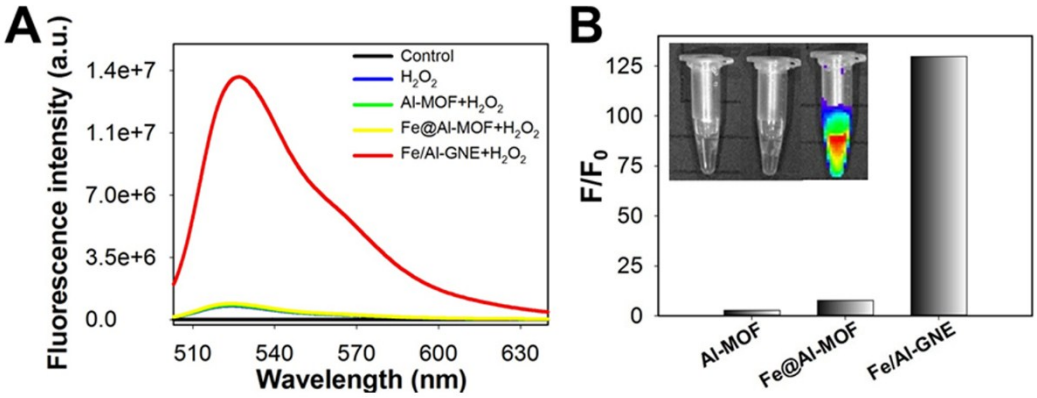

Fig. S6 (A) Fluorescence intensity of DCFH-DA (5 μM) in response to different nanozymes in the presence of H_2O_2 (13 mM) at pH 6.9. (B) The corresponding fluorescence ratios of DCFH-DA after and before being treated with nanozymes in (A).

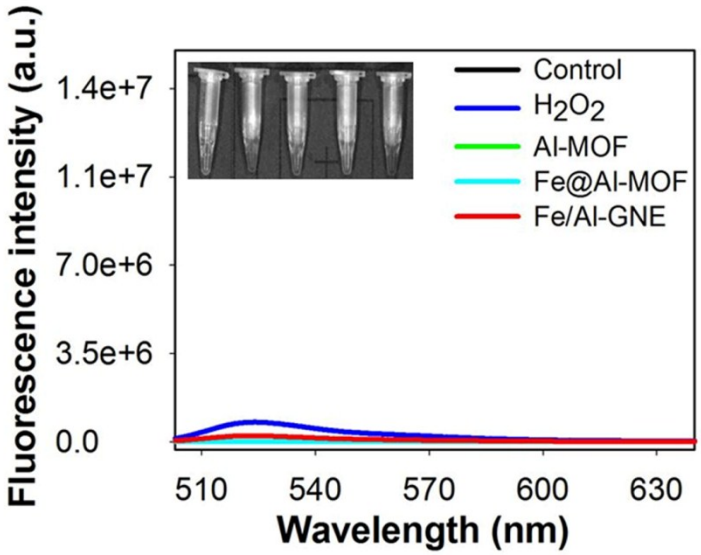

Fig. S7 Fluorescence intensity of DCFH-DA (5 μM) in response to different nanozymes without H_2O_2 at pH 6.9. Inset: the corresponding photo of samples.

