Supporting Information

A pH-responsive Ultrathin Cu-based Nanoplatform for Specific Photothermal and Chemodynamic Synergistic Therapy

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Materials and Methods

Materials. Copper nitrate hexahydrate ($Cu(NO_3)_2 \cdot 6H_2O$, >99.0%), iron nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$, >99.0%), sodium hydroxide (NaOH, >98.0%), sodium nitrate ($NaNO_3$, >99.0%), formamide and 3, 3', 5, 5'-tetramethylbenzidine (TMB, >99.0%) were obtained from Aladdin Reagent (Shanghai, China). Natural Glucose Oxidase (GOD) was from J&K Scientific (Beijing, China). Glutathione (GSH), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), terephthalic acid (TA), calcein acetoxymethyl ester (Calcein-AM), propidium iodide (PI), Annexin V-FITC & propidium iodide apoptosis detection kit were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and D-luciferin potassium were acquired from Solarbio Science &Technology Co, Ltd (Beijing, China). 0.25% trypsin-EDTA, RMPI medium 1640, high glucose Dulbecco's modified Eagles medium (DMEM), ciprofloxacin (CPFX), fetal bovine serum (FBS) were from Gibco (Invitrogen, Carlsbad, CA). 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was acquired from Shanghai Dibai Chemicals Technology Co., Ltd. (Shanghai, China), None of the chemicals were further purified. All the processes use ultrapure water from the Millipore system.

Synthesis of CuFe-LDH nanosheets. Firstly, $Cu(NO_3)_2 \cdot 6H_2O$ (0.4 mmol) and $Fe(NO_3)_3 \cdot 9H_2O$ (0.2 mmol) were dissolved in deionized water (40 mL) as solution A. Secondly, NaOH (0.5 mmol) was dissolved in another 40 mL deionized water to obtain solution B. Afterwards, $NaNO_3$ (0.2 mmol) was dissolved in deionized water containing 25% formamide (40 mL) to prepare solution C. At 80 °C, solution A and B were slowly added to solution C, with the pH value adjusted to 8.5 ± 0.3. Meanwhile, nitrogen (N_2) was injected into the drip process and stirred at room temperature for 1 h. Thirdly, the synthetic LDHs colloid was centrifuged three times with deionized water and ethanol at 6000 rpm. Then LDHs was dispersed in deionized water and centrifuged at 2000 rpm to obtain the supernatant.

Finally, dialysis was performed for 48 h to remove residual formamide, and the obtained CuFe-LDH nanosheets were stored in the N_2 environment for further use.

Synthesis of GOD/CuFe-LDH nanosheets. GOD (2 mg) was added to the CuFe-LDH (2 mg mL^{-1}, 10 mL) nanosheets suspension obtained above to slowly dissolve under magnetic stirring at 25 ^{o}C. After stirring for 12 h, the obtained suspension was centrifuged at 10000 rpm for 5 min to collect GOD/CuFe-LDH nanosheets, and then dispersed in deionized water for further use.

The photothermal effect of GOD/CuFe-LDH nanosheets. GOD/CuFe-LDH was mixed in buffer solution with pH = 7.4, 6.5 and 5.4. 1 mL GOD/CuFe-LDH suspension (100 μg mL^{-1}) at different pH conditions was taken and put into a transparent quartz vial, respectively. The vial was irradiated by NIR laser (808 nm, 1 W cm^{-2}) for a duration of 600 s. The thermal infrared imaging camera (Fluke Ti450, USA) was used to monitor the temperature change of each sample. To ensure the accuracy of the experiment, the heating/cooling process was repeated in the same vial. The photothermal effect of GOD/CuFe-LDH under pH=6.5 with various concentrations (25-200 μg mL^{-1}) was measured by the same method.

Detection of hydroxyl radical (·OH). TA can react with ·OH, and the reaction product (2-hydroxyterephthalic acid) can produce unique fluorescence at the wavelength of 425 nm. Mixing CuFe-LDH (200 μL, 500 μg mL^{-1}), H_2O_2 (20 μL, 100 mM) with TA (200 μL, 30 mM), the generation of ·OH was indirectly detected by measuring the fluorescence intensity at 425 nm. In addition, the influence of temperature on CDT efficiency was studied when the sample was in 50 ℃ water bath. The excitation wavelength was 315 nm and the slit width of excitation and emission was 2.5 nm. The fluorescence emission spectra of each sample were monitored every 1 min. When 200 μL of GOD/CuFe-LDH (500 μg mL^{-1}) and 20 μL of β-D-glucose (100 mM) were mixed with TA (200 μL, 30 mM), the produced ·OH was measured in the same way as above. Electron paramagnetic resonance spectrometer (ESR) was used to

further confirm the generation of ·OH. 200 μL of DMPO (90 mM) was added into CuFe-LDH (200 μL, 150 μg mL^{-1}) in the pH of 7.4, 6.5 and 5.4, followed by the addition of 200 μL H_2O_2 (30 mM), and then ·OH was detected by ESR. For GOD/CuFe-LDH, H_2O_2 was replaced with β-D-glucose. During the measurement, the parameters of ESR were described as follows: the scanning width is 200 G, the microwave frequency is 9.873 GHz, the microwave power is 2.015 mW and the sweep time is 41.9 s.

Michaelis–Menten kinetics test. The ·OH generated by the Fenton reaction of GOD/CuFe-LDH (200 μL, 500 μg mL^{-1}) with varied H_2O_2 or β-D-glucose concentration can have a chromogenic reaction with TMB (1 mL, 3.2 mM), and the Michaelis–Menten kinetics of GOD/CuFe-LDH was determined by measuring the TMB absorbance at λ = 650 nm. NaAc buffer solution was used to provide the environment of pH = 6.5. By plotting the initial velocities against H_2O_2 and β-D-glucose concentrations, the Michaelis–Menten kinetic curve of GOD/CuFe-LDH was acquired. Then, the Michaelis–Menten constant (K_M) and maximal velocity (V_{max}) were calculated *via* Lineweaver–Burk plotting.

Consumption of GSH. The reaction of GOD/CuFe-LDH with GSH was monitored by UV-vis spectra. DTNB solution (2 mg mL^{-1}, 200 μL), GOD/CuFe-LDH (100 μg mL^{-1}, 25, 50, 100 and 200 μL) and GSH aqueous solutions (10 mM, 200 μL) were added to deionized water (1.575, 1.55, 1.5 and 1.4 mL, respectively). After reaction for 30 min, the excessive GOD/CuFe-LDH was centrifuged and the supernatant absorbance at 412 nm was measured with a UV-vis spectrophotometer.

