Supporting Information

Bismuth Nanoclusters on Nitrogen-doped Porous Carbon Nanoenzyme for Cancer Therapy

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Chemicals

Zinc nitrate hexahydrate ($Zn(NO_3)_2·6H_2O$, 99%), 2-methylimidazole, glutathione (GSH), 3,3',5,5'-tetramethylbenzidine (TMB), 5,5'-Dithio bis-(2-nitrobenzoic acid) (DTNB) were obtained from Aladdin. Methanol (MeOH), dimethyl sulfoxide (DMSO), and N, N-Dimethylformamide (DMF) were obtained from Macklin. H_2O_2 (30%) was purchased from Wokai Biotechnology Co., Ltd. Bismuth (III) nitrate pentahydrate ($Bi(NO_3)_3·5H_2O$, 98%) was purchased from Alfa Aesar. Acetoxymethylester of calcein (Calcein-AM), propidium iodide (PI), mitochondrial membrane potential assay kit with JC-1 and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Fluorescein Isothiocyanate (FITC) was obtained from Shanghai Yisheng Bio-Technology Co., Ltd.

Characterizations

Scanning electron microscopic (SEM) images were acquired on a Nova field emission microscope (NanoSEM 450). X-ray diffraction (XRD) was measured on XPert3 powder diffractometer. Transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM) images, energy dispersive X-ray spectroscopy (EDX) and elemental mapping were acquired with a JEM-2100F transmission electron microscope. Zeta potential (Smoluchowski model) and hydrodynamic diameters were measured via dynamic light scattering (DLS) on a BECKMAN Nanosizer (DelsaMax PRO). Raman spectroscopy were measured on a confocal Raman microscope (LabRAM HR Evolution). Ultraviolet-visible absorption spectroscopy (UV-Vis) was performed on a UV-3600 Shimadzu spectrometer. Infrared spectroscopy (FT-IR) measured on Bruker VERTEX80v infrared spectrometer. Electron spin resonance (ESR) was measured using a Brucker E 500 spectrometer.

Synthesis of Bi-NC

For synthesis of ZIF-NC, 1069 mg $Zn(NO_3)_2·6H_2O$ was dis-solved in 15 mL mixed solvent of DMF and methanol (v/v= 4:1). 1,161 mg 2-methylimidazole was dissolved in 10 mL mixed solvent of DMF and methanol. The two solution was mixed at 30 °C and sit for 24 h. The obtained ZIF-8 was collected by centrifugation at 15,000 rpm for 5 min, washed with methanol and dried at 70 °C for 5 h. Then, the ZIF-8 powder was heated to 900 °C in tube furnace, with a heating rate of 5 °C min^{-1} and kept at 900 °C under flowing nitrogen gas for 3 h. After cooled down to room tempera-ture naturally, ZIF-NC was obtained.

To obtain Bi-NC, 1 mmol of the as-obtained Zn-NC and 1 mmol $Bi(NO_3)_3$ $5H_2O$ were homogeneously dispersed in 75 mL mixed solvent of glycerin and methanol (v:v= 2:1) under ultrasonication. Subsequently,

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the mixture was sealed in a 100 mL Teflon-lined autoclave, maintained at 120 °C for a pre-determined time and then cooled naturally. The obtained Bi-NC was collected by centrifugation at 9,000 rpm for 5 min, washed with methanol for three times, and finally dried in a vacuum oven overnight.

Bi-NC was subsequently surface-modified with HA by electrostatic adsorption. Briefly, Bi-NC and HA were mixed in methanol, heated under reflux at 60 °C for 3 h, and con-centrated by rotary evaporator. Then, excess HA molecules were removed through dialysis in water for 24 hours, and Bi-NC@HA was obtained by lyophilization.

POD-like and GSHOx-like activity

In the presence of H_2O_2, the color change of TMB to ox-TMB induced by •OH was monitored to detect the POD-like activity of Bi-NC@HA. In brief, Bi-NC (200 μg mL^{-1}), H_2O_2 (10 mM) and TMB (100 μg mL^{-1}) were added to 2 mL of PBS solution (pH 6.5). The absorbance of oxTMB was rec-orded by a UV-3600 Shimadzu spectrometer.

To study GSHOx-like activity, Bi-NC@HA (200 μg mL^{-1}) was mixed with GSH (0.15 mM) in 2 mL of PBS solution (pH 6.5). At desired time points, the residual GSH was de-tected using 5,5'-Dithio bis-(2-nitrobenzoic acid) (DTNB) as the probe. The yellow product from the reaction of DTNB with the thiol group (−SH) of GSH was recorded via UV-Vis spectrophotometer.

Photothermal properties.

