Supporting Information

Hypoxia-responsive liquid metal-containing nanomaterial for cancer phototheranostics

Wenlong Zhang[a], Heting Li[a], Bing Wu[a,b], Qinguan Zhang[a], and Yaning He[a]*

[a] Key Laboratory of Advanced Materials (MOE), Department of Chemical Engineering, Tsinghua University, Beijing, 100084, People's Republic of China.

[b] Emergency Research Institute, Chinese Institute of Coal Science (CICS), Beijing, 100013, People's Republic of China.

Table of Contents

Materials

4-Aminobenzophenone (Energy Chemical, 98%), zinc powder (Fu Chen Chemical Reagent Co. Ltd, AR), tetrahydrofuran (Macklin, 99.5%), titanium tetrachloride ($TiCl_4$, Macklin, 99.5%), potassium carbonate (Beijing Tong Guang Fine Chemicals Co., AR), dichloromethane (Beijing Chemical Industry Group Co., Ltd, AR), $MgSO_4$ (Tianjin Jin Ke Fine Chemicals Co., AR), petroleum ether (Greagent, AR), ethyl acetate (Sinopharm Chemical Reagent Co., Ltd, AR), $NaHCO_3$ (Beijing Chemical Industry Group Co., Ltd, 99.5%), phenyl chloroformate (J&K Chemical, 99%), n-hexane (Beijing Tongguang Fine Chemical Co., Ltd, AR), NaCl (Beijing Chemical Industry Group Co., Ltd, 99.5%), Na_2SO_4 (Greagent, 99%), (4-aminophenyl)methanol (Heowns Biochem Technologies, LLC, Tianjin, 99%), hydrochloric acid (HCl, Beijing Tong Guang Fine Chemicals Co., GR), sodium nitrite ($NaNO_2$, Beijing Tong Guang Fine Chemicals Co., 99.99%), phenol (Sigma Aldrich, 99%), NaOH (Greagent, AR), KI (J&K Chemical, 99%), 1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane (Macklin, 95%), *N*, *N*-dimethylformamide (DMF, Greagent, 99.5%), methanol (Beijing Chemical Industry Group Co., Ltd, 99.5%), dibutyltin dilaurate (DBTL, J&K Chemical, 97.5%), heptakis-(6-mercapto-6-deoxy)-beta-cyclodextrin (HS-*β*-CD, Meryer, 98%), dimethyl sulfoxide (DMSO, Greagent, 99.0%), eutectic gallium-indium (Sigma Aldrich, 99.99%), and nicotinamide adenine dinucleotide phosphate (NADPH, Sigma Aldrich, AR) were utilized as received without further purification, unless stated otherwise. Rat liver microsomes were acquired from CHI Scientific, Inc. (Jiangsu, China). Human cervical cancer cells line HeLa was available from Beijing EallBio Biomedical Technology Co., Ltd. Phosphate buffer saline (PBS, pH 7.2) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay kit were obtained from Beijing Solarbio Science & Technology Co., Ltd. AO/PI double staining kit was purchased from APExBIO Technology LLC. HeLa cells were cultured in a complete cell culture medium (Procell, China) at 37 °C and 5% CO_2. Trypsin-EDTA were purchased from Gibco. The solvents employed in the study were of analytical grade and were utilized without further purification. Ultrapure water was obtained from a Milli-Q water purification system with a resistivity exceeding 18 MΩ·cm.

Instruments

^{1}H NMR spectra were acquired using a JEOL JNM-ECZ400S NMR spectrometer. The ultraviolet–visible (UV-vis) absorption spectra were recorded on an Agilent 8453 UV-vis spectrophotometer. Fluorescence spectra were obtained with a Hitachi F-7000 fluorescence spectrophotometer. Transmission electron microscopy (TEM) observations were conducted using a Hitachi H-7650B transmission electron microscope operating at 80 kV. The dynamic light scattering (DLS) and Zeta potential graphs were captured by MALVERN Zetasizer (ZSU3200). Thermogravimetric experiments were performed on a thermogravimetric analyzer (NETZSCH TG209 F3, Germany) under a N_2 atmosphere, with a heating rate of 10 °C/min from 40 °C to 800 °C. The Fourier-transform infrared (FTIR) spectra were recorded using a Thermo-Nicolet 6700 FTIR spectrometer. X-ray photoelectron spectra were acquired with an Axis Supra$^+$ X-ray photoelectron spectrometer from Shimadzu corporation. Infrared thermometer images were captured with a Fotric 223s infrared thermal camera. A MW-GX-980 multimode fiber-coupled laser diode (maximum power: 5W) from Changchun New Industries Optoelectronics Tech. Co., Ltd. was employed to provide the 980 nm laser. Fluorescence imaging of HeLa cells and multicellular tumor spheroids was conducted using a Nikon Eclipse Ti2-E confocal laser scanning microscope (CLSM).

Experimental procedures

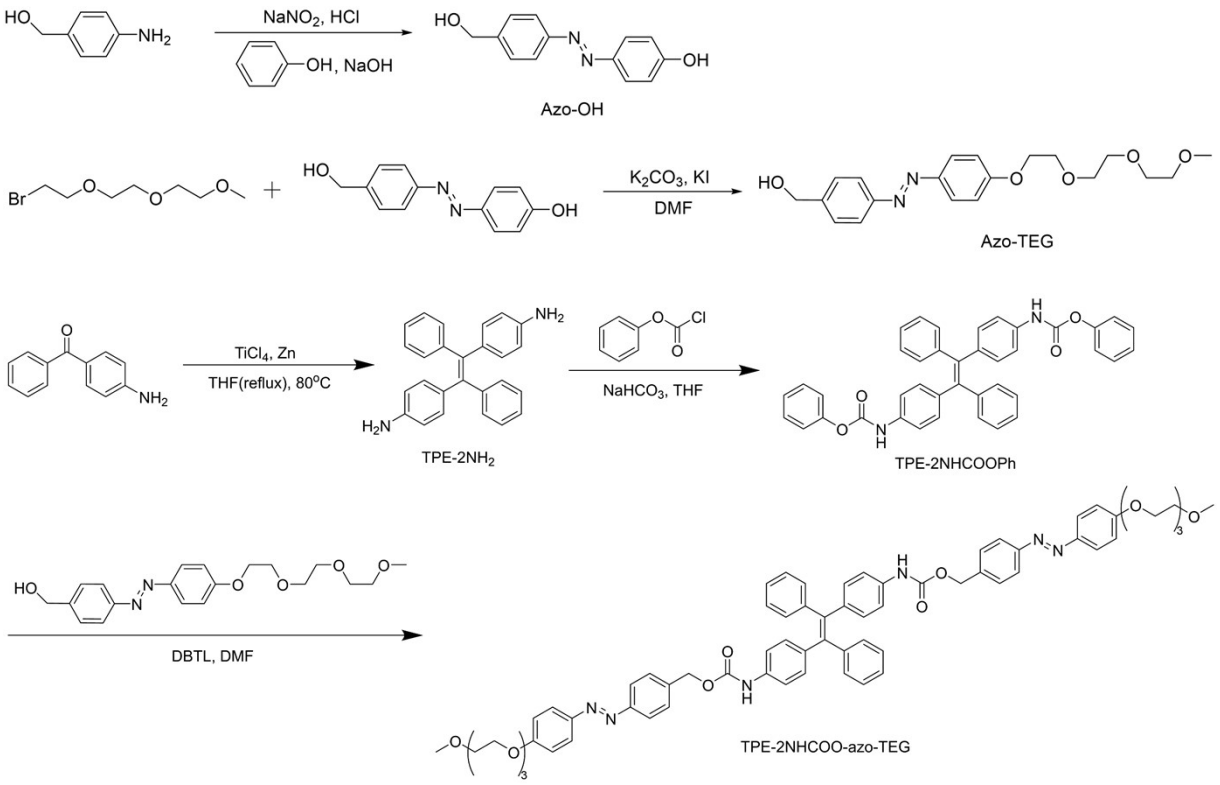

Fig. S1 The synthesis route of TPE-2NHCOO-azo-TEG.