Cell culture. Hela, HepG2 and U87 cells were acquired from the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Beijing, China). Among them, Hela cells were incubated in the media of RMPI 1640 with 10% FBS and 10 μg mL^{-1} CPFX, while HepG2 and U87 cells were cultured with DMEM containing 10% FBS and 10 μg mL^{-1} CPFX. Three kinds of cells were cultured in 25 cm^2 cell-culture flask respectively and kept in the

incubator with an atmosphere of 5% CO_2 and constant temperature of 37 °C. The cells were separated from the culture bottle by adding 1 mL of trypsin (0.25%) for 2–3 min after reaching 90% confluence, and half of the cells were subcultured for the subsequent experiment by adding fresh DMEM or RMPI 1640 medium.

***In vitro* MTT assay.** To study the biocompatibility of GOD/CuFe-LDH, Hela, HepG2 and U87 cells (1×10^4 cells/well) were inoculated into 96-well plates and cultured at 37 °C with 5% CO_2 for 24 h, respectively. Subsequently, GOD/CuFe-LDH with a serial concentration of 10, 20, 50, 100 and 200 μg mL^{-1} was added into the 10% FBS containing DMEM or RMPI 1640 medium and then inoculated into 96-well plates for another 24 h incubation. After washing with PBS, the cell viability was measured by the standard methyl thiazolyl tetrazolium (MTT) assay. As for *in vitro* cytotoxicity assays, Hela cells were incubated with 200 μL of various samples including PBS, CuFe-LDH, GOD/CuFe-LDH at pH=7.4 and 6.5 with/without irradiation, respectively, and the concentration was set from 3.125 to 50 μg mL^{-1}. The pH value was adjusted to 6.5 by adding HCl. MTT assay was also used to determine the cell survival rate.

Calcein-AM/PI staining. To further validate and visualize the MTT results, Calcein-AM/PI was applied to stain viable and necrotic cells according to the instructions of the annexin V-FITC and PI staining kit. Briefly, Hela cells (1×10^5 cells/well) were seeded into 6-well plates and incubated for 24 h, followed by incubation with 2 mL, 50 μg mL^{-1} of PBS, CuFe-LDH, GOD/CuFe-LDH at pH=7.4 and 6.5 with/without irradiation for 24 h, respectively. After irradiation with 808 nm laser (1.0 W cm^{-2}) for 10 min, Calcein-AM (2 mL, 5 μg mL^{-1}) and PI (2 mL, 10 μg mL^{-1}) were adopted to stain the cells for 30 min, then the cells were washed thoroughly with PBS and observed with a Leica fluorescence microscope.

Generation of intracellular ROS. Hela cells were cultured in CLSM-exclusive culture disks (5×10^4 cells mL^{-1}) for 24 h. Subsequently, 2 mL RMPI 1640 medium (at pH=7.4 and 6.5)

containing 50 μg mL^{-1} of CuFe-LDH and GOD/CuFDHs was added into the disks for 6 h incubation and subsequently Hela cells were stained with DCFH-DA (2 mL, 5 μg mL^{-1}) for another 30 min. Finally, the cells were washed with PBS twice, and the produced ROS was detected qualitatively *via* confocal laser scanning microscopy and quantitatively with a flow cytometer.

Animal experiments. Female Balb/c-nude mice (4–6 weeks old, ~20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, and all the animal procedures were performed by following the protocols approved by China-Japan friendship Hospital Animal Research Center. The tumor model was established by subcutaneously inoculating 1×10^7 Hela cells (suspended in 100 μL of PBS). The tumor volume reached 80 mm^3 before all the experiments started.

***In vivo* antitumor therapy.** The mice bearing Hela tumors were randomly separated into three groups (each group of six) and injected intravenously with the following samples (200 μL): (Group 1) PBS + L, (Group 2) GOD/CuFe-LDH (1 mg kg^{-1}), (Group 3) GOD/CuFe-LDH + L (1 mg kg^{-1}). The tumor size and body weight of mice were recorded every 2 days for 16 days of treatment. The tumor volume was calculated as: $Volume = tumor\ length \times tumor\ width^2 \times 0.5$

The relative tumor volume was V/V_0 (V and V_0 represent the tumor volume measured at day t and day 0, respectively).

The bioluminescent imaging (BLI) of mice inoculated with *in-situ* tumors was used to further investigate the anticancer effect *in vivo*. The mice were randomly separated into three groups (3 mice per group) and injected intravenously with the following samples (200 μL): (Group 1) PBS + L, (Group 2) GOD/CuFe-LDH (1 mg kg^{-1}), (Group 3) GOD/CuFe-LDH + L (1 mg kg^{-1}). After 24 hours, the mice were intraperitoneally injected with d-fluorescein (100 μL, 15

mg mL^{-1}) and anesthetized for 5-10 min. The anesthetized mice were placed in the imaging chamber and photographed.

***In vivo* biodistribution**. The mice were intravenously injected with GOD/CuFe-LDH and sacrificed after 4, 8, 12, 24 and 48 h post-injection. Then their tumors and major organs including heart, liver, spleen, lung, and kidneys were collected and washed with PBS. After all tissues were weighted and homogenized, the biodistributions of GOD/CuFe-LDH in different organs and tumors were obtained by measuring Cu concentration *via* ICP-AES.

Histology examination. On the 16th day post-injection, all of the mice were euthanized. The main tissues (hearts, livers, spleens, lungs, and kidneys) and tumors were dissected and preserved with a 4% paraformaldehyde solution for histology analysis. After hematoxylin and eosin (H&E) staining, all the slices were observed with a Leica digital microscope.

Statistical Analysis. Significant differences between two means were performed using a two-sided Student's t-test with $*p < 0.05$, $**p < 0.01$.

