Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

H_2O_2-triggered controllable carbon monoxide delivery for photothermal augmented gas therapy

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Experimental section

Materials

4,4'-bis(octyloxy)-2,2'-bis(tributylstannyl)-5,5'-thiazole (IN) was obtained from SunaTech Corporation Ltd. (Suzhou, China), 4,4'-diethoxy-2,2'-bis(tributylstannyl)-5,5'-thiazole (DPP) was procured from Detong Optoelectronic Materials Technology Co., Ltd. (Shenzhen, China), Griess reagent kits was acquired from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), Poly(styrene-co-maleic anhydride) (PSMA, average Mn ~ 1,900) was obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China), Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 99%), (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzene and chloroform-d were procured from J&K Chemical Ltd. (Beijing, China). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anhydrous tetrahydrofuran (THF, 99.9%), phosphate-buffered saline (PBS), acridine orange (AO), and ethidium bromide (EB) were sourced from Sigma-Aldrich (Shanghai, China). Ultrapure water with a resistance of 18.25 $M\Omega \cdot cm^{-2}$ at 25°C was used for the entire study. Unless stated otherwise, all reagents were commercially sourced and used without the need for additional purification.

Characterization. We acquired ^1H NMR spectra using a 400 MHz Bruker NMR spectrometer in Fourier transform mode, employing deuterated chloroform (CDCl3) as the solvent. We determined the molecular weight and molecular weight distribution using gel permeation chromatography (GPC) 2410, with a polystyrene standard and calibration done with chloroform ($CHCl_3$). We measured the absorption spectra from 200 to 1200 nm using a UV-vis 2600 spectrophotometer with an ISR-2600Plus integrating sphere. A standard transmission electron microscope (TEM)（Hitachi H-600, Japan）was used to obtain images of the samples. Dynamic light scattering (DLS) and measured ζ-potential were performed using the Nanobrook 90Plus Zeta instrument (Brookhaven Instruments, USA).

Synthesis of conjugated polymer IN-DPP

The IN-DPP polymer with donor-acceptor (D-A) structure was synthesized through Stille coupling polymerization. In a 50 mL flask with a single neck, 282.9 mg (0.25 mmol) DPP and 246.3 mg (0.25 mmol) IN were dissolved in 10 mL toluene. To remove air, the solution was degassed with nitrogen (N_2) using a series of five freeze-pump-thaw cycles. Following that, $Pd(PPh_3)_4$ (5 mg, 0.004 mmol) was added to the reaction as a catalyst, and the reaction mixture was heated at 110 ° C for 48 hours under a nitrogen atmosphere. To eliminate the bromine end groups, 1 mL of (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzene (20 mg, dissolved in toluene) was added into the reaction mixture to react for 4 hours. Immediately, to remove stannyl end groups, 0.2 mL of bromobenzene was added to the reaction mixture and continued to react for four hours. Afterward, the reaction mixture was cooled to room temperature and gradually added to 150 mL of methanol, resulting in the formation of a precipitate. This was followed by filtration and a sequence of washes using ammonia solution, deionized water, ethanol, and acetone. Next, the product was suspended in 150 mL of acetone and stirred continuously for 24 h. In the end, the resulting product was gathered via filtration and subjected to vacuum drying at 50°C, resulting in the formation of a brown-black solid (279.26 mg, 80%).

Synthesis of mPEG(CO) for CO donor

Synthesis of mPEG(CO): Dodecacarbonyltriiron (50 mg) and mPEG-SH(MW ≈ 2000) (200 mg) were dissolved in 50 mL tetrahydrofuran (THF). The mixture was stirred at 50 °C for a duration of 2 h under a nitrogen atmosphere. Once the solution changed from dark blue to brown, cooled to -20 °C, and n-hexane was added to get a brown precipitate, and mPEG(CO) (105.3 mg) was obtained after washing with ether and drying. mPEG(CO) is a brown solid that can be dissolved in water and chloroform.

Synthesis of Compound RCO for Detecting CO

Synthesis of Compound 1. 0.38 mmol Rhodamine B and 3.8 mmol hydrazine hydrate were mixed in 50 mL 10 mL dichloromethane. The mixture was stirred at room temperature for 6 h. The yellow solid obtained was used directly for the next step of the reaction.

Synthesis of Compound RCO. Compound 1(45.6 mg, 0.1 mmol) and 50 uL 2-

pyridinecarboxaldehyde were dissolved by 10 mL anhydrous methanol. Next, the solution was stirred for 10 h. Subsequently, the solvent was evaporated through rotary evaporation. Then the yellow solid product was obtained after chromatography (silica gel, CH_2Cl_2: CH_3OH = 25:1). Yield: 39.2 mg (72 %). Molecular formula: $C_{34}H_{35}N_5O_2$. ^1H NMR (400 MHz, CDCl3) δ 8.46 (ddd, J = 4.9, 1.8, 1.0 Hz, 1H), 8.34 (s, 1H), 8.01 (ddt, J = 7.2, 4.7, 1.0 Hz, 2H), 7.61 (td, J = 7.9, 1.8 Hz, 1H), 7.53 – 7.41 (m, 2H), 7.15 – 7.08 (m, 2H), 6.55 (d, J = 8.8 Hz, 2H), 6.45 (d, J = 2.6 Hz, 2H), 6.24 (d, J = 2.6 Hz, 1H), 6.22 (d, J = 2.6 Hz, 1H), 3.31 (q, J = 7.1 Hz, 8H), 1.14 (t, J = 7.0 Hz, 12H).