The Bi-NC@HA suspension (100 μL) with various concentrations (0, 50, 100, 200 μg mL^{-1}) was exposed to 808 nm laser (1.0 W cm^{-2}) for 10 min, and the temperature variation were monitored by a thermocouple. The photothermal conversion efficiency (η) of Bi-NC@HA can be calculated through the following formula:

The total energy input and dissipation from the system can be written as:

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = Q_{in,NP} + Q_{in,sys} - Q_{out} \qquad \text{(Eq. 1)}$$

where the terms $m_iC_{p,i}$ are products of mass and heat capacity of the system components, including NPs solution and cuvette. T is the solution temperature, $Q_{in,NP}$ is the energy absorbed by NPs, $Q_{in,sys}$ is the energy inputted to the sample cell in the absence of NPs, and Q_{out} is heat dissipation of the system.

The heat input term $Q_{in,NP}$ can be expressed as:

$$Q_{in,NP} = I(1 - 10^{-A_{808}})\eta \qquad \text{(Eq. 2)}$$

where I is incident laser power in W, η is the photothermal transduction efficiency,

and A_{808} is the absorbance of NPs at specific wavelength 808 nm.

The system heat dissipation term Q_{out} is linear with system temperature:

$$Q_{out} = hS(T - T_{surr}) \qquad \text{(Eq. 3)}$$

where h is heat transfer coefficient, S is the exposed surface area of the cuvette, and T_{surr} is the ambient temperature of the surroundings. At the system equilibrium, solution temperature reaches T_{max}, and the heat input and output are balanced:

$$Q_{in,NP} + Q_{in,sys} = Q_{out} = hS(T_{max} - T_{surr}) \qquad \text{(Eq. 4)}$$

When the light is turned off, the heat input terms become zero, Eq. 1 becomes

$$\sum_i m_i C_{p,i} \frac{dT}{dt} = -Q_{out} = -hS(T - T_{surr}) \qquad \text{(Eq. 5)}$$

rearranging Eq. 5 would give

$$dt = -\frac{\sum_i m_i C_{p,i}}{\tau} \frac{dT}{(T - T_{surr})} \qquad \text{(Eq. 6)}$$

and by integration, Eq. 6 becomes

$$t = -\frac{\sum_i m_i C_{p,i}}{\tau} \ln \frac{T - T_{surr}}{(T_{max} - T_{surr})} \qquad \text{(Eq. 7)}$$

A system time constant τ_s is defined as:

$$\tau_s = \frac{\sum_i m_i C_{p,i}}{hs} \qquad \text{(Eq. 8)}$$

and a dimensionless term θ is introduced:

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}} \qquad \text{(Eq. 9)}$$

$$t = -\tau_s \ln \theta \qquad \text{(Eq. 10)}$$

Substituting Eq. 8 and 9 into Eq. 7 gives:

$$Q_{in,sys} = hS\left(T_{max,H_2O} - T_{surr}\right) \qquad \text{(Eq. 11)}$$

Thus, the time constant for system heat transfer τ_s can be determined by linear regression of the time data *vs.* negative natural logarithm of θ.

$Q_{in,sys}$ can be measured directly as:

$$Q_{in,NP} = I(1 - 10^{-A_{808}})\eta = hS\left(T_{max} - T_{max,H_2O}\right) \qquad \text{(Eq. 12)}$$

and Eq. 4 can be rewritten as:

$$\eta = \frac{hS(T_{max} - T_{max,H_2O})}{I(1 - 10^{-A_{808}})} \qquad \text{(Eq. 13)}$$

With τ_s = 392.54 s obtained by fitting (Fig. 2i), m is 1.0 g and the C is 4.2 J/g, hS is calculated to be 8.26 mW/°C. Substituting I = 1.766 W, A_{808} = 0.443, T_{max}-$T_{max,H2O}$ = 37.3 °C into Eq. 13, the photothermal transduction efficiency is determined to be 35.34%.

Cell Culture

4T1 murine breast cancer cells were originally obtained from American Type Culture Collection (ATCC). The 4T1 cells were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM, Gibco) medium supplemented with 10% fetal bovine serum (FBS, Gibco), 1% streptomycin, and 1% penicillin and cultured at 37 °C in a thermostatic incubator (ThermoFisher) with 5% CO_2.

Cytocytotoxicity

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate the cytotoxicity of different treatment groups (Control, Laser, Bi-NC@HA, and Bi-NC@HA+Laser). The cells were seeded in 96-well plates at a density 1×10^4 cells per well and cultured overnight. Then, the fresh acidified medium (pH=6.5) containing different concentrations of Bi-NC@HA was added and incubated with the cells for 4 h. For the Laser Bi-NC@HA+Laser groups, the cells were irradiated under 808 nm NIR laser for 3 min (1.0 W cm^{-2}). The cells were further incubated for another 24 h. The relative cell viabilities were measured using the standard MTT assay.