Sample characterization. The crystalline structure of CuFe-LDH was studied by powder X-ray diffraction (XRD) patterns (Shimadzu XRD-6000 diffractometer, Cu Kα radiation source). The morphology of CuFe-LDH and GOD/CuFe-LDH were investigated on a high-resolution transmission electron microscope (HRTEM) images (JEOL, JEM-2100, accelerating voltage: 200 kV). The thickness of CuFe-LDH was recorded by the atomic force microscope (AFM, Veeco, NanoScope IIIa) in tapping mode. X-ray photoelectron spectrometer (PHIQ2000, Al K X-ray source) was adopted to research the XPS spectrum of samples. The hydrodynamic diameters and zeta potential of CuFe-LDH and GOD/CuFe-LDH in water were measured by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments, U.K.). Inductively coupled plasma atomic emission spectroscopy (ICP-AES, Shimadzu ICPS-7500) was used to determine the chemical composition of CuFe-LDH. Fourier transform infrared (FT-IR) spectra were recorded from 4000 to 400 cm^{-1} in 2 cm^{-1} resolutions on a Vector 22 (Bruker) spectrophotometer. Shimadzu U-3000 spectrophotometer (slit width: 1.0 nm) was applied to

record UV-vis-NIR absorption spectra in the range from 200 to 900 nm. ESR spectrum was obtained by the Bruker EMX1598 spectrometer. Cellular fluorescence images were obtained by Leica confocal laser scanning microscopy (Leica DM6000M, Germany). Flow cytometer (MoFlo XDP, Beckman Coulter) was used to perform the *in vitro* cellular fluorescence quantity. An automated hematology analyzer (Bayer Advia 2120) was applied for serum biochemistry analysis, and an automatic biochemical analyzer (Olympus AU400) was used for blood cell counts.

Table S1. Comparison of photothermal conversion efficiency (PCE) of nanomaterials.

Nanomaterials	**PCE**	**Reference**
NdNP@PB	60.8%	*Angew. Chem. Int. Ed.* **2019**, *58*, 8536–8540
QDI-NPs	64.7 ± 4%	*Angew. Chem. Int. Ed.* **2019**, *58*, 1638–1642
BSA-IrO_2 NPs	67.8%	*Angew. Chem. Int. Ed.* **2018**, *57*, 10309–10313
Bi_2S_3-Au NRs	51.06%	*Angew. Chem. Int. Ed.* **2018**, *57*, 246–251
CNTR@AuNP	76%	*J. Am. Chem. Soc.* **2016**, *138*, 7005−7015
Bi cluster	39.67%	*Adv. Funct. Mater.* **2019**, *29*, 1900017
Zr-FeP MOF	33.7%	*Adv. Funct. Mater.* **2018**, *28*, 1804634
SP NP	62.3%	*Adv. Funct. Mater.* **2017**, *27*, 1605094
PTIGSVS NPs	74%	*Adv. Funct. Mater.* **2018**, *28*, 1800135

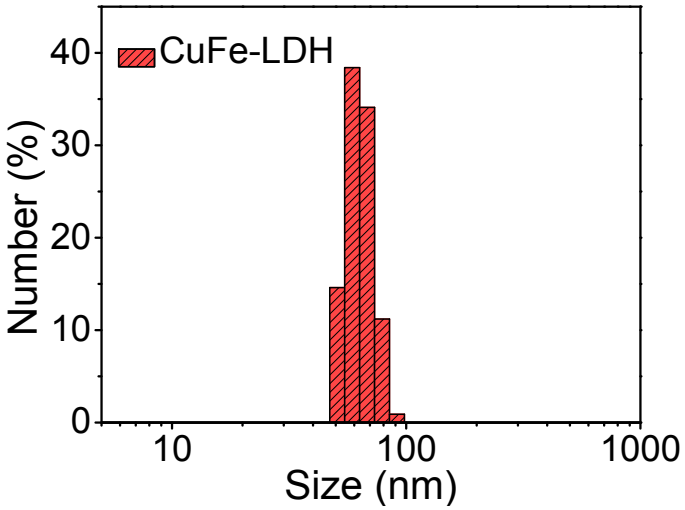

Figure S1. The size distribution of CuFe-LDH in water.

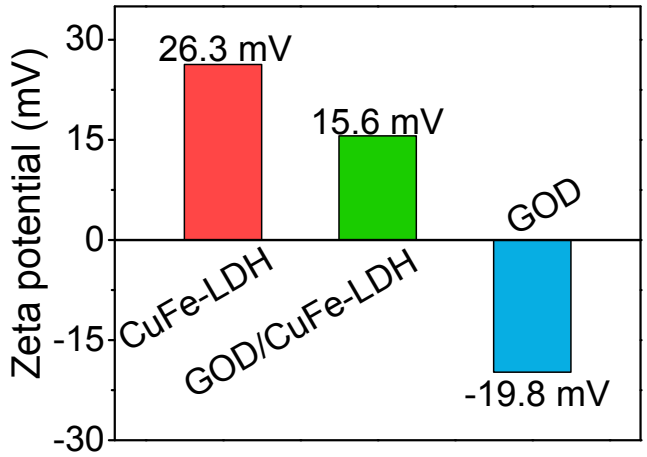

Figure S2. Surface zeta potential of CuFe-LDH, GOD and GOD/CuFe-LDH.

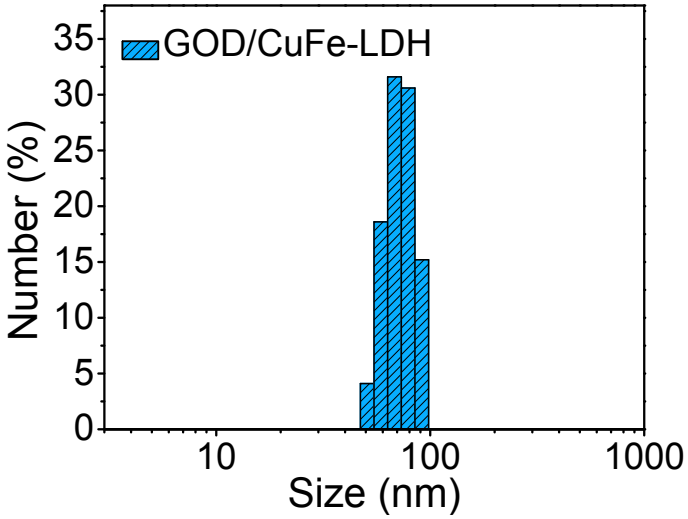

Figure S3. The size distribution of GOD/CuFe-LDH in water.