Synthesis of 4,4'-(1,2-diphenylethene-1,2-diyl)dianiline (TPE-2NH_2)

TPE-2NH_2 was synthesized following a previously reported literature procedure.[1] A mixture of 4-aminobenzophenone (16.0 g, 81.2 mmol) and zinc powder (15.0 g, 229 mmol) was dissolved in tetrahydrofuran (THF, 300 mL) and cooled to 0 ℃. Subsequently, $TiCl_4$ (25.2 mL, 229 mmol) was added dropwise over a period of 30 minutes. The reaction mixture was stirred at 0 ℃ for 30 minutes and then refluxed at 80 ℃ for 6 h under an Ar atmosphere. Upon completion, an aqueous solution of potassium carbonate (400 mL, 10 wt%) was added to the reaction mixture, which was subsequently filtered. The filtrate was collected, extracted with 300 mL dichloromethane for three times and dried over $MgSO_4$. After removing the solvent, the desired product was isolated as a yellow solid in 70% yield by column chromatography (petroleum ether/ethyl acetate = 1:1.5). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (TMS, ppm): 7.12-7.06 (m, 10H), 6.77-6.76 (m, 4H), 6.41-6.39 (m, 4H), 3.57 (s, 4H).

Synthesis of diphenyl ((1,2-diphenylethene-1,2-diyl)bis(4,1-phenylene))dicarbamate (TPE-2NHCOOPh)

TPE-2NH_2 (724 mg, 2.0 mmol) and $NaHCO_3$ (15.0 g, 229 mmol) were sequentially suspended in THF (30 mL). Subsequently, phenyl chloroformate (640 mg, 4.1 mmol) was added slowly over a period of 5 minutes. Upon completion of the reaction, as monitored by thin-layer chromatography (TLC, n-hexane/ethyl acetate = 5:1), the mixture was diluted with 70 mL of ethyl acetate and 100 mL of water. The products in the aqueous phase were extracted with 200 ml ethyl acetate for twice. The organic phase was merged, washed with 200 mL saturated NaCl solution, and dried over Na_2SO_4. After removing the solvent, the desired product was isolated as a beige solid in 63% yield by column chromatography (n-hexane/ethyl acetate = 1:1). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (TMS, ppm): 7.40-7.38 (m, 4H), 7.24-7.21 (m, 6H), 7.18-7.16 (m, 4H), 7.09 (m, 6H), 7.02-7.00 (m, 8H), 6.88 (s, 2H).

Synthesis of 4-((4-(hydroxymethyl)phenyl)diazenyl)phenol (Azo-OH)

(4-Aminophenyl)methanol (6.16 g, 50.0 mmol) was dissolved in 4 mol·L^{-1} HCl (50 mL) and cooled to 0°C in an ice-water bath. Subsequently, an aqueous solution (25 mL) of sodium nitrite (4.14 g, 60.0 mmol)

was added dropwise to obtain the diazonium salt of (4-aminophenyl)methanol. Phenol (4.71 g, 50.0 mmol) was dissolved in 2.5 mol·L^{-1} NaOH (40 mL) and cooled to 0 °C with vigorous stirring for 15 min. The phenol solution was then added dropwise to the aqueous diazonium salt solution. The mixture was kept at room temperature for 12 h to afford the crude product. The crude product was recrystallized twice from THF to obtain a dark yellow solid in 84% yield. ^{1}H NMR (400 MHz, DMSO-d_6), δ (TMS, ppm): 10.27 (s, 1H), 7.80-7.78 (dd, 4H), 7.50-7.48 (d, 2H), 6.95-6.93 (d, 2H), 5.33 (s, 1H), 4.59 (s, 2H).

Synthesis of (4-((4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)diazenyl)phenyl)methanol (Azo-TEG)

The synthesis route of Azo-TEG has been previously reported.[2] Azo-OH (2.37 g, 10.4 mmol), K_2CO_3 (5.75 g, 41.6 mmol), KI (0.17 g, 1.0 mmol), and 1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane (4.09 g, 18.0 mmol) were dissolved in DMF (80 mL). The reaction mixture was stirred at 100 ℃ for 24 h, and the reaction was quenched by the addition of water. Subsequently, the solution was extracted with dichloromethane. Further purification was performed using column chromatography (dichloromethane/methanol = 25/1) to afford the orange solid product in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (TMS, ppm): 7.89-7.87 (d, 2H), 7.83-7.81 (d, 2H), 7.52-7.50 (d, 2H), 7.15-7.14 (d, 2H), 5.35 (s, 1H), 4.60 (s, 2H), 4.22-4.21 (t, 2H), 3.79-3.78 (t, 2H), 3.61-3.42 (m, 8H), 3.24 (s, 3H).

Synthesis of bis(4-((4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)diazenyl)benzyl) ((1,2-diphenylethene-1,2-diyl)bis(4,1-phenylene))dicarbamate (TPE-2NHCOO-azo-TEG)

TPE-2NHCOOPh (325 mg, 0.54 mmol) was dissolved in anhydrous DMF (10 mL) and stirred for 15 minutes at 90 ℃. Subsequently, Azo-TEG (404 mg, 1.08 mmol) and dibutyltin dilaurate (DBTL, 68 mg, 0.11 mmol) were added to the reaction mixture. The reaction was monitored by TLC using n-hexane/ethyl acetate (3:1) as the eluent. Upon completion, the solvent was removed from the mixture by dialysis (MWCO = 500). The desired product was lyophilized and isolated as an orange-yellow solid. Further purification was performed using column chromatography (n-hexane/ethyl acetate = 1:1 to pure ethyl acetate) to afford the orange solid product in 53% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (TMS, ppm): 9.81 (s, 2H), 7.90-7.85 (m, 8H), 7.60-7.58 (m, 4H), 7.29-7.24 (m, 4H), 7.14-7.08 (m, 10H), 6.95-6.93 (m, 4H), 6.89-6.87 (m, 4H), 5.22 (s, 4H), 4.21-4.19 (t, 4H), 3.79-3.77 (t, 4H), 3.61-3.59 (m, 4H), 3.55-3.50 (m, 8H), 3.43-3.41 (m, 4H), 3.23 (s, 6H).

Synthesis of HS-*β*-CD/TPE-2NHCOO-azo-TEG complex

TPE-2NHCOO-azo-TEG (5.3 mg) and HS-*β*-CD (11.3 mg, 1:1 molar ratio with respect to the azo group) were initially dissolved in 4 mL DMSO. Subsequently, 20 mL water was slowly injected into the DMSO solution of TPE-2NHCOO-azo-TEG and HS-*β*-CD. The mixture was stirred for 12 h at room temperature and for another 12 h at 60 ℃ to obtain the solution of the HS-*β*-CD/TPE-2NHCOO-azo-TEG complex. Finally, the concentration of the HS-*β*-CD/TPE-2NHCOO-azo-TEG complex was 0.69 mg/mL.

Synthesis of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG

EGaIn (300 mg) was added to a 50 mL centrifuge tube, which was filled with a total volume of 20 mL DMSO/H_2O mixture (v/v = 1/5) containing 13.8 mg HS-*β*-CD/TPE-2NHCOO-azo-TEG complex. After sonication, the largest particles precipitated within seconds, and the slurry was removed from the tube. The remaining dispersion was further centrifuged to obtain the resulting nanospheres.

Degradation of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG with enzyme treatment

0.2 mL of rat liver microsomal suspension (protein concentration: 20 mg/mL) and 0.1 mg of NADPH were mixed in 4 mL of PBS solution containing 2 mg LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG, which was purged with argon for 30 minutes. The reaction mixture was maintained at 37 ℃ in an isolated quartz cuvette (4 mL) for 48 h.

Three-dimensional multicellular tumor spheroid formation

The preparation of three-dimensional multicellular tumor spheroids (3D MCTS) in agarose-coated 96-well plates has been previously reported.[3-5] Briefly, a single cell suspension of 50,000 cells/mL (100 μL) was seeded onto sterile agarose-coated (1.5% w/v in PBS) 96-well imaging plates. After centrifuging the plates for 15 minutes at 1500 rcf, the HeLa cells were allowed to aggregate for 96 hours without disturbance, leading to the formation of a single spheroid with a diameter of 400-500 μm in each well.

Cellular imaging

HeLa cells were seeded onto a 35 mm glass-bottomed dish at a density of 2×10^5 cells per well and cultured overnight before the assay. Subsequently, the cells were incubated under normoxic (21% O_2, v/v) or different hypoxic (10%, 5%, 1% O_2, v/v) conditions for 24 h as a preculture. The cells were then treated with LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG at a final concentration of 500 mg/L for an additional 24 h under the same condition. Fluorescence investigation was performed using a Nikon ECLIPSE Ti2-E confocal laser scanning microscope.

3D MCTS were prepared after 4 days of growth. LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG was added to the 3D MCTS at a final concentration of 500 mg/L and investigated at 0, 12, 24, 48 and 72 h using a Nikon ECLIPSE Ti2-E confocal laser scanning microscope with an excitation wavelength of 405 nm and an emission wavelength range of 480-530 nm.