Cellular uptake and lysosomal co-staining

The intracellular distribution of Bi-NC@HA was studied by CLSM. First, 4T1 cells were seeded on glass-bottom dishes and cultured for 24 h. Subsequently, FITC-labelled Bi-NC@HA (50 μg mL^{-1}) was added to the

wells and incu-bated for 12 hours. Cells were then washed three times with PBS and incubated with Lyso-Tracker Red probe (100 nM) for 1 h to stain the lysosomes. Finally, CLSM was used to visualize the cells.

Cell Living-Dead Staining

The 4T1 cells were seeded on glass-bottom dishes and cul-tured for 24 h. After that, the old medium was replaced by fresh acidified medium (pH=6.5) containing Bi-NC@HA and sequentially incubated for 4 h. For the Laser Bi-NC@HA + Laser groups, the cells were irradiated under 808 nm NIR laser for 3 min (1.0 W cm^{-2}). After 24 h, the cells were washed for three times with PBS, followed by staining with Calcein-AM and PI for 15 min. The stained cells were analyzed by confocal laser scanning microscopy (CLSM).

Intracellular ROS Detection

2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect the intracellular ROS level. After different treatments, the cells were stained with DCFH-DA (10 μM) for 15 min. The stained cells were analyzed by CLSM.

JC-1 staining

After different treatments, the cells were stained with 2 μM 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) for 20 min. The stained cells were analyzed by CLSM.

Hemolysis assay

Hemolysis assay were performed by dispersing different concentrations of Bi-NC@HA (25, 50, 100, 200, 400 and 800 μg/mL) in PBS and then the suspension was added to the red blood cells of ICR mice. Water and PBS were used as positive and negative controls, respectively. The mixture was allowed to stand at room temperature for 4 hours and the supernatant was collected by centrifugation at 3500 rpm for 5 minutes. The absorbance at 540 nm was then measured and the hemolytic ratio was calculated by Eq. 14:

$$\text{Hemolysis} = \frac{\text{A}_{sample} - \text{A}_{negative}}{\text{A}_{positive} - \text{A}_{negative}} \qquad (\text{Eq. } 14)$$

Animals

The mice were provided by Beijing Charles River Laborato-ries, and the procedures for handling the animals firmly stick to the national standards “Laboratory Animal Re-quirements of Environment and Housing Facilities (GB 14925-2001)”. The animal experiments were approved by the Committee on Ethics of Beijing Institute of Nanoenergy and Nanosystems (Approval Number: 2021A041).

In vivo systematic toxicity

Sixteen ICR mice (female, 8 weeks, body weight 30–40 g) were randomly divided into 4 groups, and 0, 50, 100, and 200 mg kg^{-1} of Bi-NC@HA were intravenously injected through the tail vein. The activity, health status, food and water intake of the mice were recorded over a 14-day evaluation period and the body weight of the mice was recorded every 2 days. After 14 days, the eyeballs were enucleated and the mice were sacrificed by painless cervical dislocation, and the blood samples were subjected to blood routine and blood biochemical analysis. The major organs of mice (heart, liver, spleen, lung, kidney) were stained with Hematoxylin-eosin (H&E) for pathological analysis.

In vivo cancer therapy

The BALB/c mice (female, 8 weeks, 18-22 g body weight) were purchased from Vital River Corp. Beijing. 8 × 10^6 4T1 cells in PBS (100 μL) was injected into the abdomen of BALB/c mice and the tumors were allowed to growth to approximately 60 mm^3. Then, the tumor-bearing mice were randomly divided into 4 groups (n= 6), I) PBS group, II) Laser group, III) Bi-NC@HA group, and IV) Bi-NC@HA+Laser group. For the I and II groups, 100 uL PBS solution was intravenously injected and for the III and IV groups, Bi-NC@HA were intravenously injected with a dose of 20 mg kg^{-1}. For the II and IV groups, after 48 h of NP injection, the mice tumors were irradiated with NIR laser (1 W cm^{-2}) for 5 min for a single time. The body weight and tumor size of the mice were recorded every two days individually. The tumor volume (*V*) was calculated as following formula:

$$V = \frac{\text{Length} \times Width^2}{2} \qquad \text{(Eq. 15)}$$

After 16 days, all the mice were sacrificed and the tumors and the main organs (liver, spleen, kidney, heart, and lung) were collected. Hematoxylin-eosin (H&E) staining and Ki-67 immunohistochemistry staining were performed for the tissues. Tumor growth inhibition (TGI) for each group was calculated from the weight of the tumour at the end of treatment (Eq. 16):

$$TGI = \frac{W_{control} - W_{treatment}}{W_{control}} \times 100\% \qquad \text{(Eq. 16)}$$