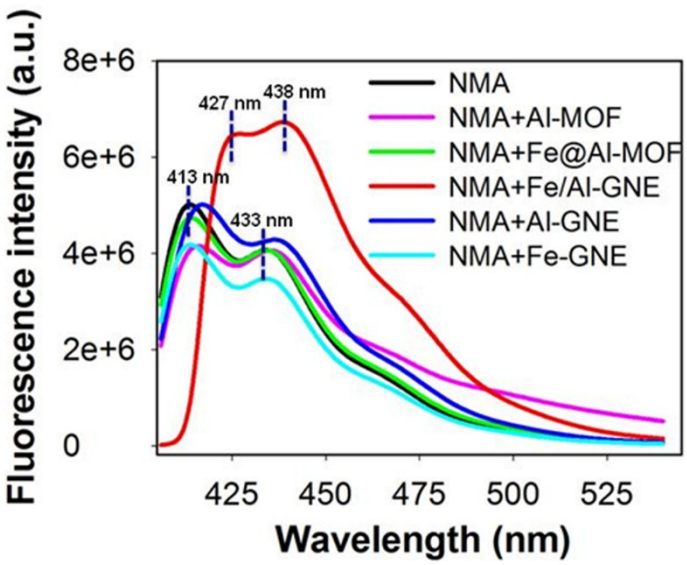

Fig. S8 Fluorescence emission spectra of NMA in response to different nanozymes in MeCN. The λ_{max} of NMA red-shifts upon binding to Lewis acidic metal sites, and the extent of the red-shift correlates to the Lewis acidity.[3]

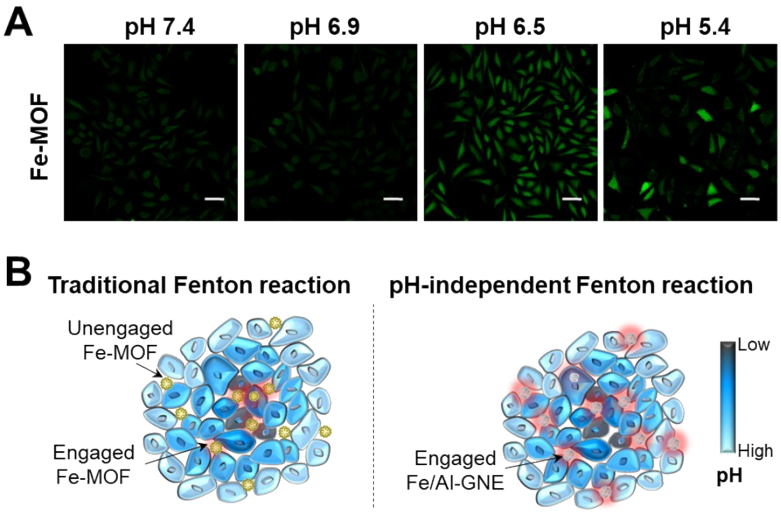

Fig. S9 (A) Confocal imaging of DCFH-DA pre-stained SMMC-7721 cells after treating with Fe-MOF under different pH values. (H_2O_2: 50 μM) (B) Schematic illustration of Fenton reaction mediated by Fe-MOF and Fe/Al-GNE, respectively. Fe-MOF could initial efficient Fenton reaction only in strongly acidic area, but didn't work at the tumor surface with nearly neutral pH. Fe/Al-GNE could initial efficient Fenton reaction in various locations of tumor. Scale bars: 20 μm.

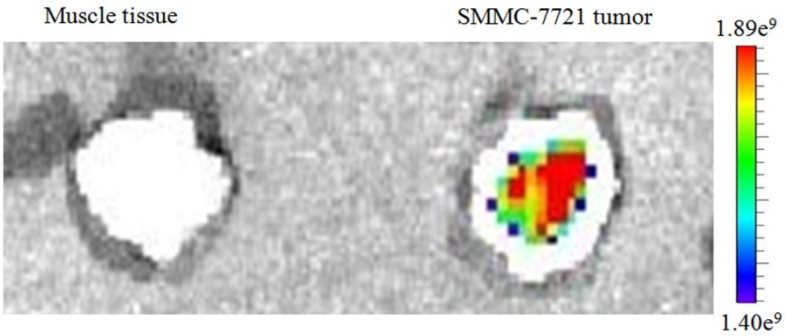

Fig. S10 Images of the isolated muscle tissue and SMMC-7721 tumor tissue after treating with Fe/Al-GNE for 1 h.