Cytotoxicity Assay

The cytotoxicity of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG was evaluated using an MTT assay. HeLa cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 24 h under normoxic (21% v/v O_2) or hypoxic (1% v/v O_2) conditions. Subsequently, LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG was added at concentrations ranging from 10 to 500 mg/L, and the cells were incubated for an additional 24 h. The medium was then replaced with 10 μL of MTT solution (5 mg/mL), and after 4 h, the supernatant was removed and replaced with 110 μL of DMSO. The absorbance was measured at 570 nm using an Infinite M200 microplate reader.

***In vitro* photothermal effects evaluation**

LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG was diluted with DMEM complete cell culture medium to concentrations ranging from 10 to 500 mg/L. HeLa cells were seeded into a 96-well plate at a density of 3×10^4 cells per well and cultured for 24 hours under normoxic (21% v/v O_2) or acute hypoxic (1% v/v O_2) conditions. Subsequently, the cell culture medium was removed, and 200 μL of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG solutions were added to each well of the plate, followed by an additional 24-hour incubation. Following this, the HeLa cells were irradiated with a 980 nm laser at a power density of 2 W/cm^2 for approximately 5 min. HeLa cells treated with different concentrations of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG without laser irradiation served as a negative control.

To visualize cell viability immediately after photothermal therapy, a standard double-staining kit (APExBIO Technology LLC) with acridine orange (AO) and propidium iodide (PI) was employed to assess metabolic activity and evaluate cell membrane integrity. After the laser irradiation process, the tumor cells were gently washed with PBS twice and co-stained with a mixture of 3 μL AO and 3 μL PI in 90 μL of 1X staining buffer for 10 minutes at 37 °C. Cells that excluded PI (red) and retained green color were considered viable, while those stained with PI (red) were classified as dead.

***In vivo* imaging study and photothermal therapy of tumors**

Female BALB/c nude mice (5 weeks, SPF Biotechnology Co., Ltd., Beijing) were subcutaneously inoculated with SK-OV-3 ovarian cancer cells (Fuheng Biology Co., Ltd.). When tumor volumes reached approximately 75 mm^3 (7 days post-inoculation), the 20 mice were randomly allocated into four experimental groups: untreated control group (n = 5), only laser group (n = 5), only LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG group (n = 5), and LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG with laser group (n = 5). Each mouse in the latter two experimental groups was administered 200 μL of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG solution (500 mg/L) through intratumoral injection. All mice were anesthetized with isoflurane (Shenzhen Ruiwode Lift Technology Co.,Ltd.) using a portable rodent

anesthesia system (Beijing Zhongshi Dichuang Technology Development Co., Ltd.) before photothermal treatment.

In vivo imaging of mice was performed using a Tanon ABL X5 imaging system with an excitation wavelength of 470 nm and an emission wavelength of 535 nm at 48 h post injection. Body weights and tumor volumes of mice were tracked across all groups. Tumors were measured with orthogonal diameters (major axis 'a' and minor axis 'b') using vernier calipers. Volumes were calculated as $V = (a \times b^2)/2$.

All experimental procedures were conducted in KWT Animal Co., Ltd. in accordance with protocols approved by the Animal Ethical and Welfare Committee (AEWC) of Beijing. The approval number for the laboratory is SYXK(Jing)2020-0050. Data are presented as mean ± SEM from ≥3 independent experiments. Statistical comparisons were performed using Student's two-tailed *t*-tests in Excel and Origin, with $p < 0.05$ considered significant.

Characterization

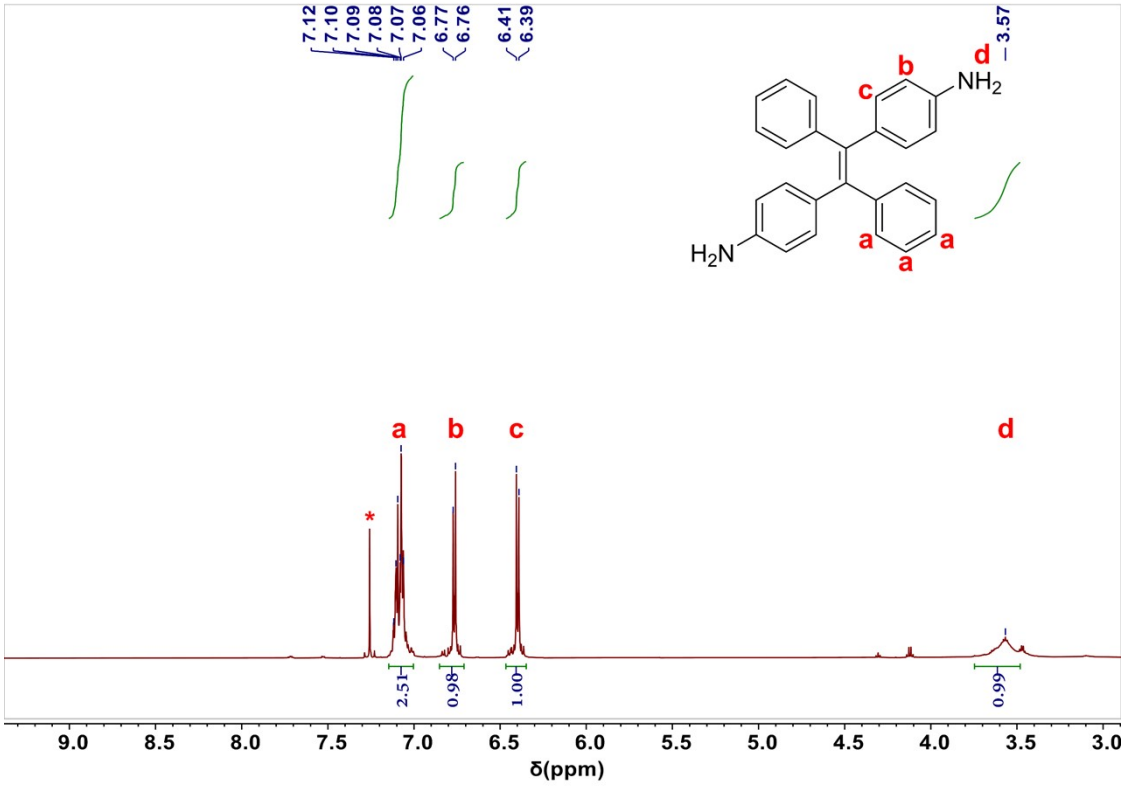

Fig. S2 ^{1}H NMR spectrum of TPE-2NH_2. Red asterisk (*) indicates the solvent peak.

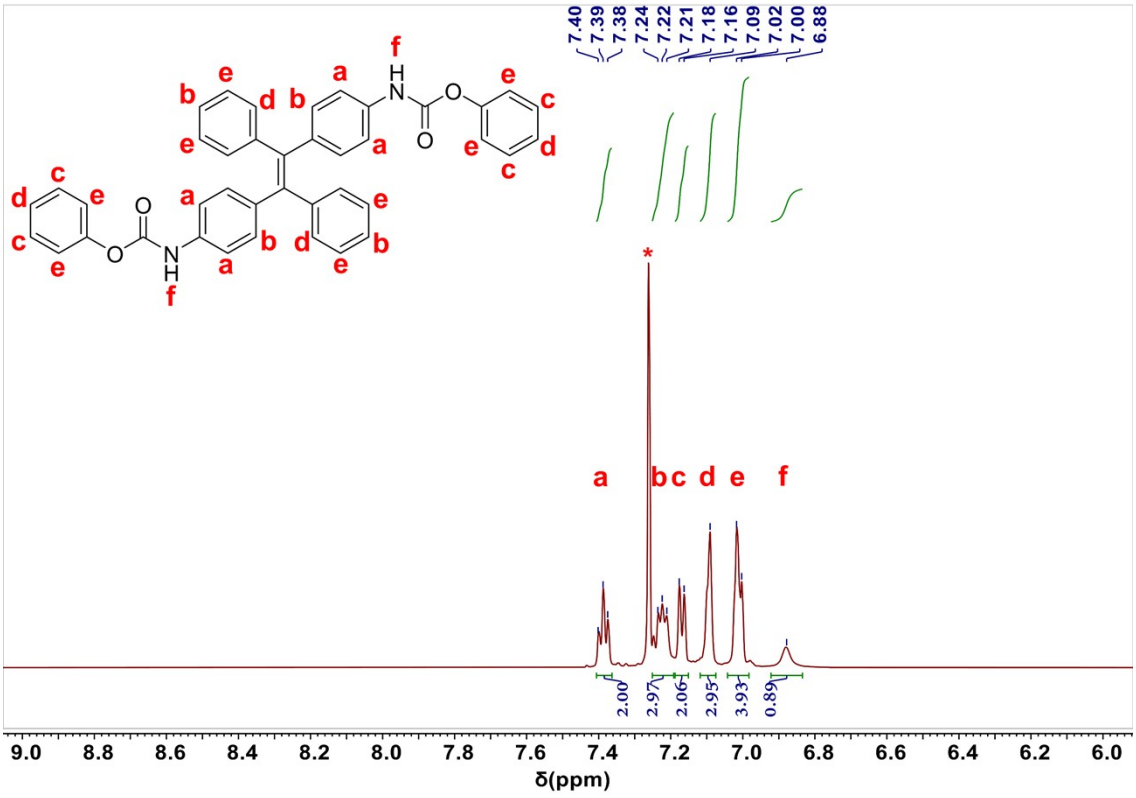

Fig. S3 ^{1}H NMR spectrum of TPE-2NHCOOPh. Red asterisk (*) indicates the solvent peak.