Statistical Analysis

Data in this work were expressed as mean ± standard deviation. Statistical difference was determined using One-Way ANOVA. The data is considered statistically significant when $p < 0.05$. (* represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$)

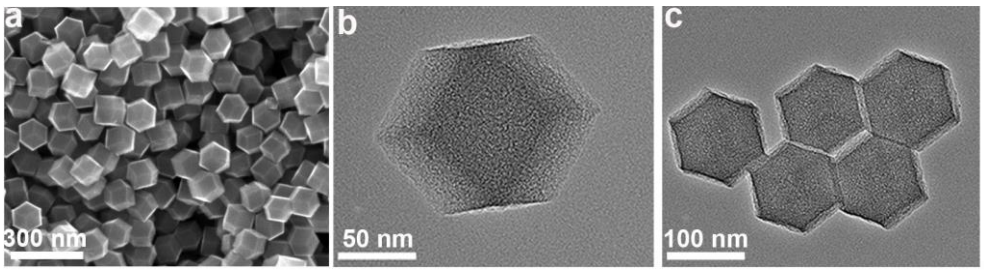

Fig. S1 a) SEM image of ZIF-NC. b, c) TEM images of ZIF-NC.

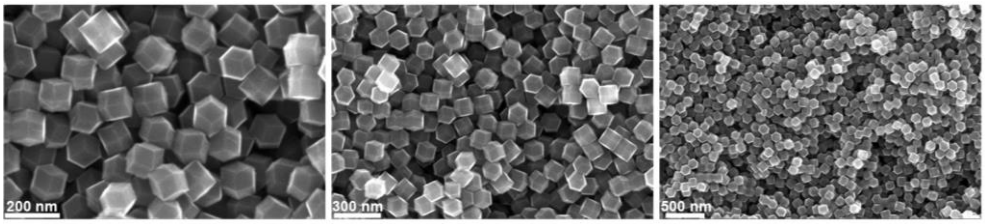

Fig. S2 SEM images of Bi-NC with different magnifications.

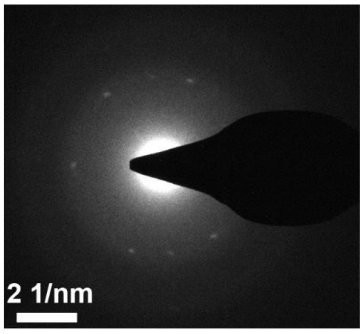

Fig. S3 Selected area electron diffraction (SAED) pattern of Bi-NC.

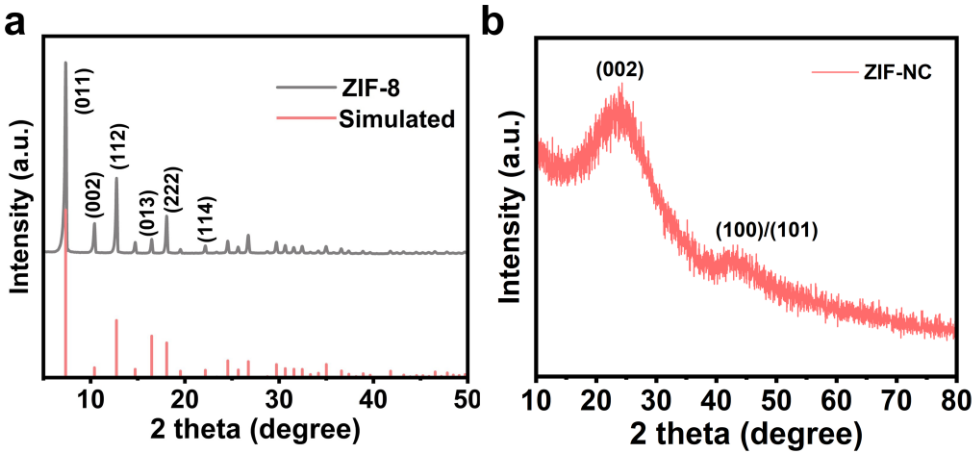

Fig. S4 a) XRD patterns of ZIF-8 and b) ZIF-NC.