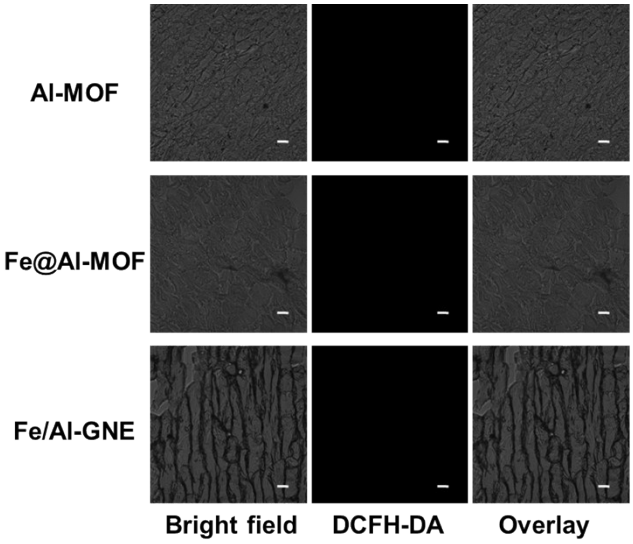

Fig. S11 Confocal fluorescence images of the muscle slices after treating with different nanozymes for 1 h. Scale bar: 20 μm.

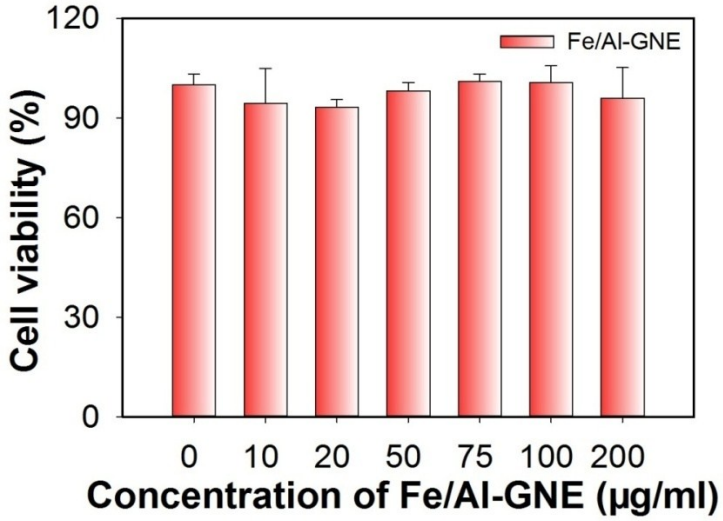

Fig. S12 Cell viability of SMMC-7721 cells after incubation with Fe/Al-GNE at different concentrations (without H_2O_2), respectively.

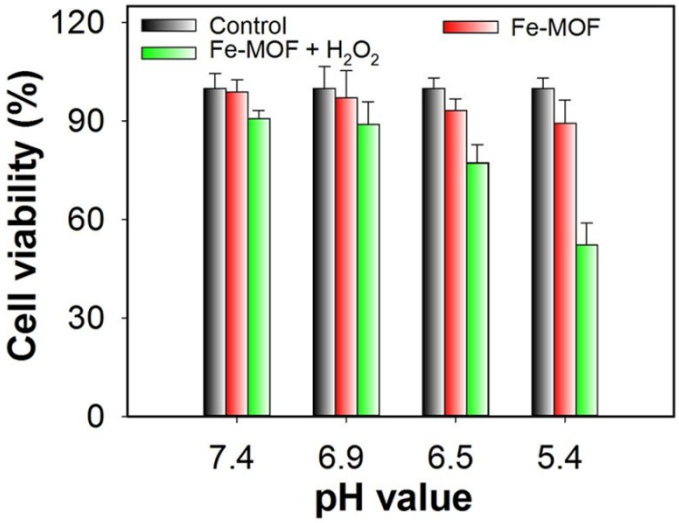

Fig. S13 Viability of SMMC-7721 cells treated with Fe-MOF under different pH value. (H_2O_2: 100 μM).