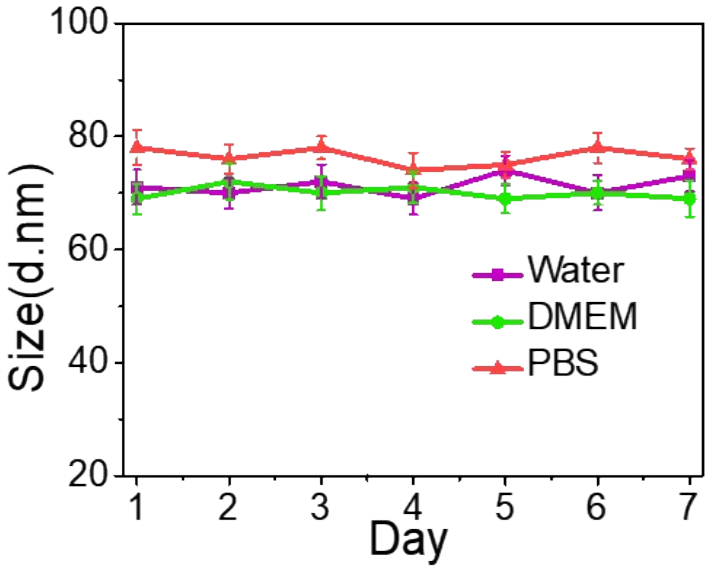

Figure S4. The size distribution of GOD/CuFe-LDH in water, DMEM, PBS within one week.

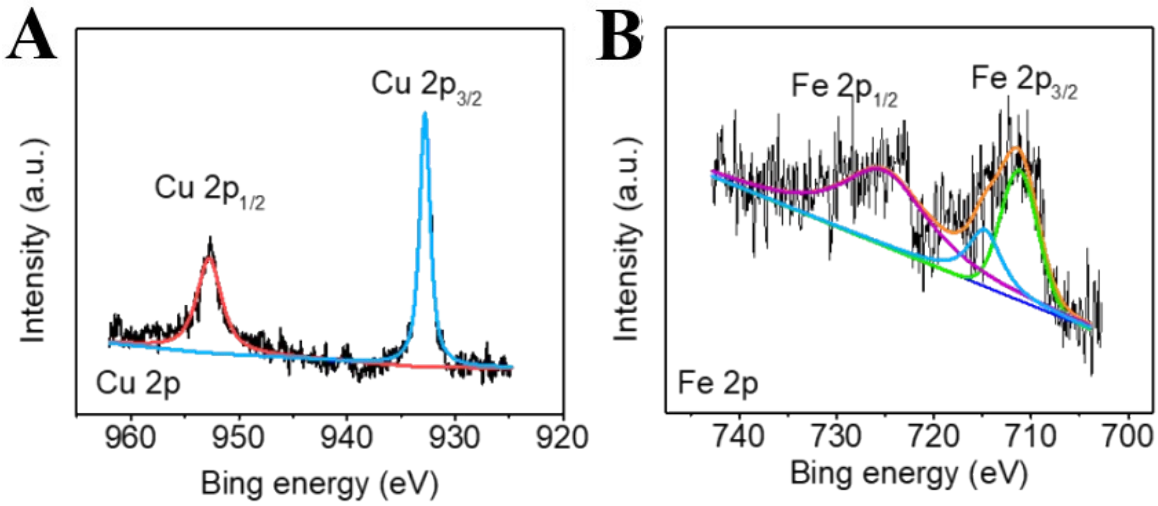

Figure S5. XPS spectra of (A) Cu 2*p* and (B) Fe 2*p* in CuFe-LDH after the reaction with GSH.

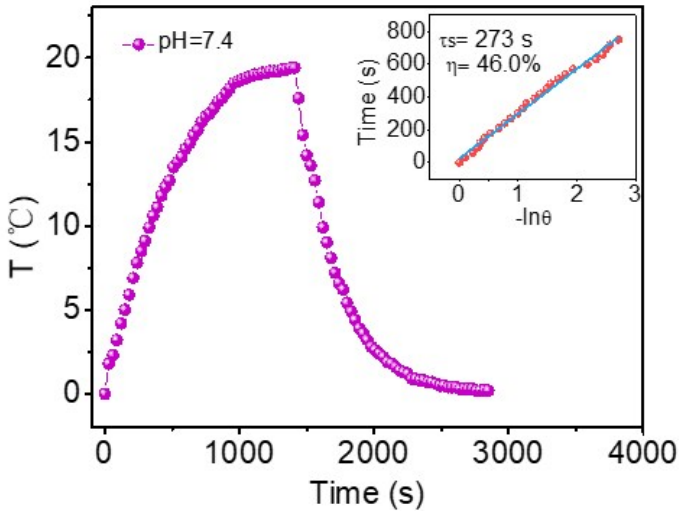

Figure S6. Calculation of the photothermal-conversion efficiency under 808 nm irradiation at pH 7.4.