Preparation of IN-DPP NPs and IN-DPPCO NPs

According to the previously reported method, all of the NPs were prepared by the reprecipitation. IN-DPP and PSMA were first dissolved in THF to prepare a 1 mg/mL IN-DPP solution and a 1 mg/mL PSMA solution, respectively. A mixture was prepared by combining 0.8 mL of the IN-DPP solution with 0.2 mL of the PSMA solution in 19 mL of THF. The mixture underwent vigorous stirring to guarantee a complete and consistent blend. Under ultrasonication, 3 mL of the mixed solution was swiftly added to 9 mL of deionized water, and then subjected to an additional 5 minutes of ultrasonication. Afterward, THF was removed using a stream of nitrogen gas. The nanoparticles were purified by passing them through a 0.22 μm membrane filter via filtration and then concentrated to a concentration of 1 mg/mL, and stored for later use. The preparation of IN-DPPCO NPs is the same as above, except the amphiphilic functional polymer PSMA is replaced by mPEG(CO).

Photothermal performance of IN-DPP NPs and IN-DPPCO NPs

To evaluate the efficiency of photothermal conversion, 0.5 mL of an aqueous solution containing IN-DPP NPs (referred to as IN-DPPCO NPs) was introduced into an EP tube. Next, the nanoparticle solution underwent irradiation with an 808 nm laser（1 W/cm²）until it achieved thermal equilibrium. After shutting down the laser, the temperature of the solution was allowed to cool down naturally to reach room temperature. The photothermal conversion efficiency (η) was calculated using the following formula:

$$\eta = \frac{hs\left(T_{max} - T_{min}\right) - Qdis}{I\left(1 - 10^{-A_{808}}\right)} \quad (1)$$

In this formula, h represents the heat transfer coefficient of IN-DPP NPs (IN-DPPCO NPs); S denotes the surface area of the container; T_{max} and T_{min} are the maximum and minimum (room) temperatures during the process, respectively; Q_{dis} signifies the heat dissipation of water. I stand for the laser power density, which was fixed at 1 W/cm², while A represents the absorbance of IN-DPP NPs (IN-DPPCO NPs) at 808 nm. The value of hs was computed according to:

$$hs = \frac{cm}{\tau_s} \quad (2)$$

Where τ_s is the thermal time constant for heat transfer of the solution, which can be obtained from the measured value of τ_s in Figure 16 ($\tau_{s,\text{ IN-DPPCO NPs}} = 176.6$, $\tau_{s,\text{ IN-DPP NPs}} = 225.4$); m represents the mass of deionized water used to dissolve the nanoparticles (0.5 g), and c denotes the heat capacity of water (4.2 J/g). The calculated photothermal conversion efficiencies for IN-DPPCO NPs and IN-DPP NPs were found to be 41.5% and 29.1%, respectively.

CO detection

The release of CO from IN-DPPCO NPs under various concentrations of H_2O_2 was studied using the hemoglobin assay. To quantify the released CO, UV-vis spectrophotometry was utilized to measure the transformation of hemoglobin (Hb) into carboxyhemoglobin (HbCO). Initially, Hb from bovine red blood cells was dissolved in phosphate buffered saline (PBS, pH=7.4). Subsequently, it was subjected to reduction using sodium dithionite (1.6 mg) within a nitrogen-rich environment. The aqueous solution containing IN-DPP-mPECO was purged with nitrogen and subsequently combined with the Hb solution. Without delay, 3 μL H_2O_2 was mixed with 3 mL of the mixture solution and thoroughly blended. UV-vis absorption spectra (350-600 nm) were collected under near-infrared irradiation. To enhance accuracy and eliminate potential interferences, we monitored two distinct stable absorption peaks at 410 nm and 430 nm, corresponding respectively to HbCO and Hb, and quantified the

transformation of Hb into HbCO. The released CO concentration was then calculated:

$$C_{CO}=\frac{528.6\times I_{410nm}-304\times I_{430nm}}{216.5\times I_{410nm}+442.4\times I_{430nm}}\times C_{Hb} \qquad (3)$$

Fluorescence analysis of CO release in cells. The Confocal fluorescence analysis assay of IN-DPPCO NPs was conducted on 4T1 cells and 293T cells respectively. The cells were cultivated in a 24-well plate (100,000 cells per well, 150 μL of DMEM medium). Add 4 groups (PBS, IN-DPP NPs, mPEG(CO) and IN-DPPCO NPs with 60 μg/mL to the above medium. After 40 mins, add RCO of 40 μg/mL and incubate for another 20 mins. Wash off the residual culture medium with PBS, then record the fluorescence signal between 560-600 nm under a fluorescence microscope.

In vitro cytotoxicity

MTT Assay： MTT assays were performed to determine the in vitro cytotoxicity of the nanoparticles. Four cell lines, MCF-7, HeLa, HepG2, and 293T were individually cultured in 96-well plates using DMEM cell medium at 37°C in a 5% carbon dioxide incubator for a duration of 24 h. This medium consisted of 10% FBS, 1% penicillin, and 1% streptomycin. Following that, DMEM was replaced by DMEM culture medium containing nanoparticles (20 - 150 μg/mL). Following an additional 12 h of incubation, the cells underwent irradiation for 10 minutes (808 nm laser, 1.0 W/cm²). some cells were exposed to the laser as experimental groups while others were not as control groups. After incubating for 24 hours at 37°C in a light-free environment, Each well received an addition of 20 μL of MTT solution, which was prepared at a concentration of 5 mg/mL in PBS. Four hours later, the medium was substituted for 100 μL of dimethyl sulfoxide. Cell viability was assessed by measuring the absorbance value at 490 nm using a microplate reader (BioTeK PowerWave XS, USA).