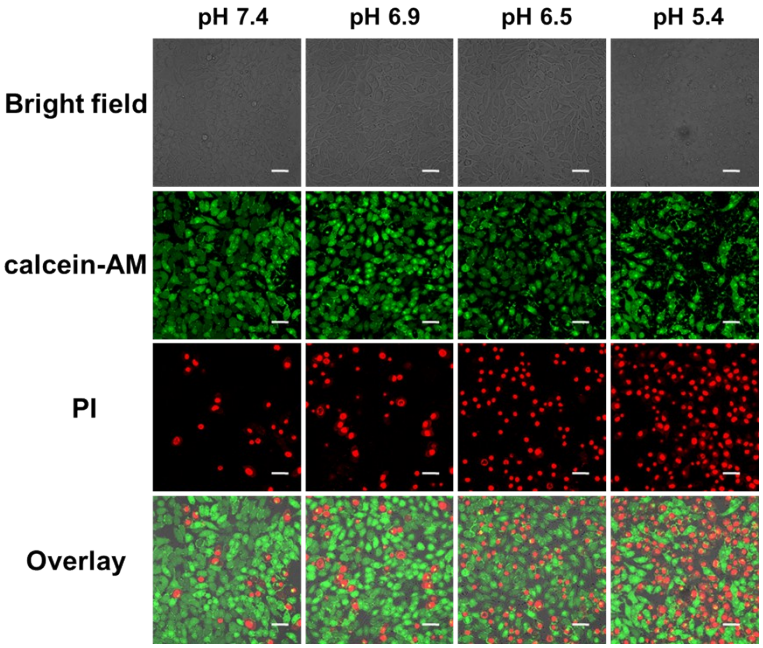

Fig. S14 Confocal imaging of calcein-AM/PI co-stained SMMC-7721 cells after incubation with Fe-MOF at different pH values. (H_2O_2: 100 μM). Scale bar: 20 μm.

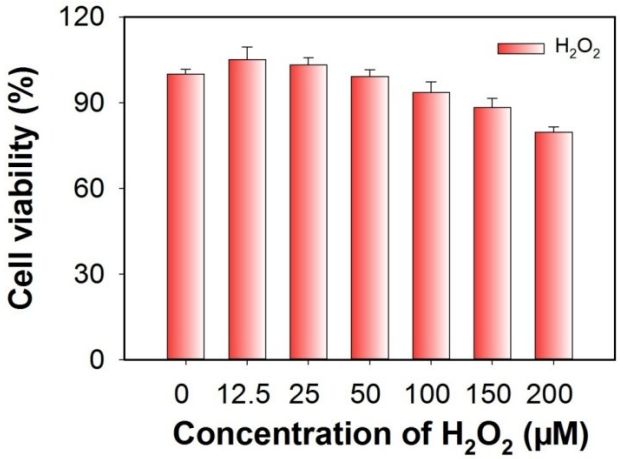

Fig. S15 Cytotoxicity of H_2O_2 with different concentration to SMMC-7721 cells.

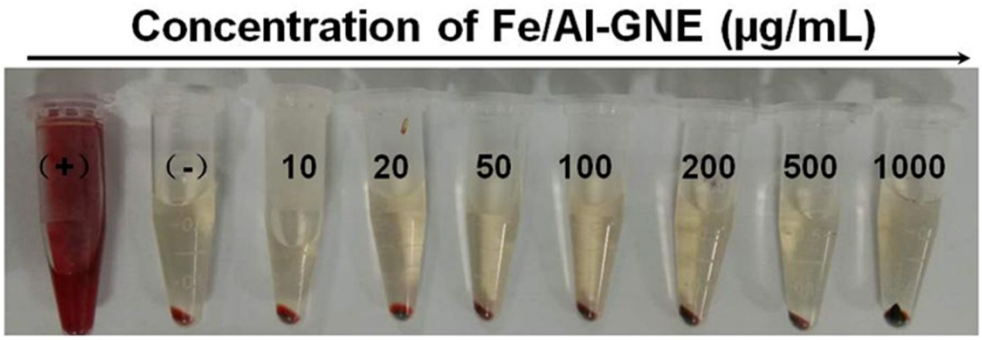

Fig. S16 Photographs of RBCs incubated with ultrapure water, PBS and Fe/Al-GNEs at different concentration for 3 h.