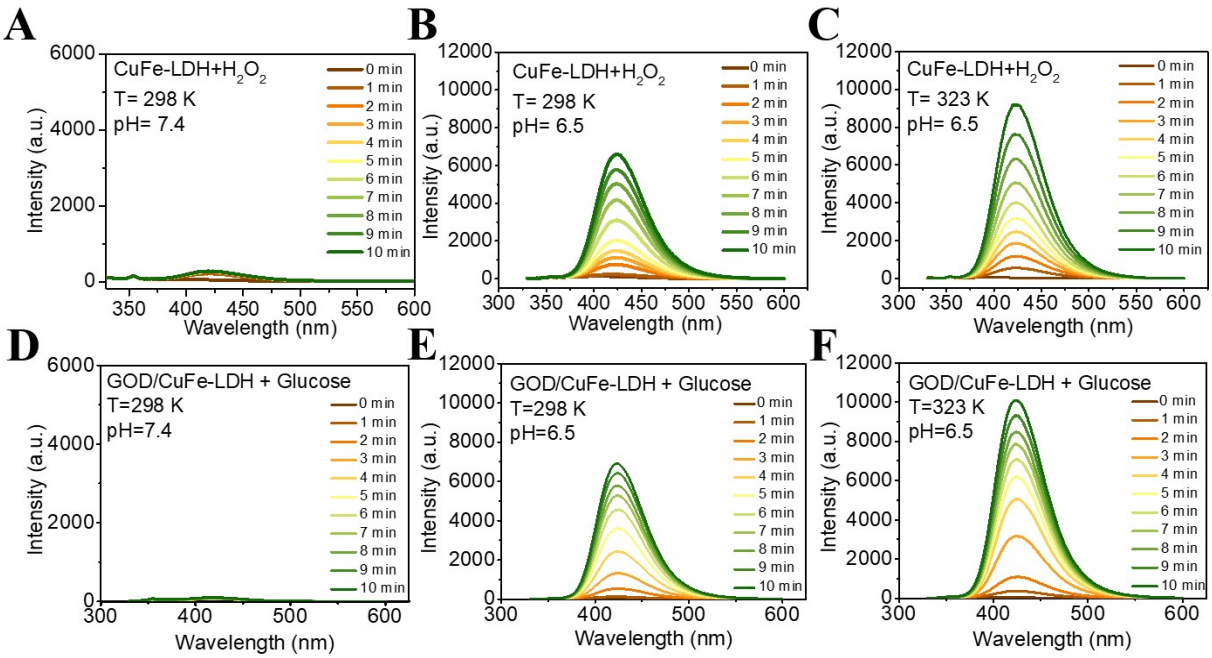

Figure S7. The reaction of TA with the ·OH-induced enhancement of fluorescence showing ·OH generation from the Fenton-like reaction between CuFe-LDH and H_2O_2 at pH = 7.4 (A), 6.5 (B), 6.5 + 323 K (C). Fluorescence enhancement showing ·OH generation from the cascaded reaction between GOD/CuFe-LDH and glucose at pH = 7.4 (D), 6.5 (E), 6.5 + 323 K (F).

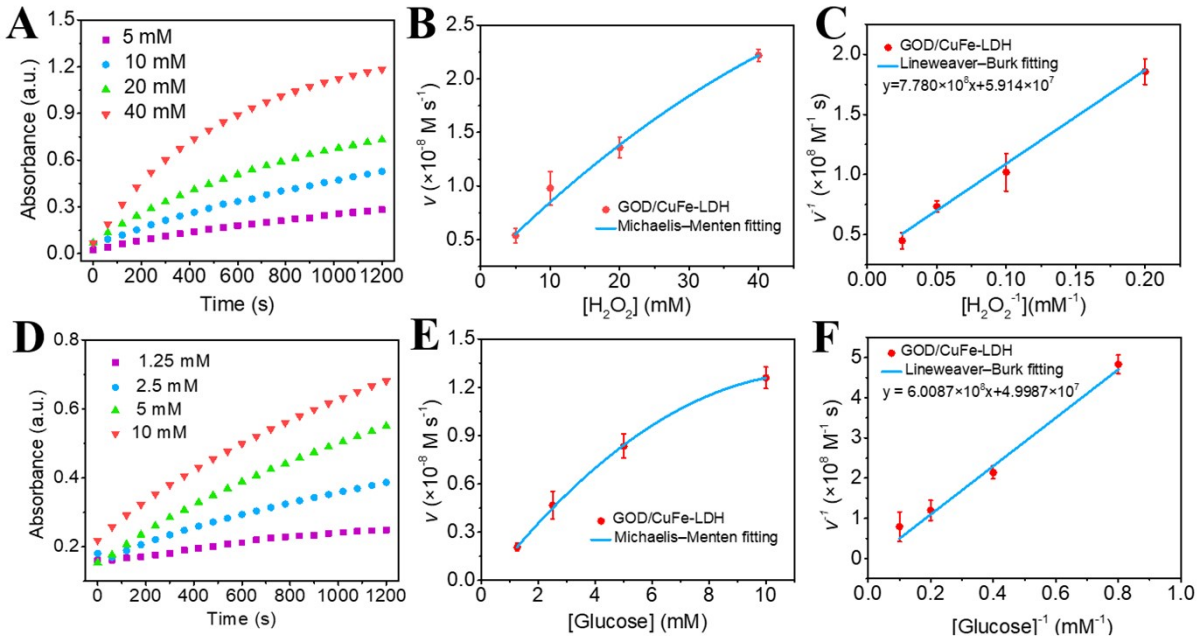

Figure S8. (A) The absorbance of GOD/CuFe-LDH (50 μg mL^{-1}) with the addition of varying concentrations of H_2O_2, measured at λ = 650 nm *via* TMB assay. (B) Michaelis-Menten steady-state kinetics by plotting reaction velocity (C) against H_2O_2 concentration. (D) The absorbance of GOD/CuFe-LDH (50 μg mL^{-1}) with the addition of varying concentrations of glucose. (E) Michaelis-Menten steady-state kinetics by plotting reaction velocity (F) against glucose concentration.

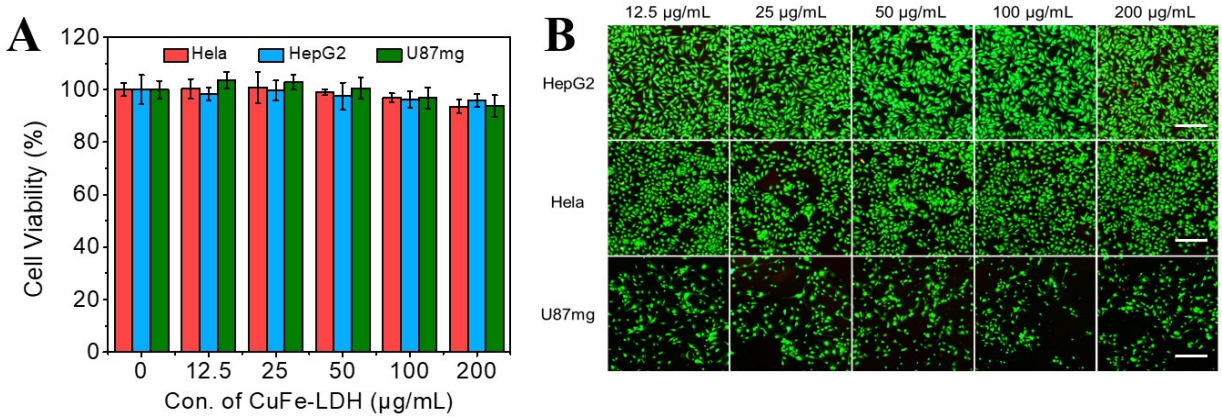

Figure S9. (A) Cell viability of Hela, HepG2 and U87mg cells incubated with various concentrations of GOD/CuFe-LDH. (B) Calcein-AM/PI staining of Hela, HepG2 and U87mg cells incubated with various concentrations of GOD/CuFe-LDH. Scale bars are 200 μm.