AO&EB Tests: HeLa cells were maintained in 12-well plates at a consistent cell density for a duration of 12 h. Following that, the cells underwent a series of treatments, which included PBS; PBS + Laser; IN-DPPNPs; IN-DPPNPs + Laser; IN-DPPCO NPs; and IN-DPPCO NPs + Laser (laser: 808 nm, 1.0 W/cm^2, 10 min). Subsequently, these treated cells were cultured for a further 12 hours, and then washed three times with PBS. The pre-prepared AO/EB solution was added into each well, and the plates were

violently shaken to achieve uniform distribution. In the end, a fluorescence microscope was used to capture the images of stained cells.

In vivo antitumor activity and biosafety

All animal experiments were approved by the Animal Management and Ethics Committee of Xinxiang Medical University. Before starting the treatment, a tumor mouse model was established through subcutaneous injection of 4T1 cells into the right hind limb of each mouse two weeks in advance. When the tumors had grown to a size ranging between 90-120 mm^3, Mice were randomly and equally divided into six groups(n=5). Each group was subjected to a distinct treatment regimen as follows: (1) PBS; (2) PBS with Laser; (3) IN-DPP NPs; (4) IN-DPP NPs with Laser; (5) IN-DPPCOs; and (6) IN-DPPCOs with Laser. The laser irradiation parameters were configured to utilize 808 nm wavelength and an intensity of 1.0 W/cm^2 for 10 minutes. We maintained a constant nanoparticle concentration of 100 μg/mL, and each mouse received an injection dose of 1.5 mg/kg by intratumoral injection. The laser irradiation employed a wavelength of 808 nm, an intensity of 1.0 W/cm^2, and a duration of 10 min. We maintained a consistent nanoparticle concentration of 100 μg/mL, and each mouse received an injection dosage of 1.5 mg/kg. Throughout the treatment, both the tumor volumes and the body weights of the mice were regularly assessed. The tumor volumes were calculated using the formula: $V = (L/2) \times (W^2)$. In the end, the mice were humanely euthanized to facilitate subsequent evaluation. Tumor tissues and vital organs were extracted and subjected to H&E staining analysis. The images of the tissue sections were captured by a fluorescence microscope.

Supporting Figures

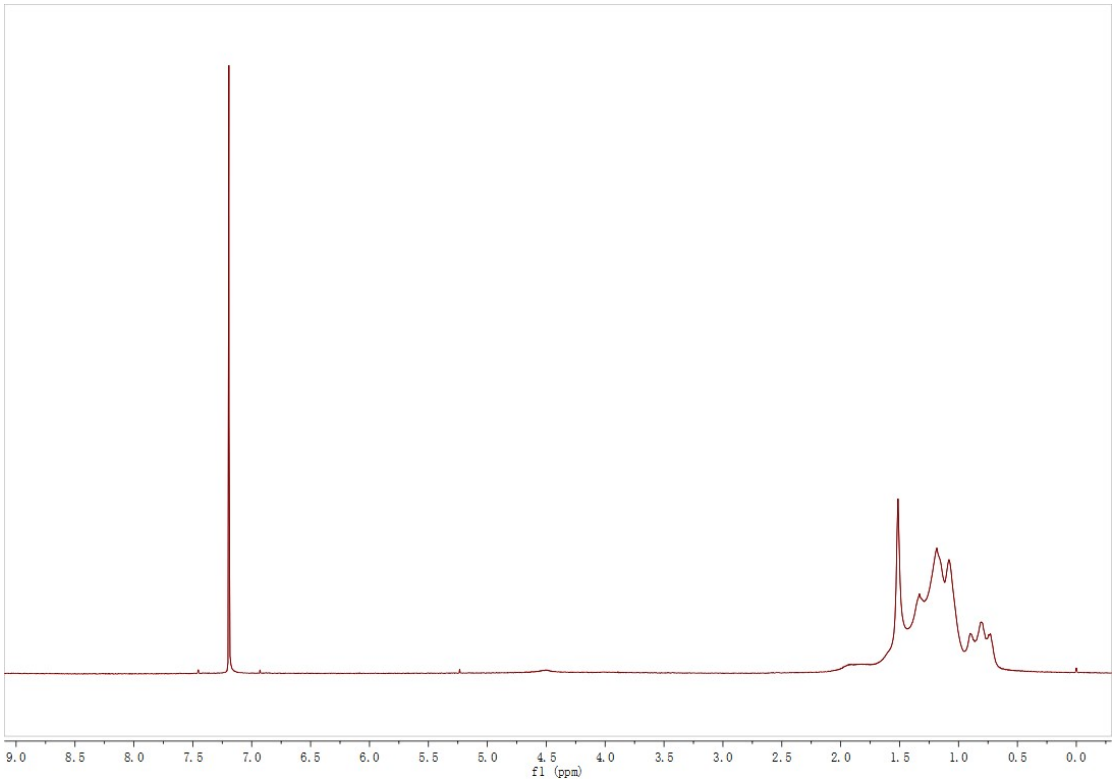


Figure S1. ^{1}H NMR spectra of IN-DPP polymer.