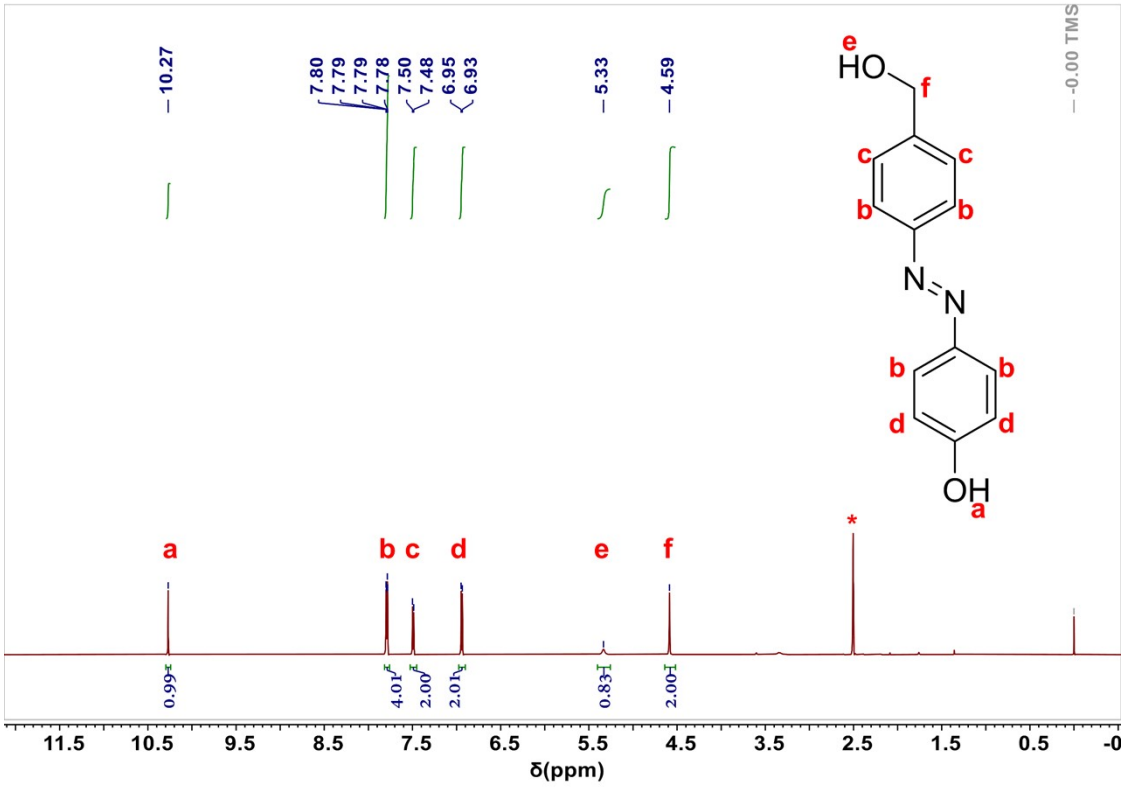

Fig. S4 [1]H NMR spectrum of Azo-OH. Red asterisk (*) indicates the solvent peak.

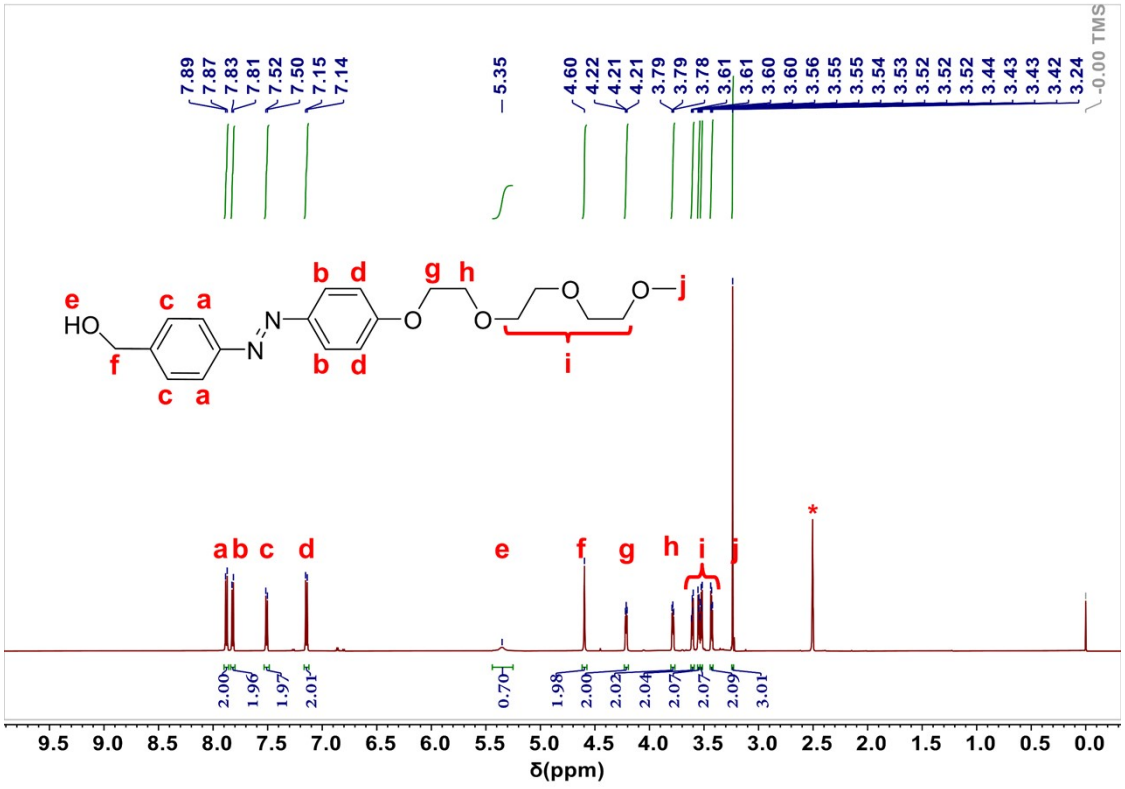

Fig. S5 [1]H NMR spectrum of Azo-TEG. Red asterisk (*) indicates the solvent peak.