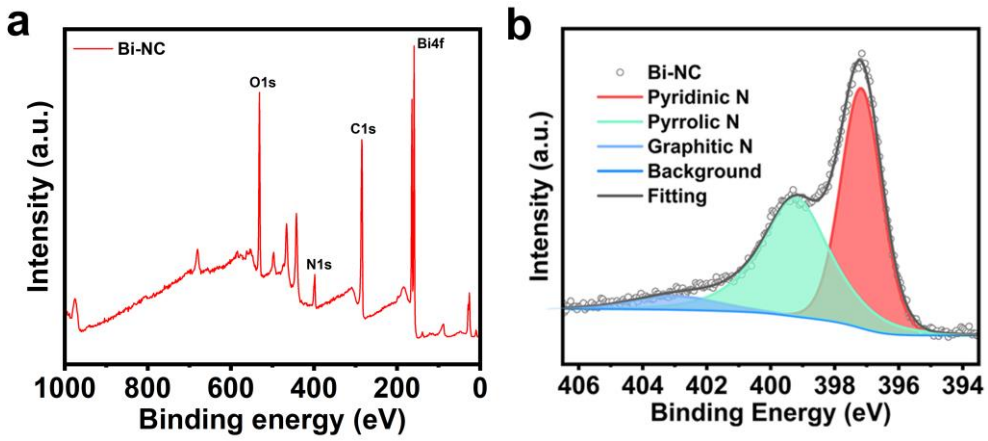

Fig. S5 XPS spectra of a) survey scan, b) N 1s region of Bi-NC.

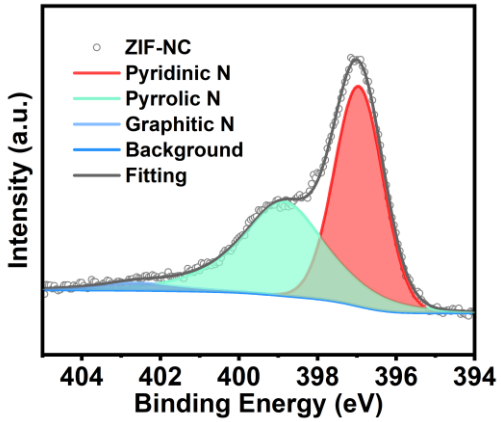

Fig. S6 N 1s X-ray photoelectron spectroscopy (XPS) spectrum of ZIF-NC.

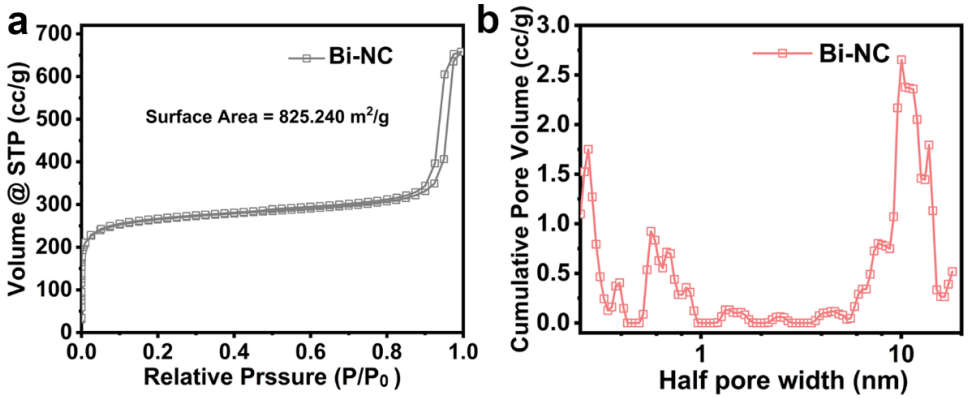

Fig. S7 N_2 adsorption isotherm and pore size distribution of Bi-NCs. The surface area was extracted by the Brunauer–Emmett–Teller (BET) method.

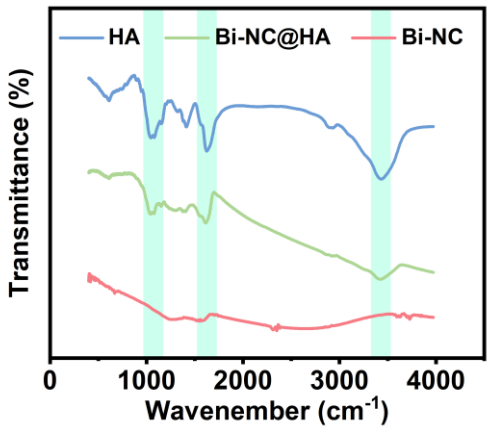

Fig. S8 Fourier transform infrared (FT-IR) spectra of HA, Bi-NC and Bi-NC@HA.