GPC Results

	Distribution Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Mz/Mw	Mz+1/Mw
1		33683	94934	54141	228575	422842	2.81843	2.407742	4.454083

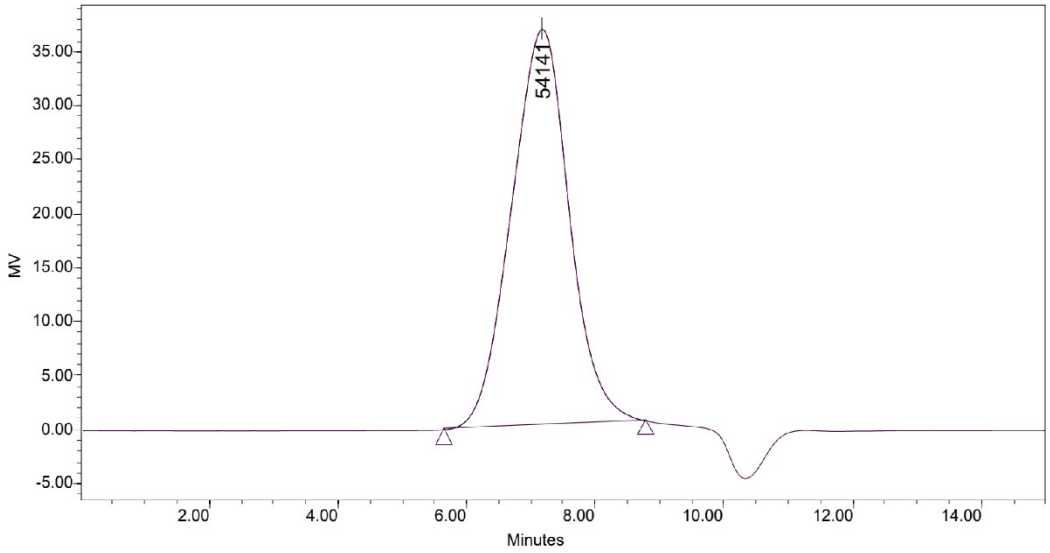


Figure S2. Gel permeation chromatography (GPC) of IN-DPP polymer.

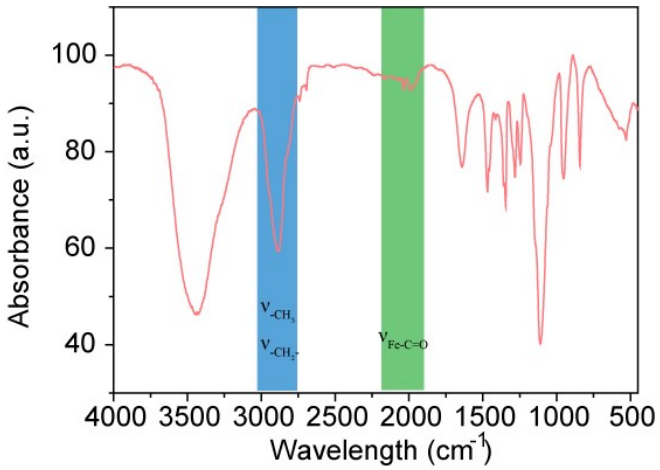


Figure S3. Fourier transform infrared spectroscopy of mPEG(CO) and its components.(ν_{-CH3}/ ν_{-CH2-}≈ 3020-2760 cm^{-1},$\nu_{Fe-CO=O}$ ≈ 2150 -1900 cm^{-1}).

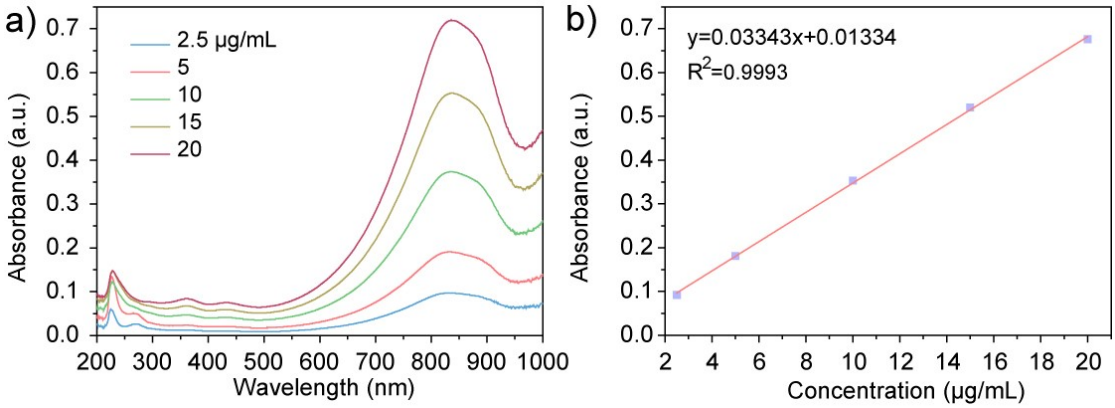


Figure S4. a) the absorption spectra of different concentrations IN-DPP. b) Linear relationship between absorption and concentration of IN-DPP at 808 nm.