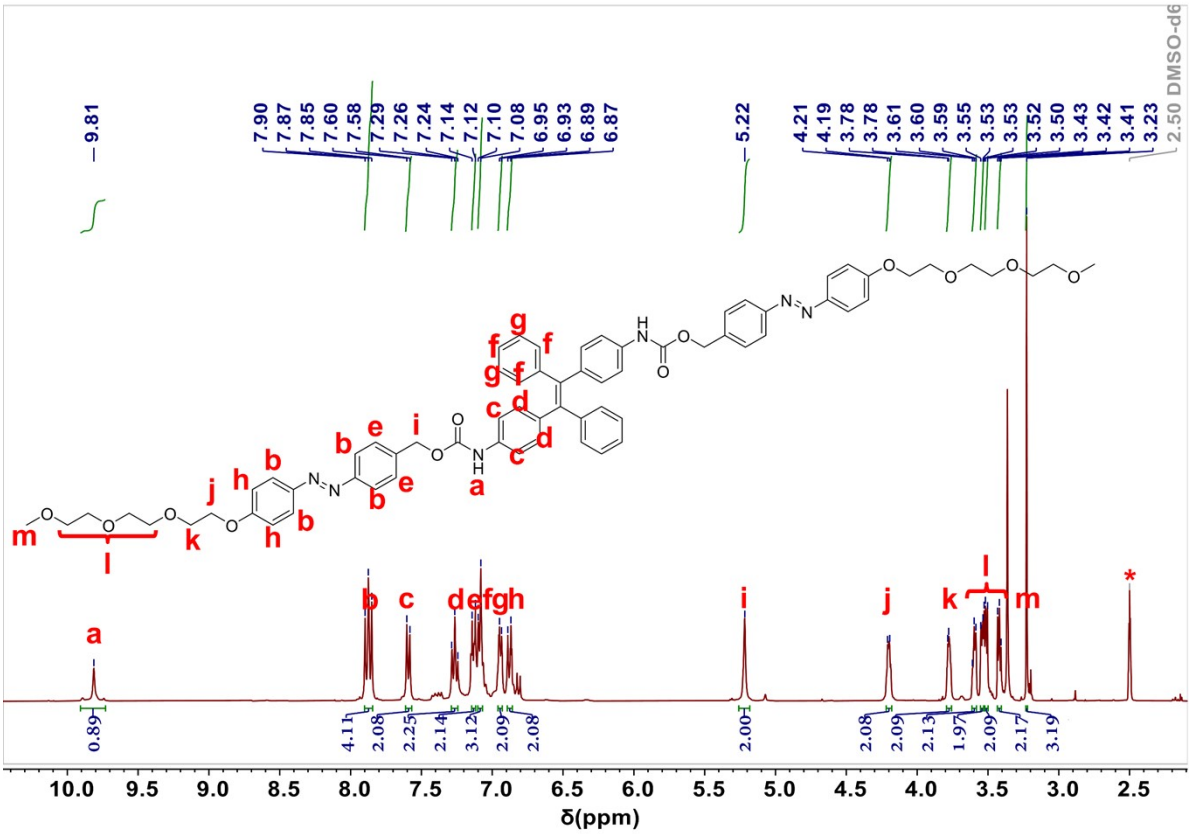

Fig. S6 ^{1}H NMR spectrum of TPE-2NHCOO-azo-TEG. Red asterisk (*) indicates the solvent peak.

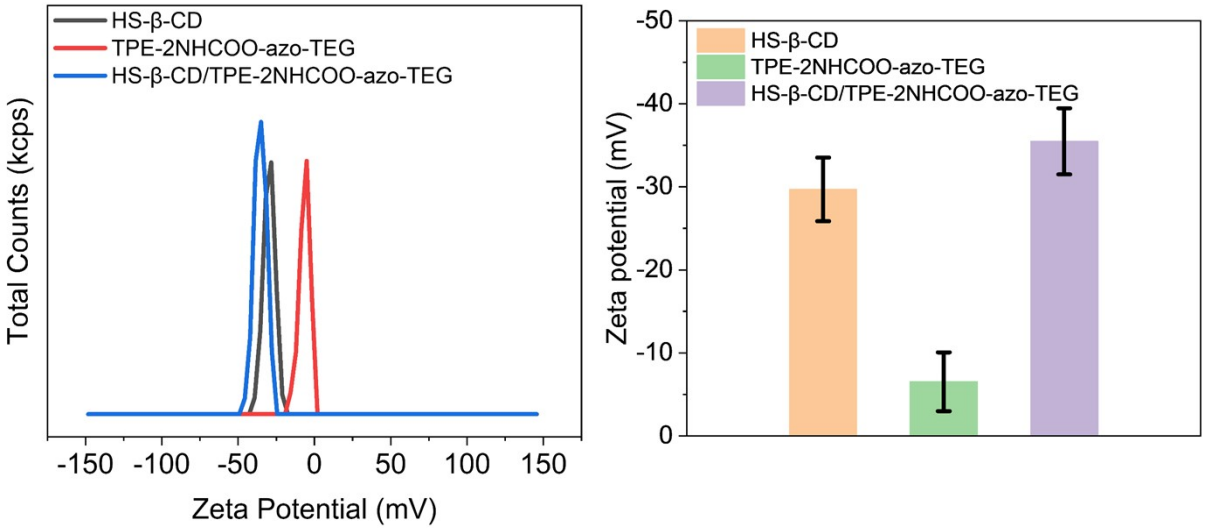

Fig. S7 Zeta potentials of HS-*β*-CD, TPE-2NHCOO-azo-TEG and HS-*β*-CD/TPE-2NHCOO-azo-TEG in water. The pH of the HS-*β*-CD and HS-*β*-CD/TPE-2NHCOO-azo-TEG solutions was approximately 6.3, while the pH of the TPE-2NHCOO-azo-TEG solution was approximately 6.8.

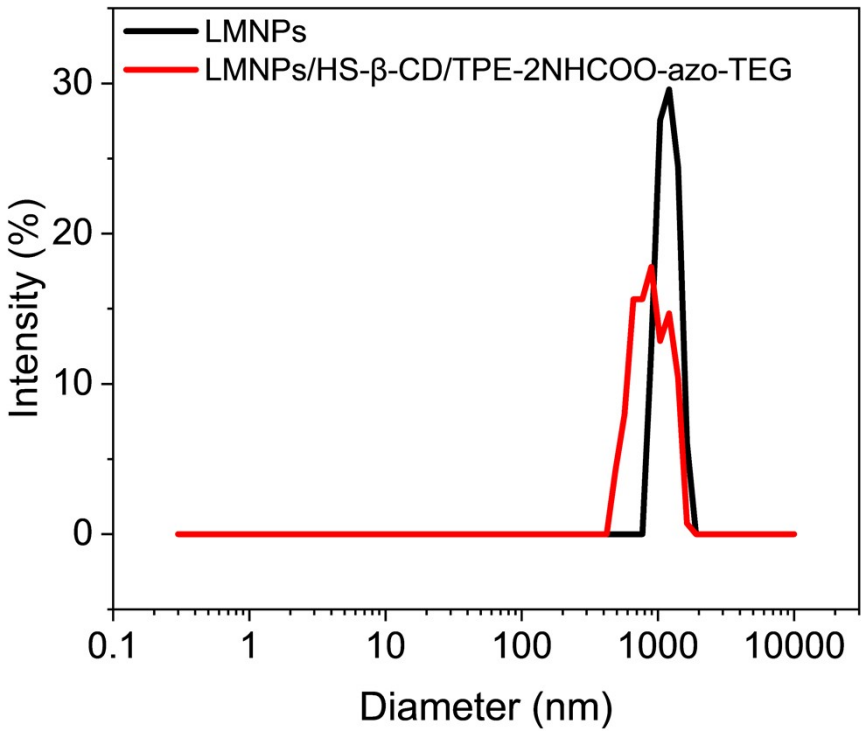

Fig. S8 DLS curves of LMNPs and LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG.

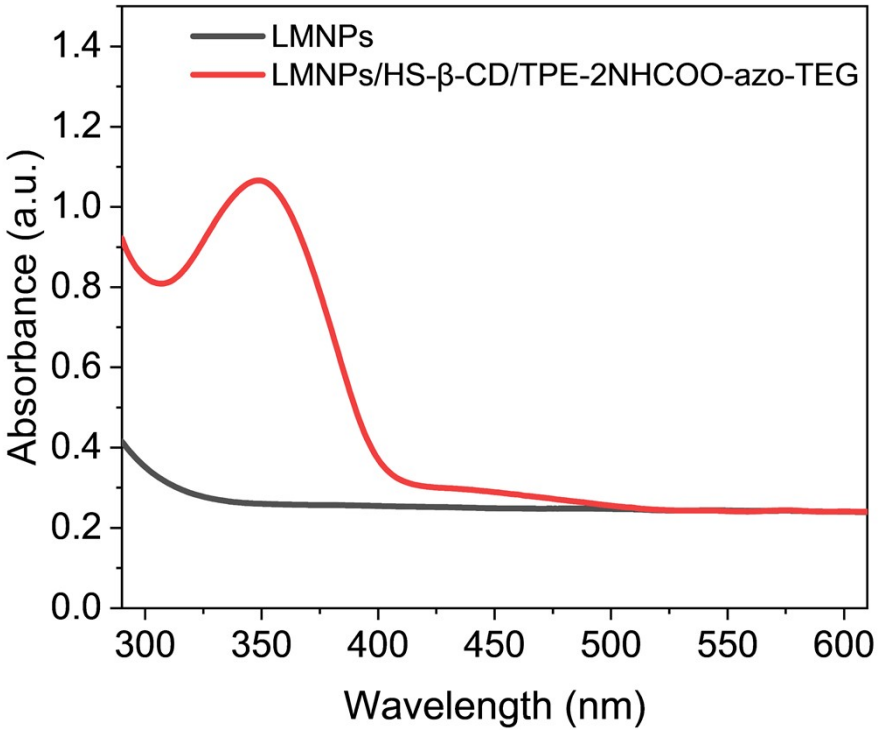

Fig. S9 The UV-vis absorption spectra of LMNPs and LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG.