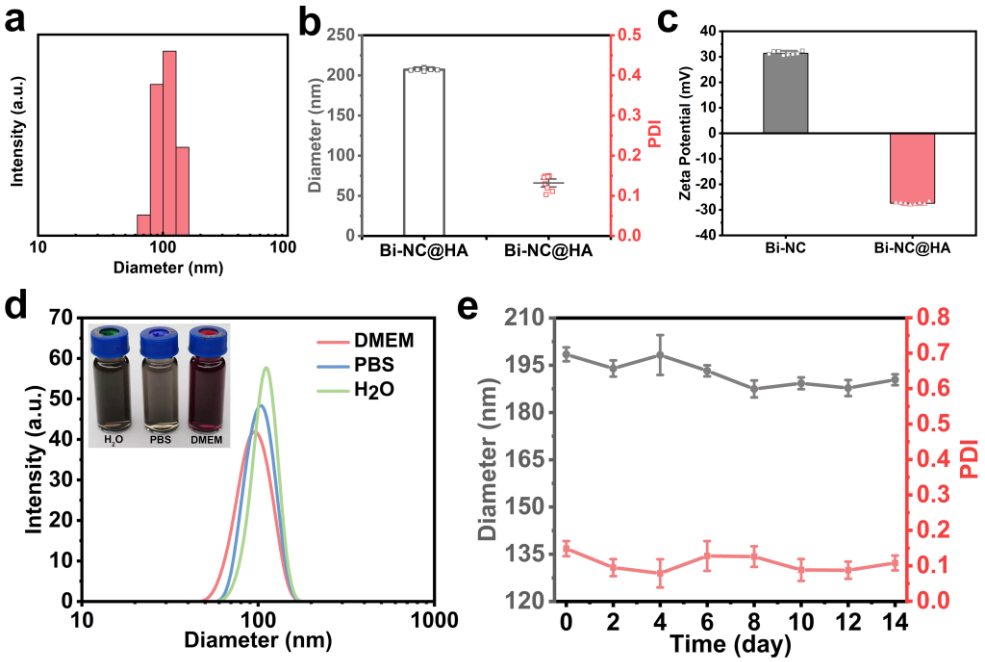

Fig. S9 a) Hydrodynamic diameter distribution of Bi-NC@HA measured in water. b) Hydrodynamic size and PDI of Bi-NC@HA in water (n=8). c) Zeta potentials of Bi-NC and Bi-NC@HA. d) Hydrodynamic size distribution of Bi-NC@HA measured in DMEM, PBS and water. Inset is the corresponding photograph of Bi-NC@HA dispersed in different media. e) Hydrodynamic size and PDI of Bi-NC@HA during 14 days of dispersion in DMEM.

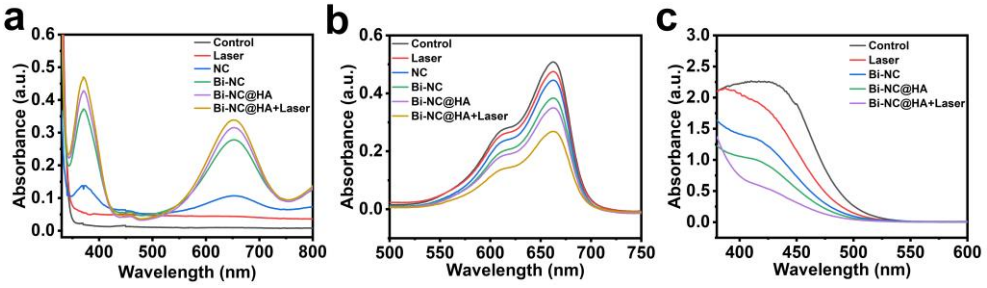

Fig. S10 a) POD-like activity under various conditions using TMB as a probe. b) MB degradation by ROS generated under different conditions. c) GSH consumption after treatments under different conditions.

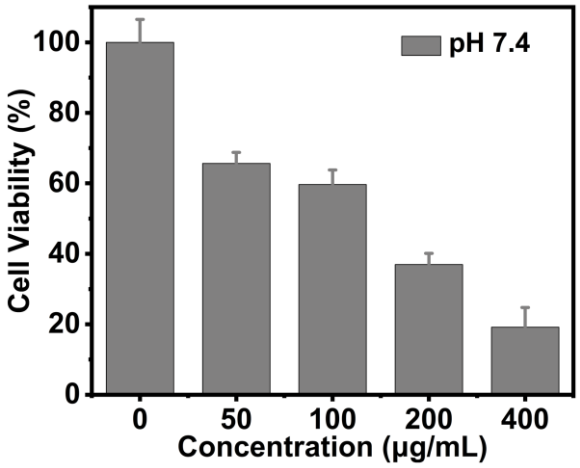

Fig. S11 Relative cell viabilities of L929 cells incubated with various concentration of Bi-NC.