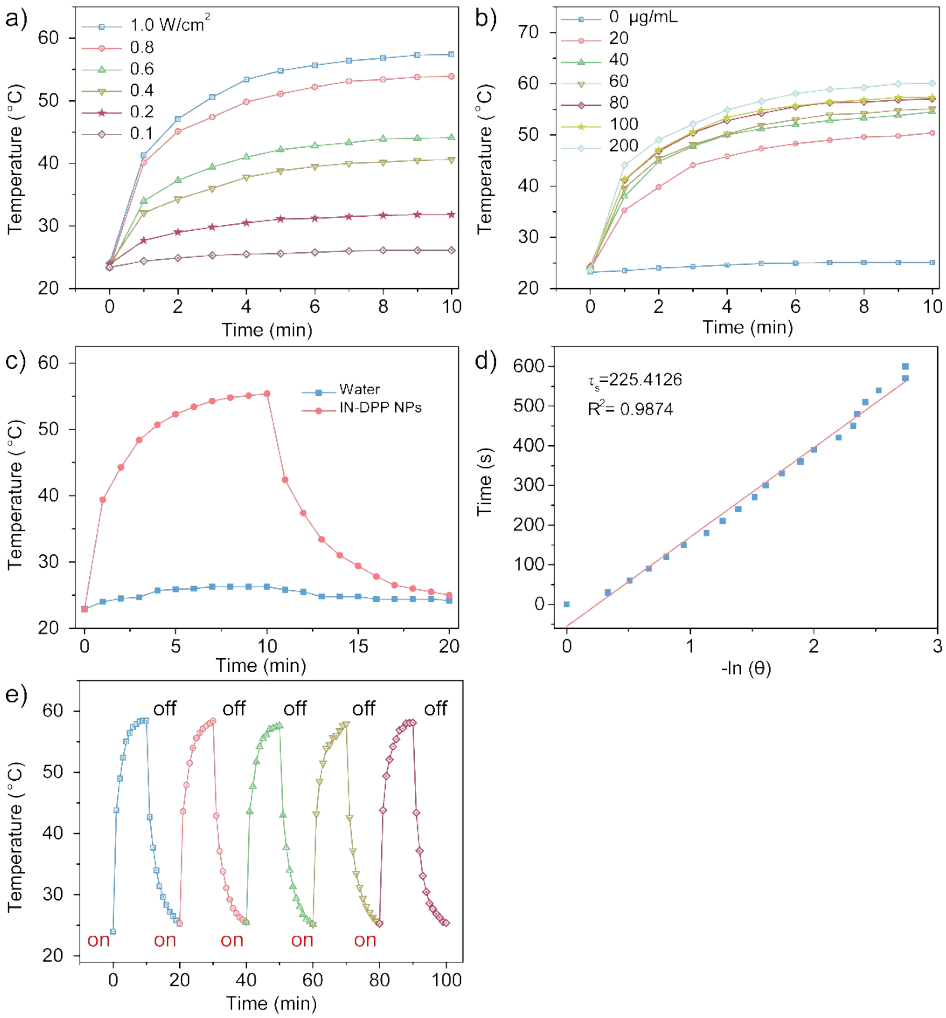
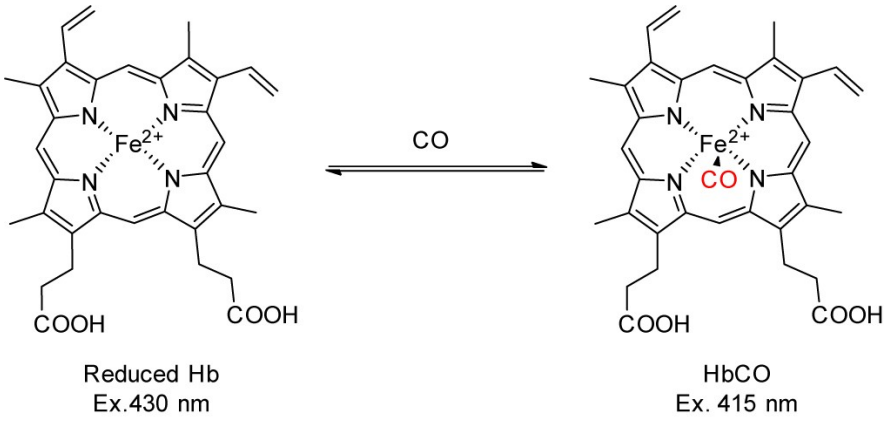


Figure S5. Photothermal properties of IN-DPP NPs. (a) Concentration-dependent photothermal curves of IN-DPP NPs under 808 nm laser irradiation. (b) Photothermal heating curves of IN-DPP NPs dispersions (100 μg/mL) irradiated using an 808 nm laser at varied power densities (0.1,0.2,0.6,0.8,1.0 W/cm^2). (c) The photothermal effect of IN-DPP NPs aqueous solution (100 μg/mL) excited with 808 nm laser. (d) Linear relationship curves between time (s) versus − ln θ based on panel e. (e) Temperature elevation of CPNPs dispersion under five on/off cycles.



Scheme S1. Detection of CO release of IN-DPPCO NPs.

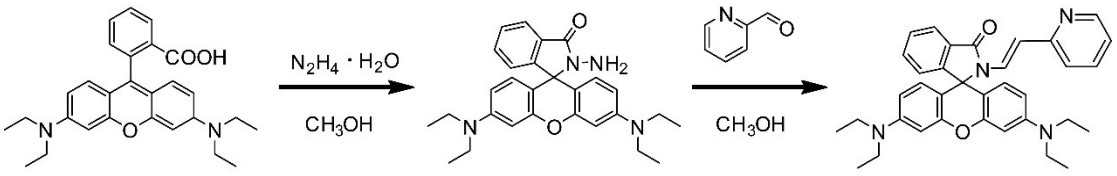


Figure S6. The synthetic route of RCO.