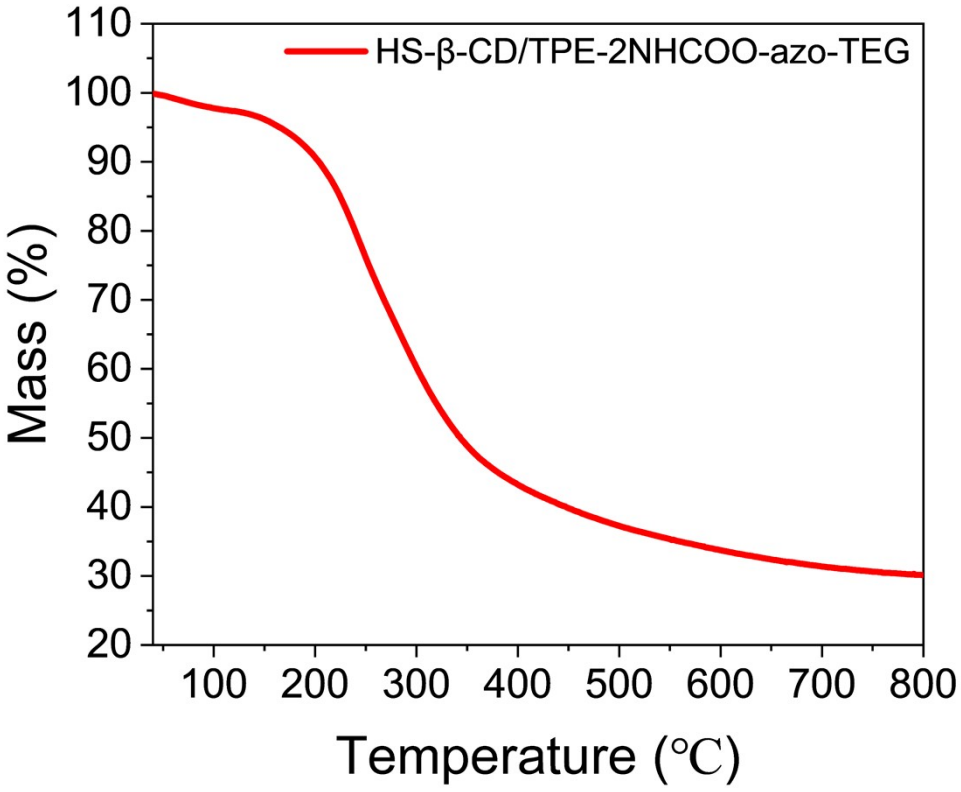

Fig. S10 TGA curve of HS-*β*-CD/TPE-2NHCOO-azo-TEG.

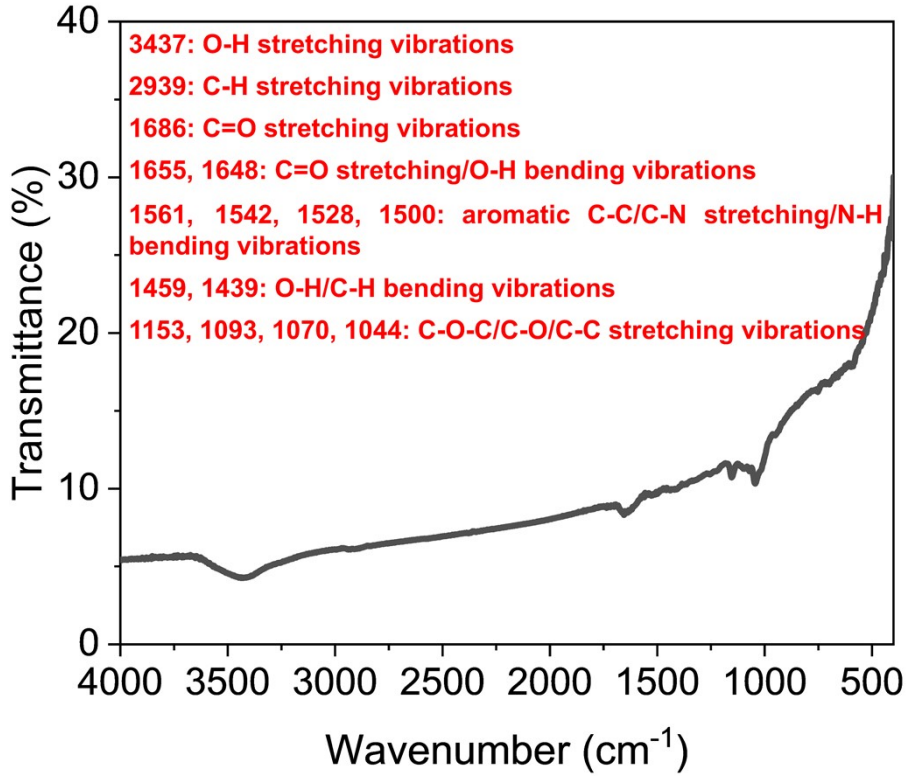

Fig. S11 FTIR spectrum of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG.

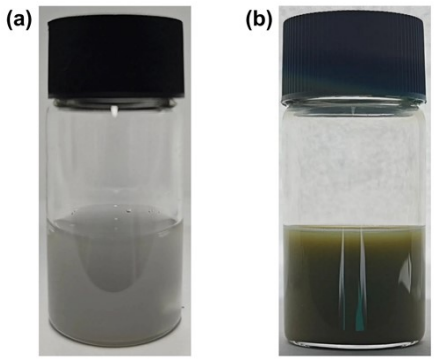

Fig. S12 (a) LMNPs dispersed in PBS solution; (b) LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG dispersed in PBS solution.

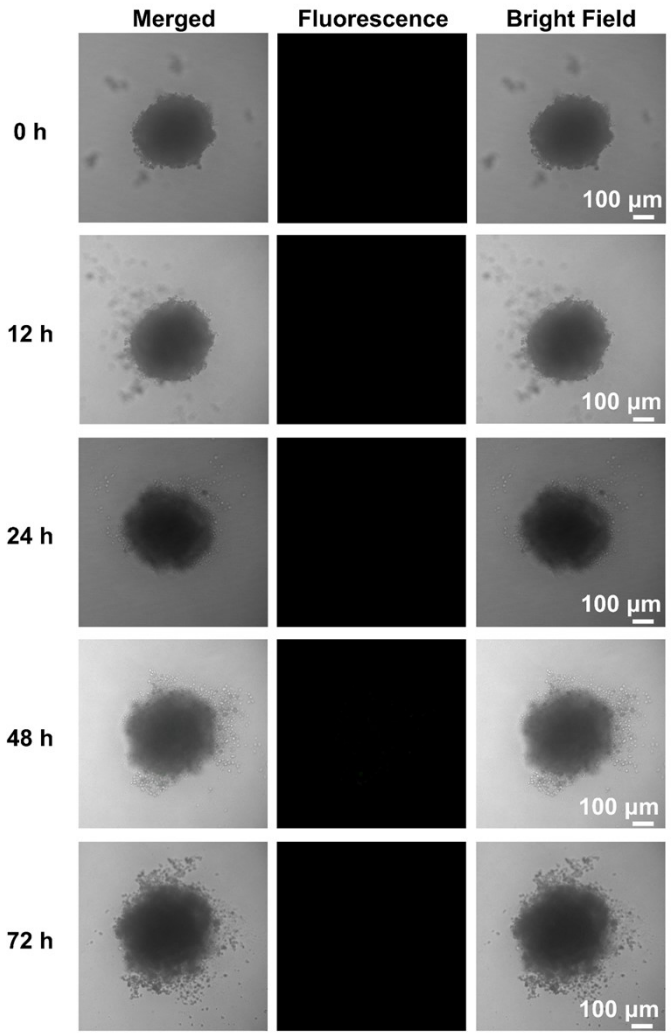

Fig. S13 CLSM images of 3D MCTS incubated with PBS. λ_{ex} = 405 nm, λ_{em} = 480-530 nm. Scale bars: 100 μm.

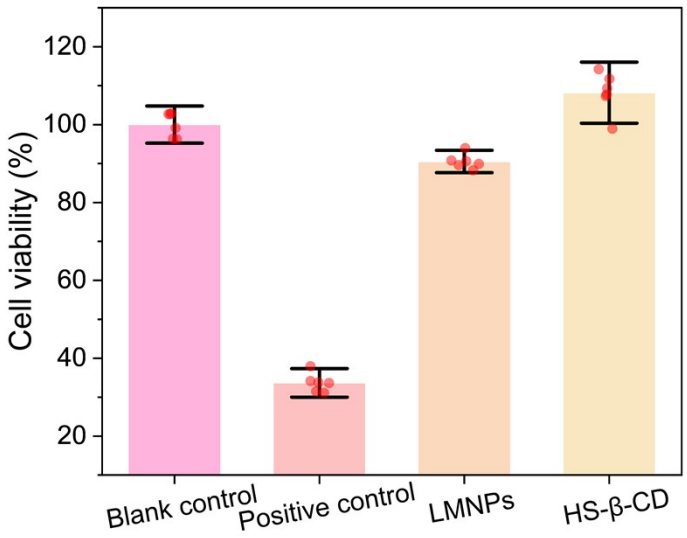

Fig. S14 Cell viability of HeLa cells treated with 500 mg/L of LMNPs and HS-*β*-CD for 24 hours. DMEM complete culture medium was used as a blank control; DMEM complete culture medium containing 10% DMSO was used as a positive control.