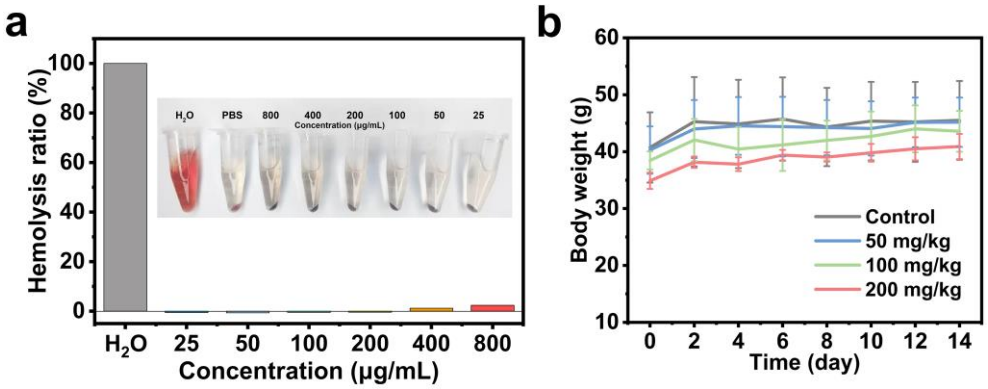

Fig. S12 a) Hemolytic results of Bi-CN@HA, negative control of PBS, and the positive control of H_2O. b) body weights of ICR mice after 14 days of intravenous injection of varying doses of Bi-NC@HA (50 mg kg^{-1}, 100 mg kg^{-1} and 200 mg kg^{-1}).