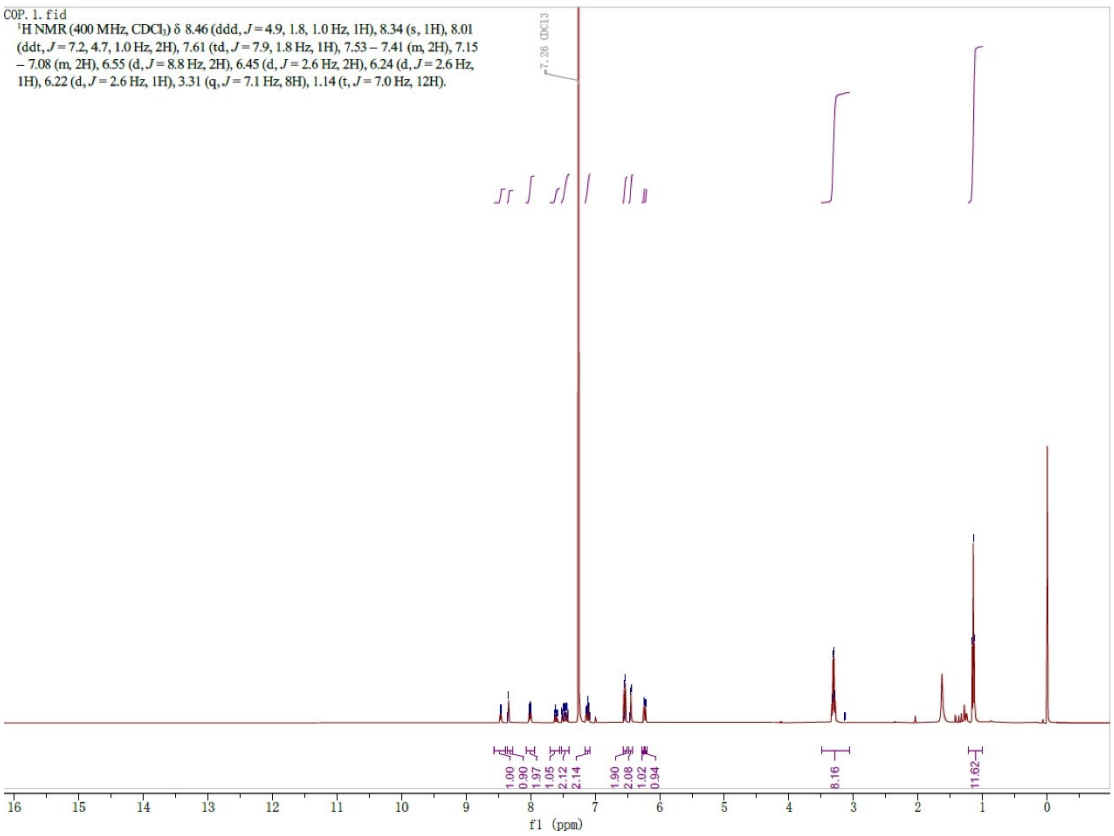
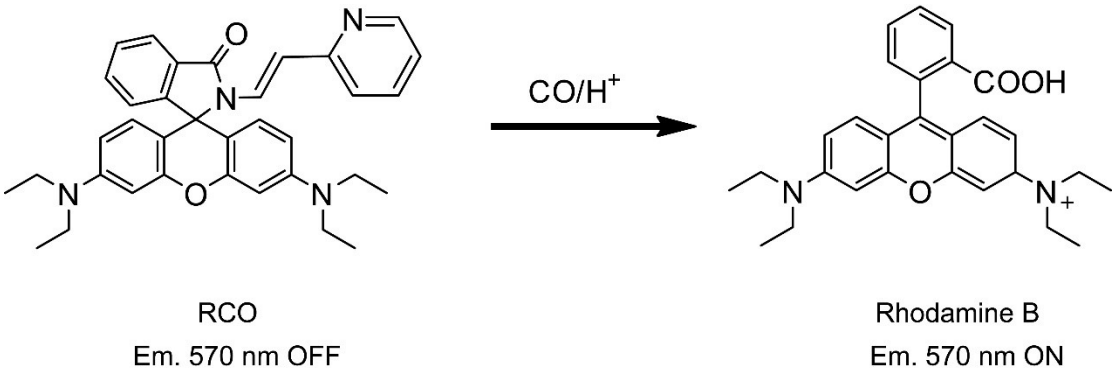


Figure S7. ^{1}H NMR spectra of RCO.



RCO
Em. 570 nm OFF

Rhodamine B
Em. 570 nm ON

Scheme S2. Detection of CO release of IN-DPPCO NPs in cells.

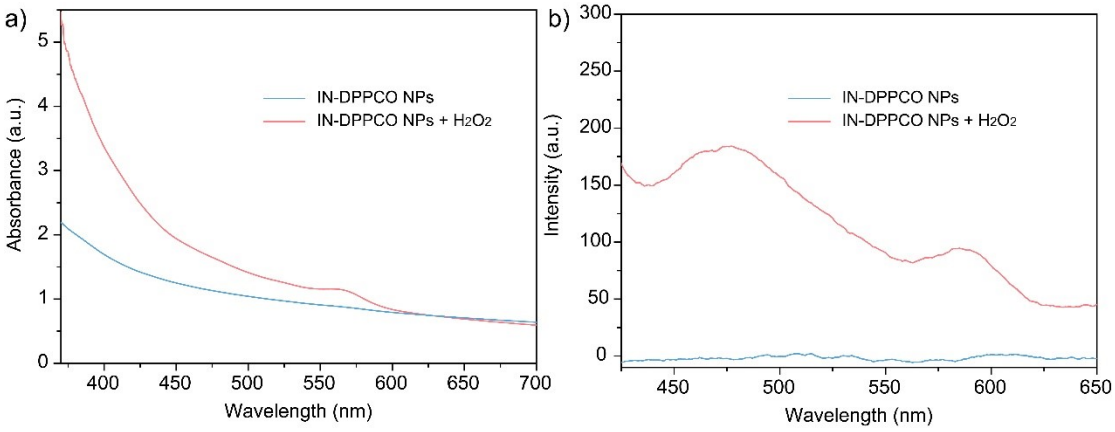


Figure S8. (A) The change of UV spectrum and (B) the FL intensity of RCO shows the release of CO in the aqueous IN-DPPCO NPs solution.