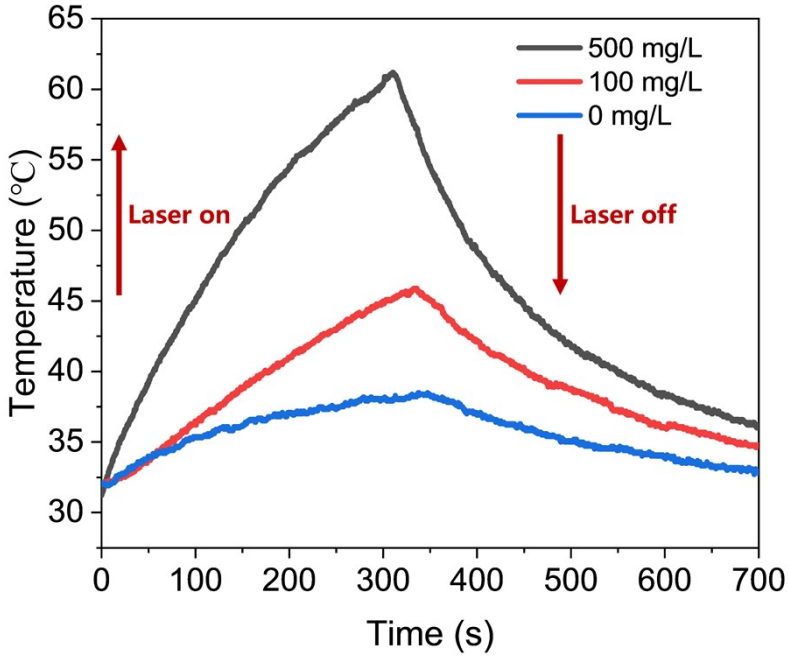

Fig. S15 Laser-induced temperature change of different concentrations of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG in PBS solution.

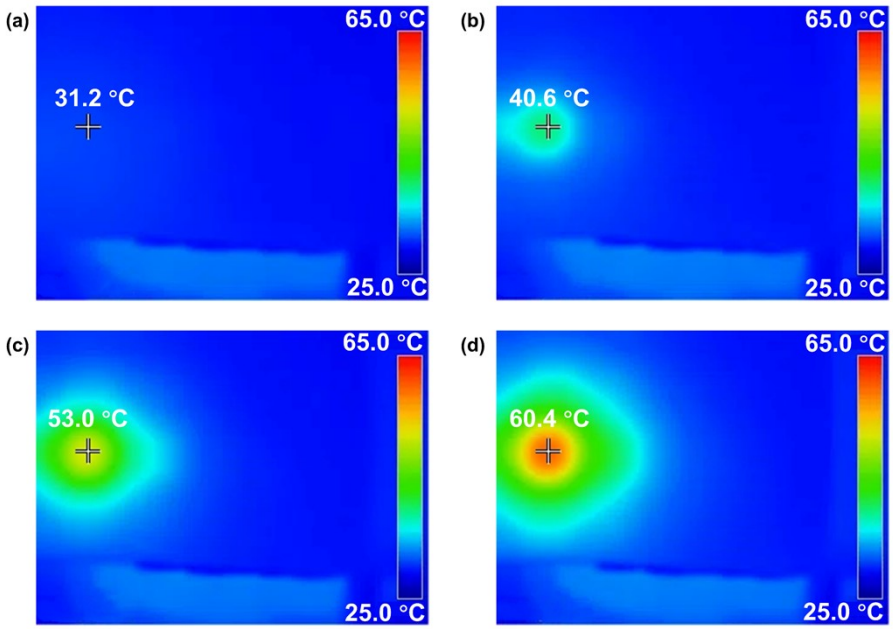

Fig. S16 Infrared thermometer images of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG solution (500 mg/L) under irradiation with 980 nm laser at different times: (a) before irradiation; (b) irradiation 1 min; (c) irradiation 3 min; (d) irradiation 5 min.

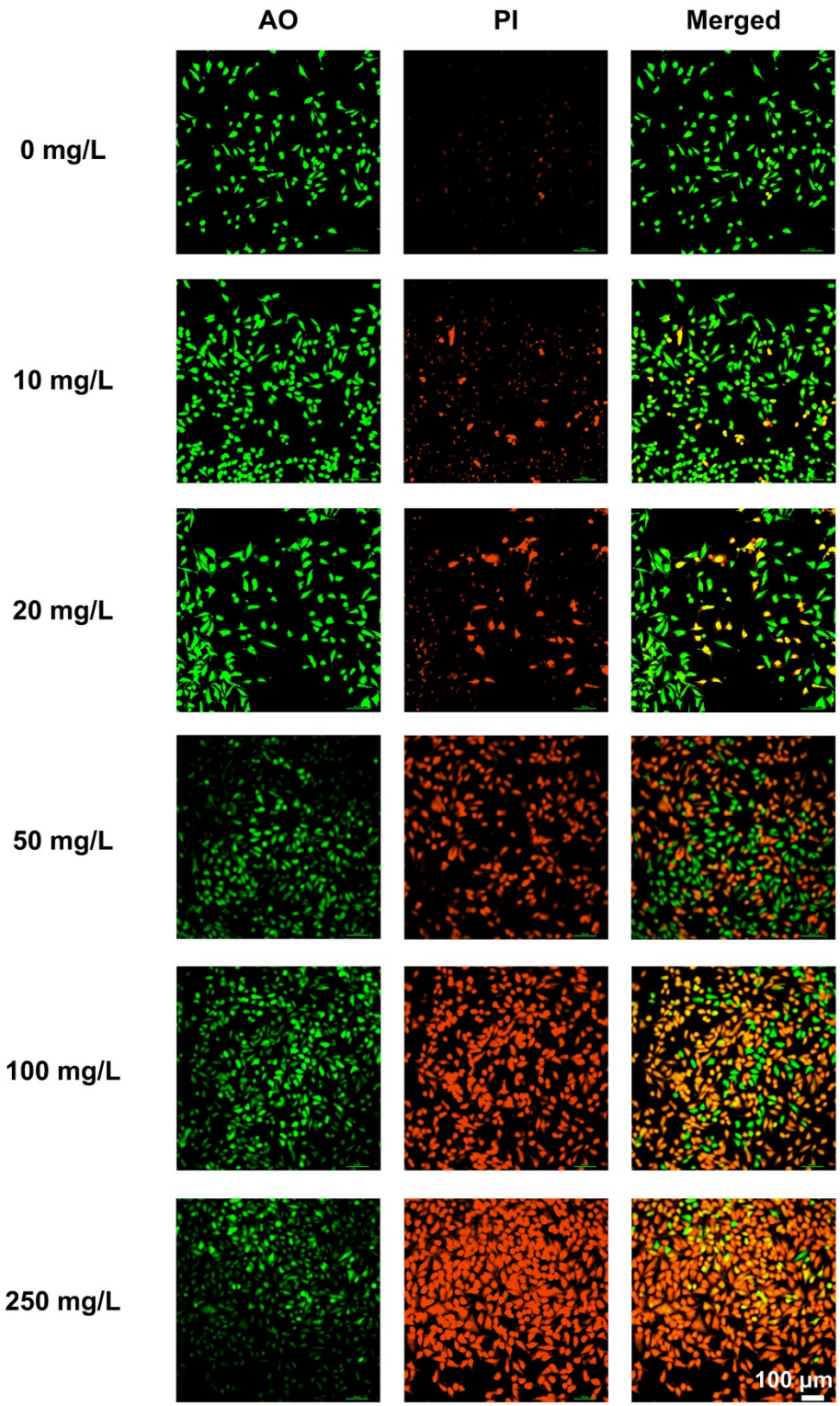

Fig. S17 Representative confocal laser scanning microscope images of HeLa cells co-stained with AO and PI after being cultured with different concentrations of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG for 24 hours under hypoxic condition with a 980 nm laser irradiation for approximately 5 min. For AO, λ_{ex} = 488 nm, λ_{em} = 500-550 nm; for PI, λ_{ex} = 562 nm, λ_{em} = 570-620 nm; scale bar = 100 μm.