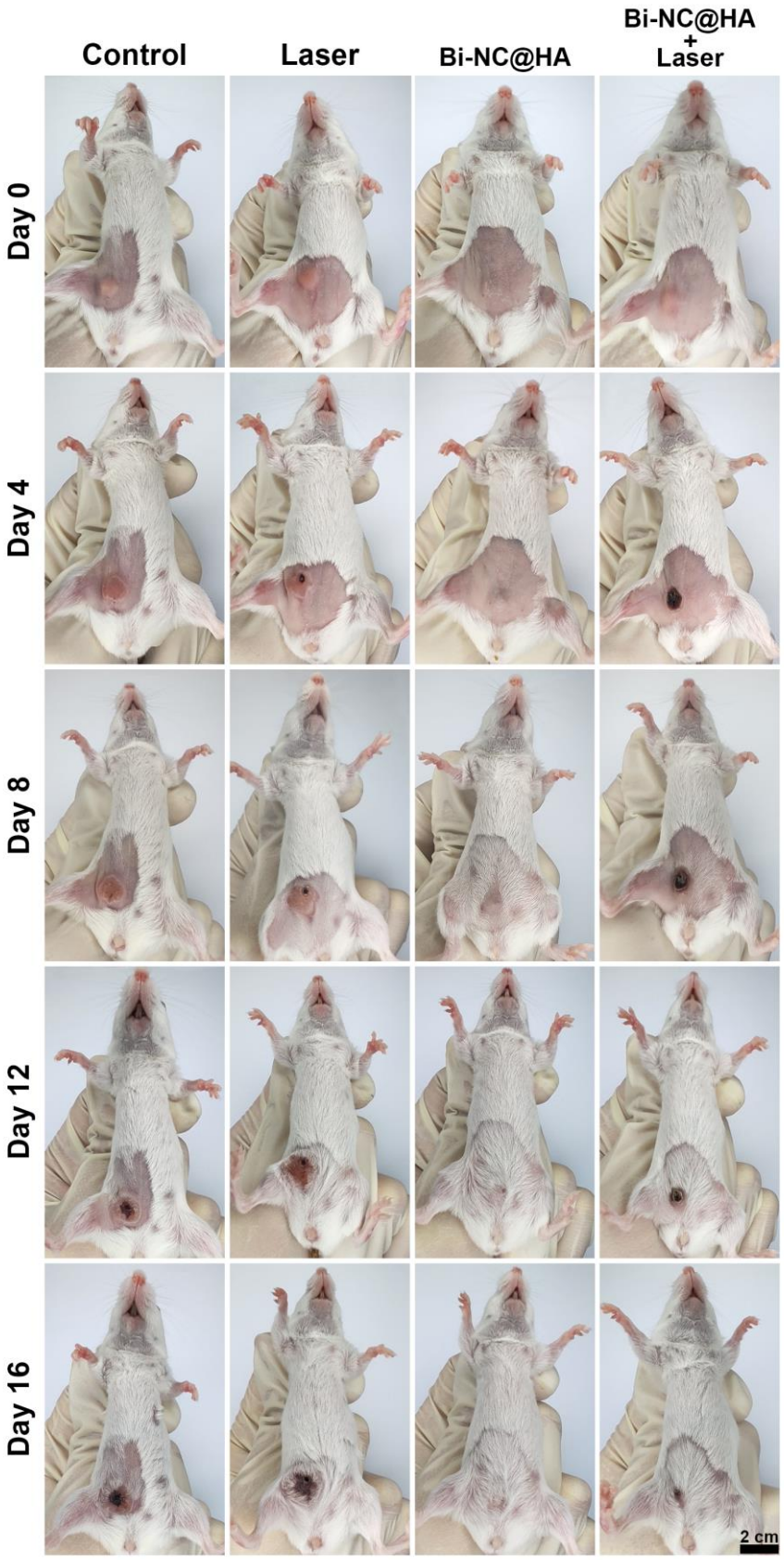

Fig. S13 Photographs of the 4T1 tumor-bearing mice during 16 days of post-injection. NIR irradiation was conducted using 808 nm laser for 5 minutes in 1.0 W cm^{-2}**.**

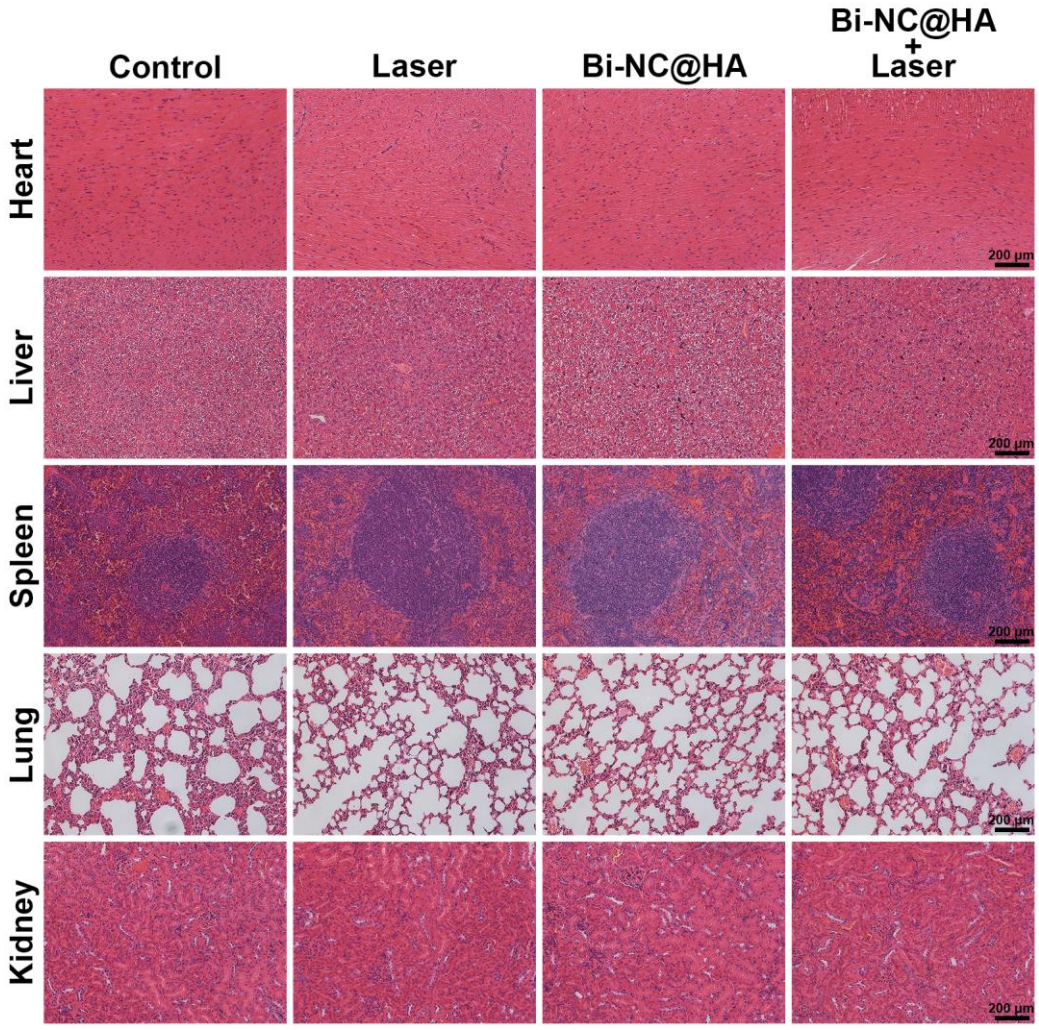

Fig. S14 H&E stained images of the major organs (heart, liver, spleen, lung, kidney) of 4T1-tumor bearing mice after different treatments.