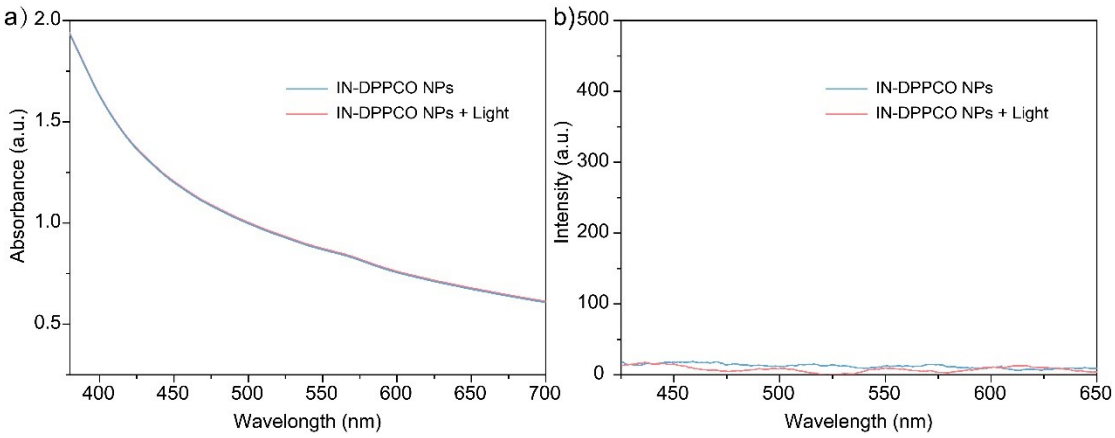


Figure S9. (A) The UV absorption and (B) the fluorescence emission curve of the CO release profiles with and without light exposure.

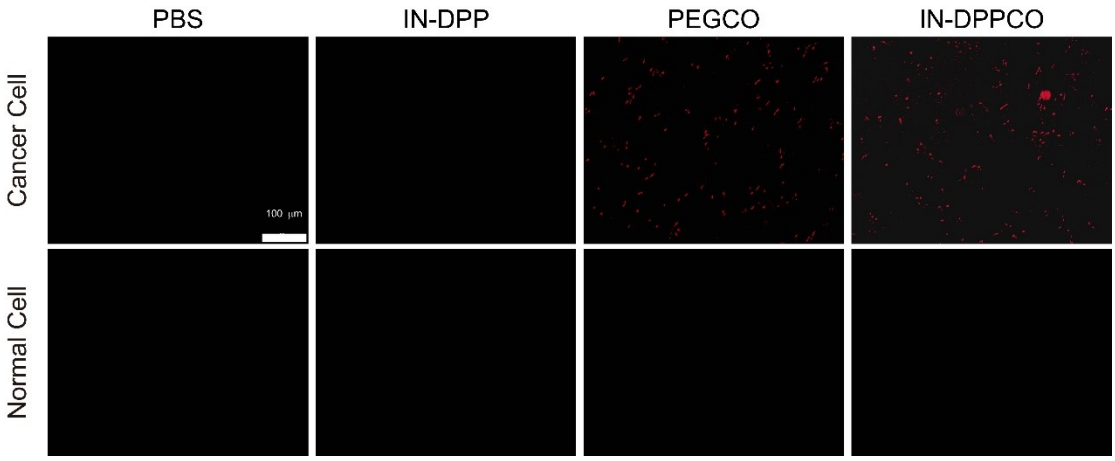


Figure S10. The fluorescence microscope in cancer cells and normal cells. Scale bar = 100 μm.