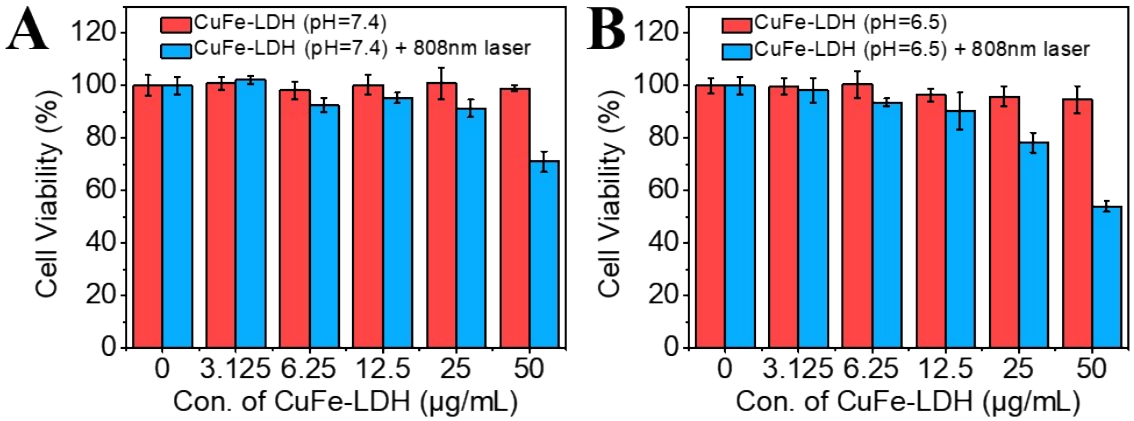

Figure S10. Relative viabilities of Hela cells incubated with CuFe-LDH under (A) neutral (pH = 7.4) and (B) acidic (pH = 6.5) conditions with and without 808 nm laser irradiation at the power density of 1.0 W cm^{-2} for 10 min.

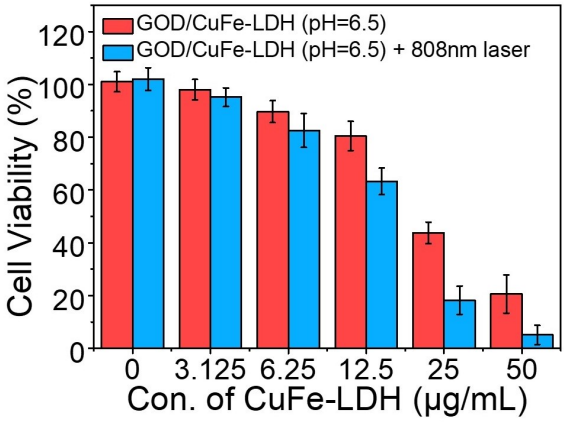

Figure S11. Relative viabilities of normal cells (Cos-7) incubated with GOD/CuFe-LDH under acidic (pH = 6.5) condition with and without 808 nm laser irradiation at the power density of 1.0 W cm^{-2} for 10 min.

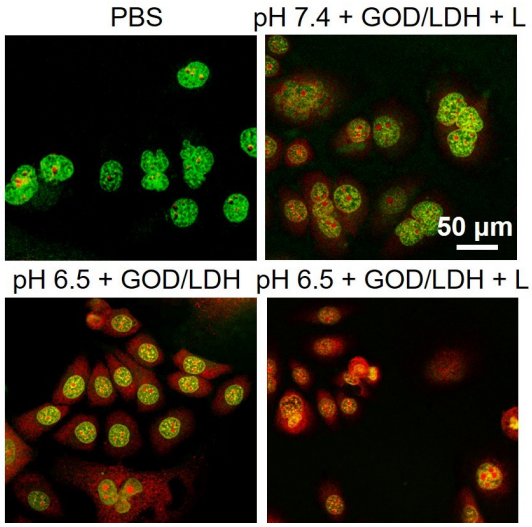

Figure S12. CLSM images of AO staining for lysosomal integrity after different treatments. (Scale bar: 50 μm).

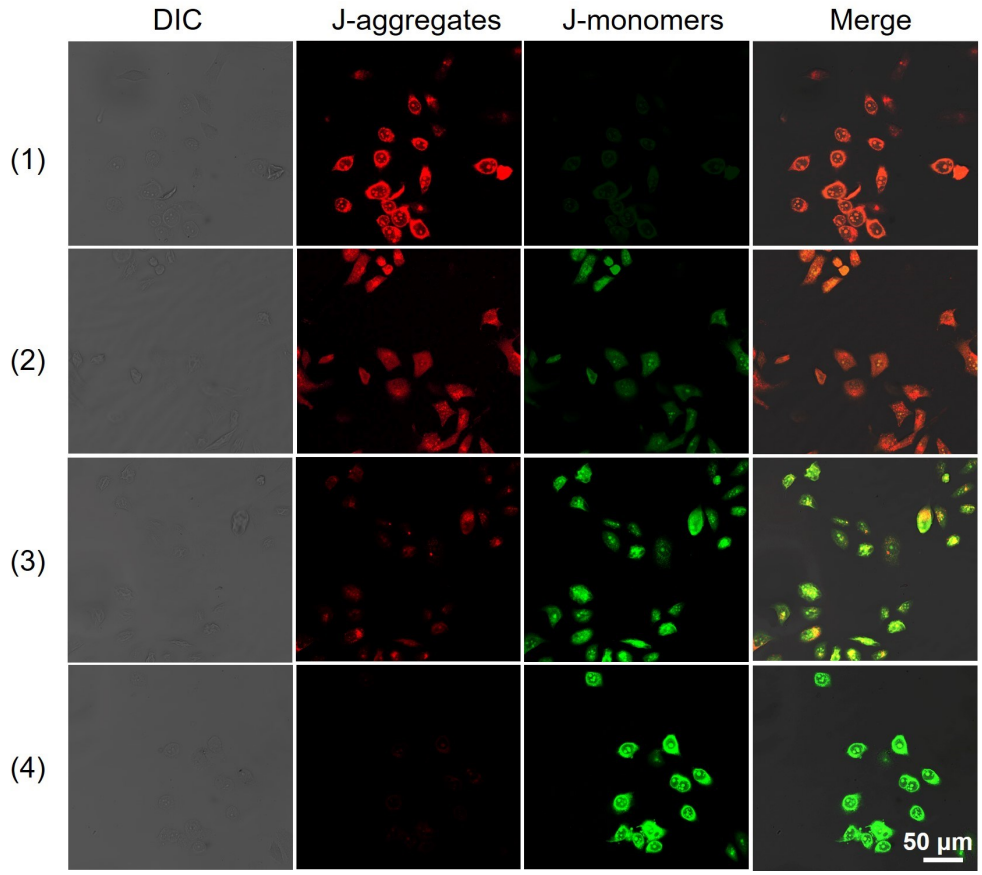

Figure S13. Mitochondrial depolarization of Hela cells indicated by the fluorescence of JC-1 aggregates and monomers in CLSM images: (1) PBS, (2) pH 7.4 + GOD/CuFe-LDH (50 μg mL^{-1}) + NIR (1.0 W cm^{-2}, 10 min), (3) pH 6.5 + GOD/CuFe-LDH (50 μg mL^{-1}), (4) pH 6.5 + GOD/CuFe-LDH (50 μg mL^{-1}) + NIR (1.0 W cm^{-2}, 10 min). (Scale bar: 50 μm).