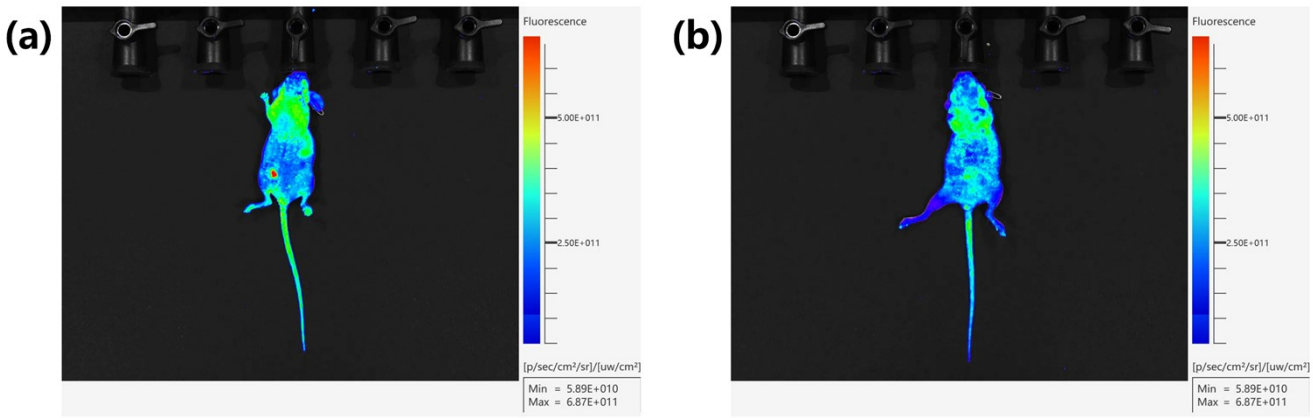

Fig. S18 *In vivo* fluorescence imaging of the SK-OV-3 tumor-bearing nude mice (a) at 48 h after intratumoral injection of LMNPs/HS-*β*-CD/TPE-2NHCOO-azo-TEG; (b) in the control group.

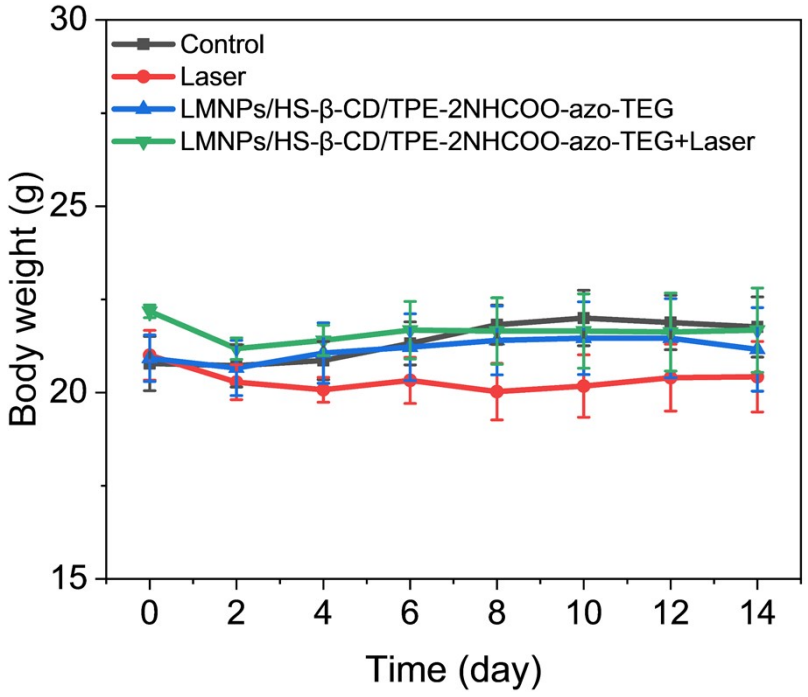

Fig. S19 The change of body weight of mice in different groups after treatments.

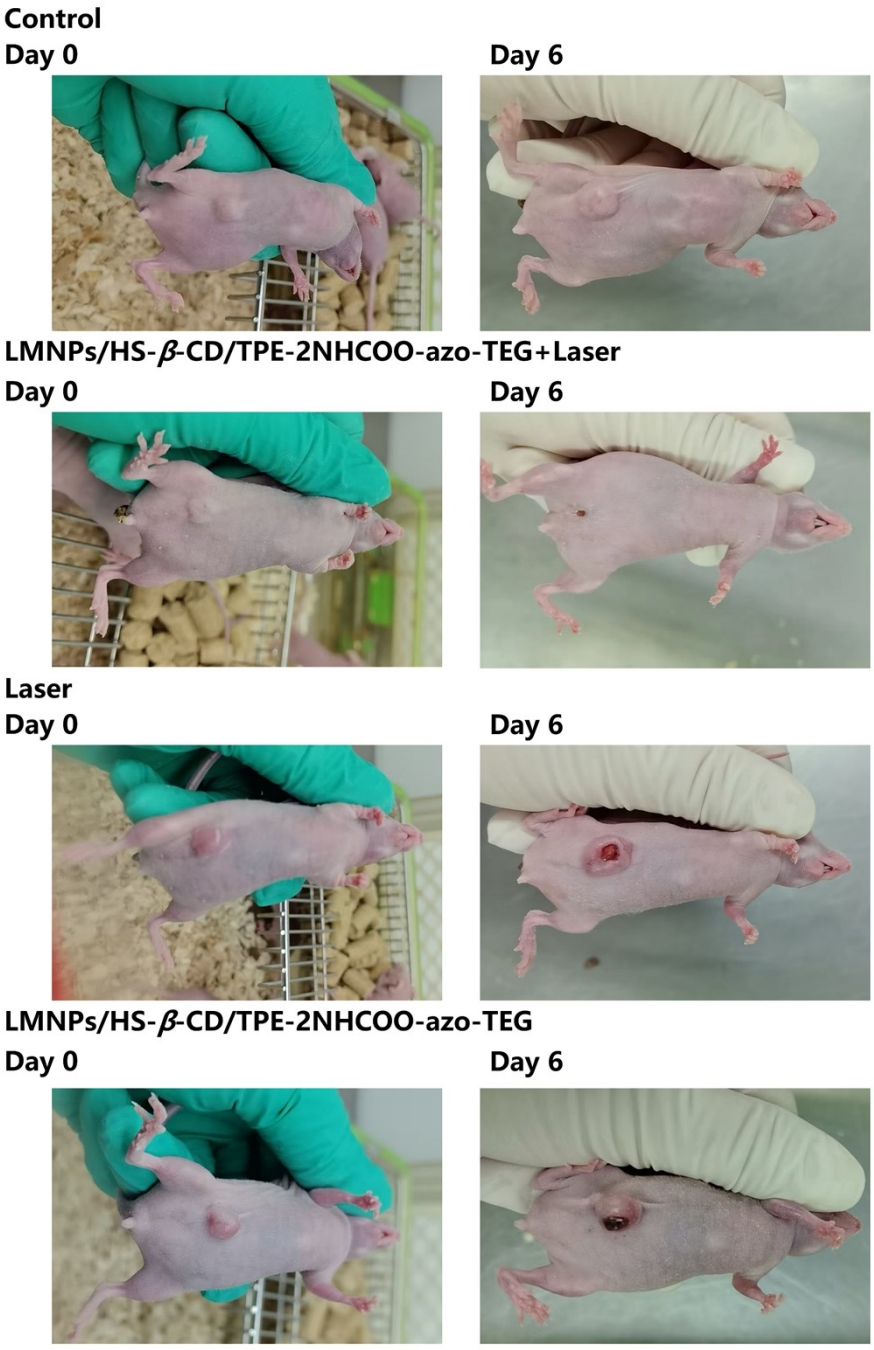

Fig. S20 Representative photos of tumor-bearing mice for different groups.

References

1 B. Wu, Y. Gao, W. Zhang and Y. He, *J. Polym Sci.*, 2024, **62**, 2539-2548.
2 T. Xue, K. Shao, J. Xiang, X. Pan, Z. Zhu and Y. He, *Nanoscale*, 2020, **12**, 7509-7513.
3 C. Fischbach, R. Chen, T. Matsumoto, T. Schmelzle, J. S. Brugge, P. J. Polverini and D. J. Mooney, *Nat. Methods*, 2007, **4**, 855-860.
4 L. Sun, G. Li, X. Chen, Y. Chen, C. Jin, L. Ji and H. Chao, *Sci. Rep.*, 2015, **5**, 14837.
5 H. Huang, P. Zhang, H. Chen, L. Ji and H. Chao, *Chemistry – A European Journal*, 2015, **21**, 715-725.