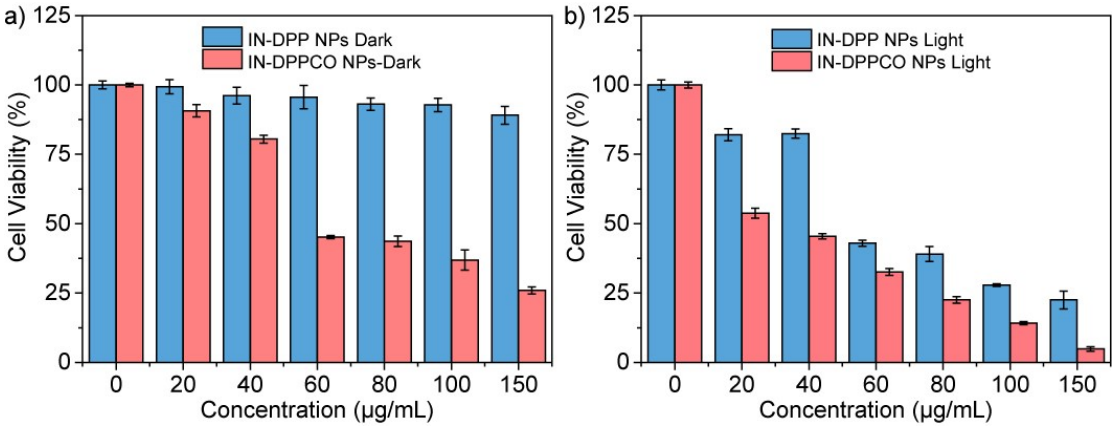


Figure S11. The cellular test of IN-DPP NPs and IN-DPPCO NPs. a) MTT assay of HepG2 cells treated with nanoparticles at various concentrations of 0, 20, 40, 60, 80 and 100 μg/mL for dark to assess the viability of the cells. Error bars denote the standard deviation (n = 6). b) HepG2 cells were treated with different concentrations of nanoparticles (0~100 μg/mL) for light to assess the cells' viability. Error bars denote the standard deviation (n = 6).

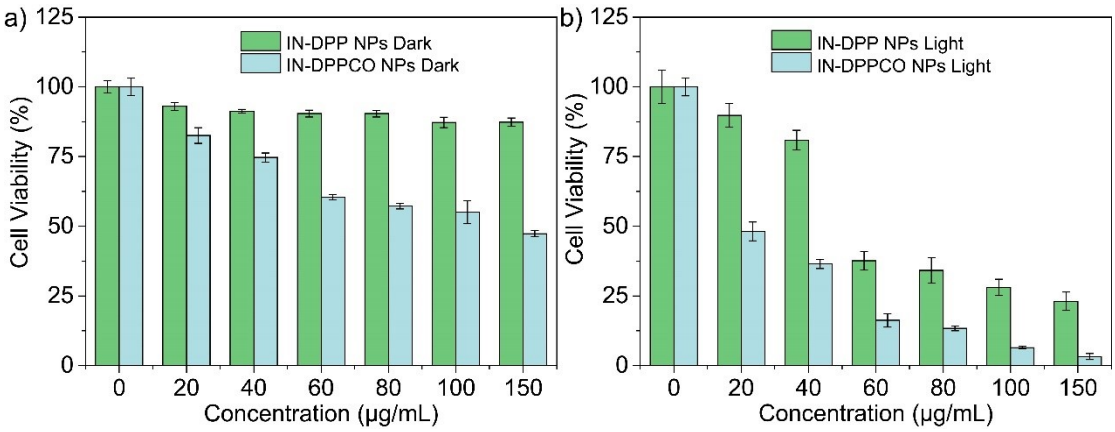


Figure S12. The cellular test of IN-DPP NPs and IN-DPPCO NPs. a) MTT assay of MCF-7 cells treated with nanoparticles at various concentrations of 0, 20, 40, 60, 80 and 100 μg/mL for dark to assess the viability of the cells. Error bars denote the standard deviation (n = 6). b) MCF-7 cells were treated with different concentrations of nanoparticles (0~100 μg/mL) for light to assess the cells' viability. Error bars denote the standard deviation (n = 6).

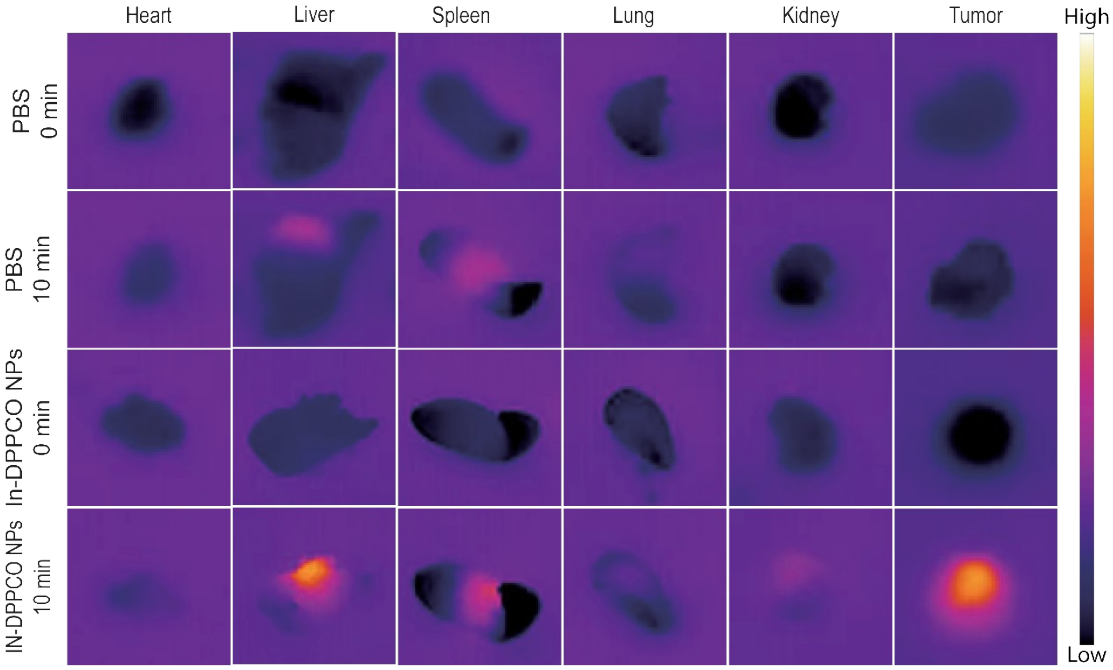


Figure S13. Ex vivo thermal imaging of dissected main organs and primary tumors of the mice sacrificed after intratumor injection of IN-DPPCO NPs and PBS.