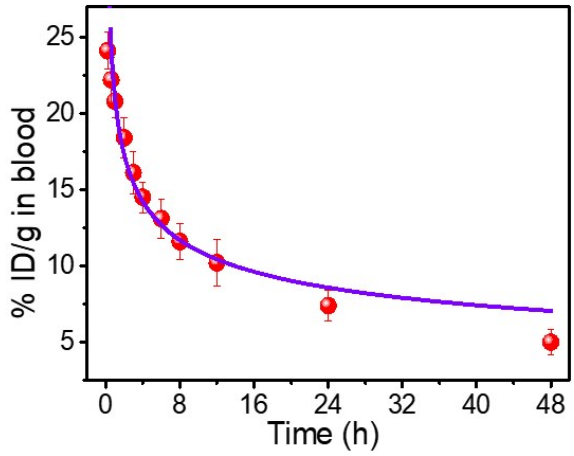

Figure S14. Blood circulation curve of GOD/CuFe-LDH determined by Cu concentration in the blood of mice at different time points post-injection.

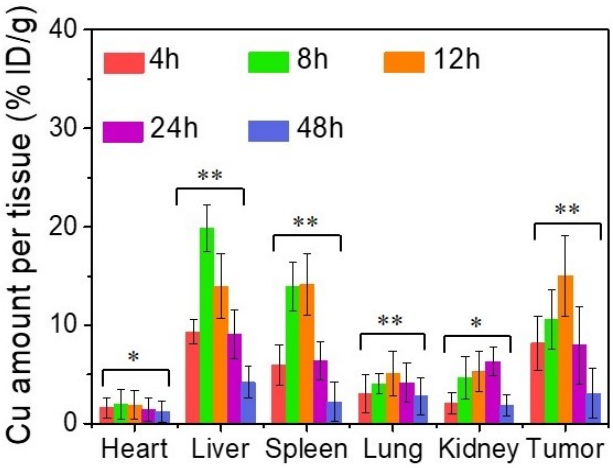

Figure S15. Time-dependent distribution of GOD/CuFe-LDH at various organs by measuring Cu concentration (*$p < 0.05$, **$p < 0.01$).

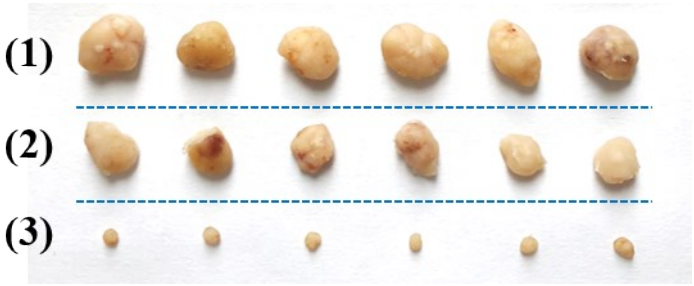

Figure S16. Digital photographs of the dissected tumors from each group in 16 days of therapies.

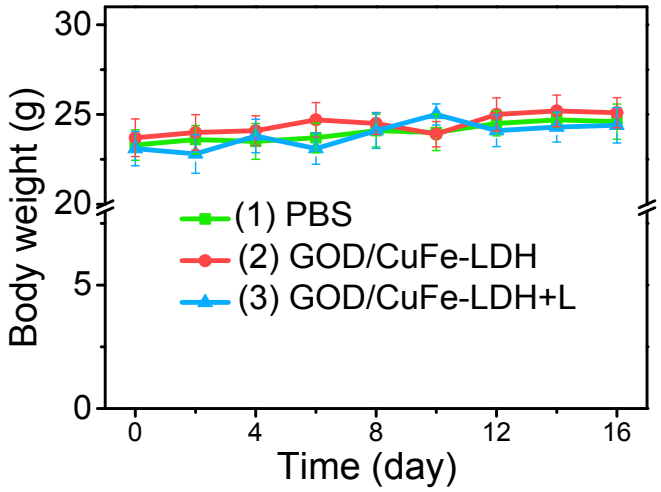

Figure S17. Body weight change of Hela tumor-bearing mice after various treatments as a function of time.

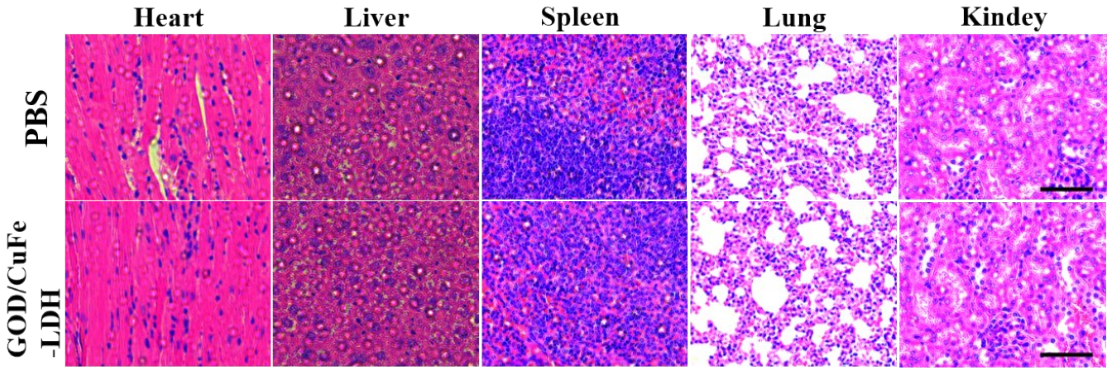

Figure S18. Histological images of major organs collected on day 16 treated with PBS and GOD/CuFe-LDH, respectively. Scale bars are 100 μm.