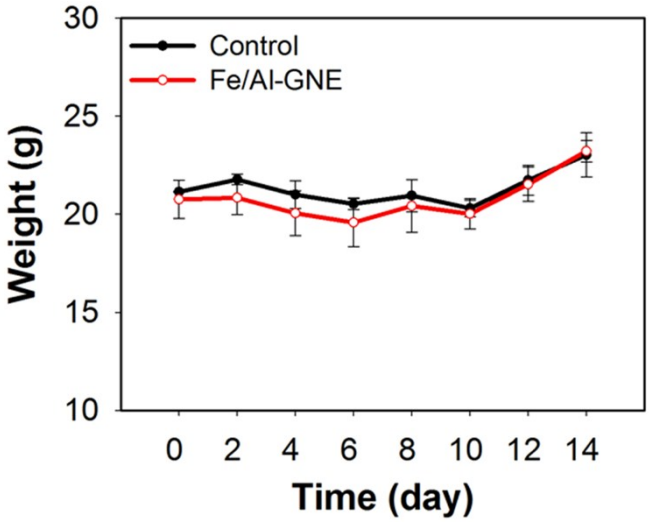

Fig. S17 Time curve of mice body weight change after different treatments.

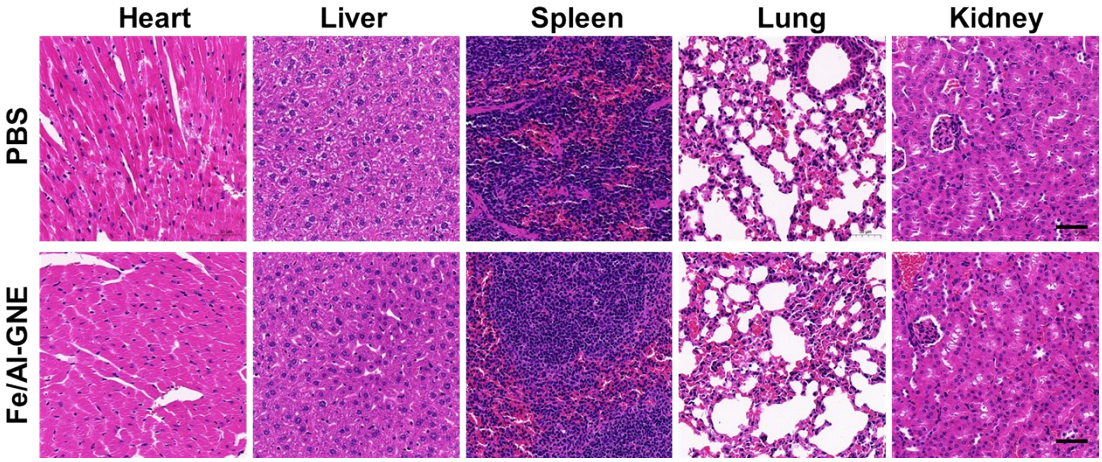

Fig. S18 H&E staining of major organs, including the heart, liver, spleen, lung, and kidney, collected from BALB/c nude mice after different treatments. Scale bar: 50 μm.

References

(1) Zhu, Q. L.; Xia, W.; Akita, T.; Zou, R. Q.; Xu, Q. *Adv. Mater.* **2016**, 28, 6391-6398.

(2) Zhang, J. W.; Zhang, H. T.; Du, Z. Y.; Wang, X. Q.; Yu, S. H.; Jiang, H. L. *Chem. Commun.* **2014**, 50, 1092-1094.

(3) Ji, P.; Drake, T.; Murakami, A.; Oliveres, P.; Skone, J. H.; Lin, W. *J. Am. Chem. Soc*, **2018**, 140, 10553-10561.