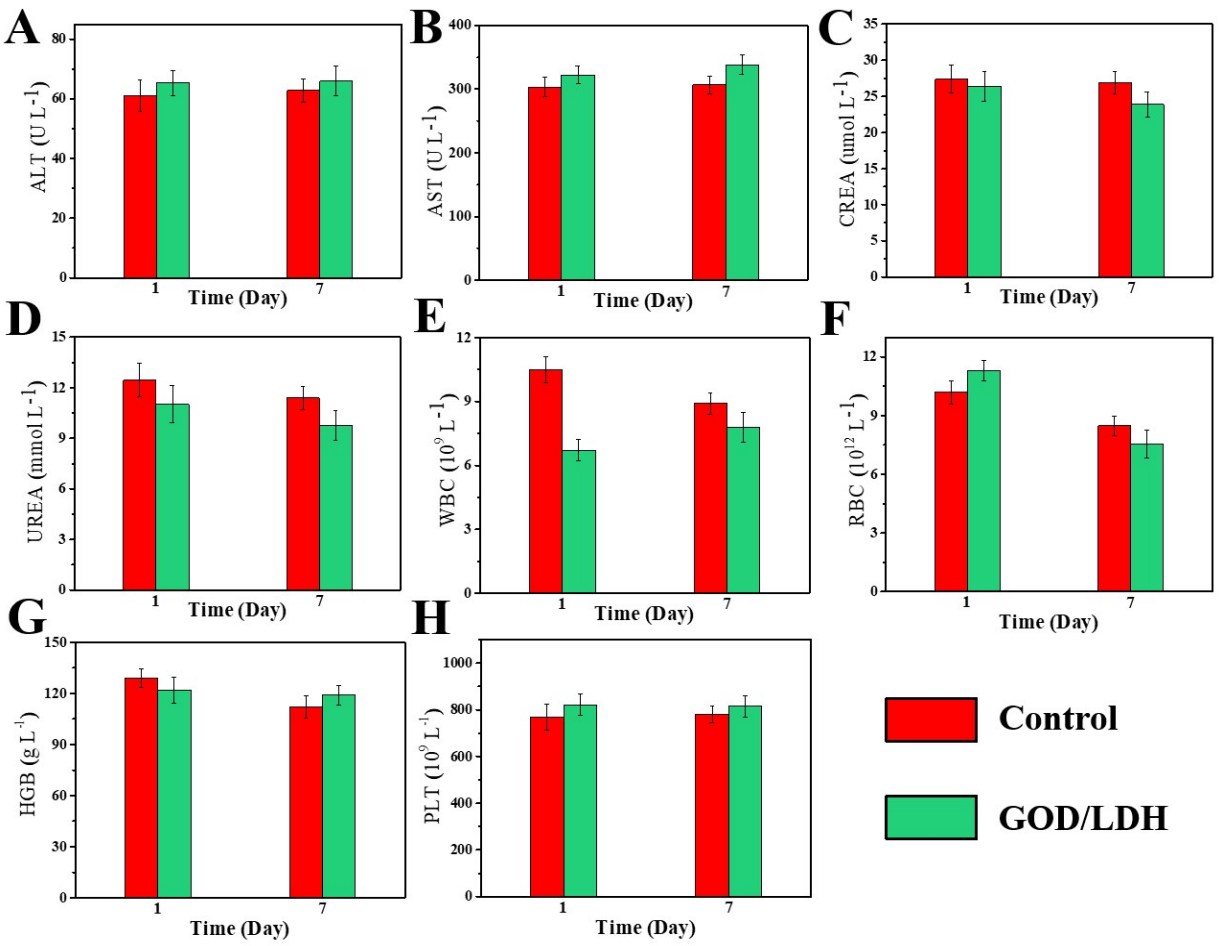

Figure S19. Kidney, liver function markers and blood cell counts of the nude mice bearing Hela tumors were detected after injection of saline (control) and GOD/CuFe-LDH at Day 1 and Day 7, respectively.

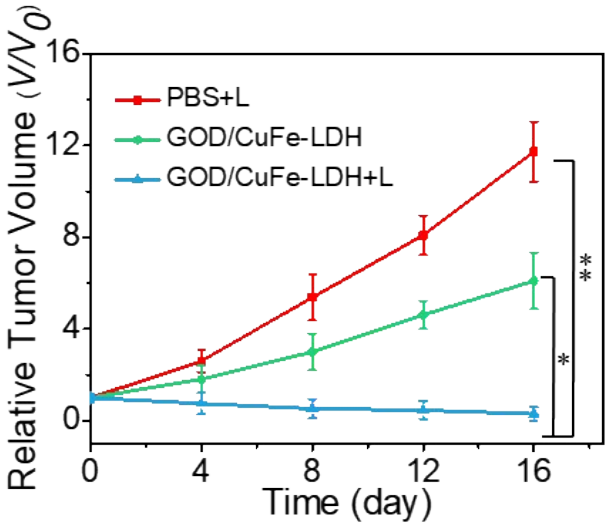

Figure S20. Relative Tumor Volume (V/V_0) of mice were monitored for 16 days (*p < 0.05, **p < 0.01).

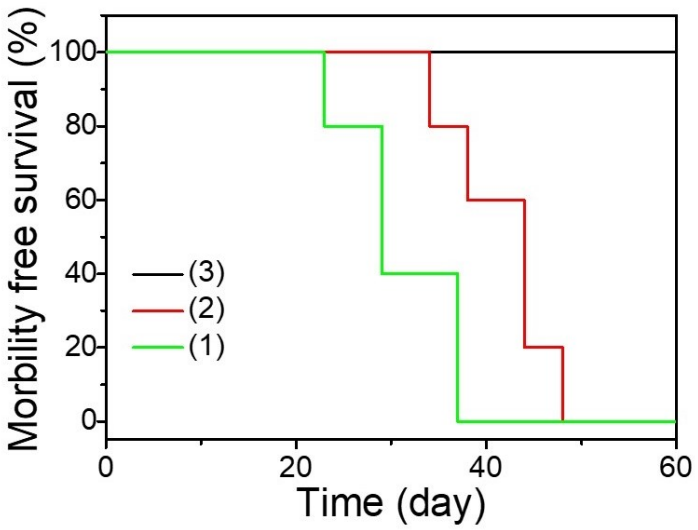

Figure S21. Morbidity free survival of different groups of mice after various treatments (n = 10 per group): (1) PBS + NIR, (2) GOD/CuFe-LDH, and (3) GOD/CuFe-LDH + NIR.