Electronic Supplementary Information

Choline phosphate lipid-hitchhiked near-infrared BODIPY nanoparticles for enhanced phototheranostics

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Materials

2-Thenaldehyde and 2-methyl-1H-pyrrole were purchased from Suzhou Boke Chemistry Co., Ltd.. 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 4-(diethylamino) benzaldehyde were purchased from Tianjin Seans Biochemical Technology Co., Ltd.. Trifluoroacetic acid (TFA) was purchased from Adamas Reagent Co., Ltd.. Reactive Oxygen Species Assay Kit and Calcein/PI Cell Viability were purchased from Beyotime Biotechnology Co., Ltd.. Annexin V-FITC/PI double staining cell apoptosis detection kit was purchased from Jiangsu KeyGEN Biotechnology Co., Ltd..

Characterization

Analytical balance (XS105DU) and Rainin Pipettes from METTLER TOLEDO were used to quantify solid and liquid, respectively. The morphology of the nanoparticles was measured by transmission electron microscopy (TEM) performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV. Size and size distribution of the nanoparticles were determined by Malvern Zeta-sizer Nano. Absorption spectra were obtained using a Shimadzu UV-2450 PC UV-vis spectrophotometer. Fluorescence intensity tests were performed using Perkin Elmer LS-55 fluorospectrophotometer. Confocal laser scanning microscope (CLSM) images were obtained from a Zeiss LSM 700 (Zurich, Switzerland).

Methods

Synthesis of NIR BODIPY. Compound 1 was synthesized according to the reported method.[1] In detail, 2-thenaldehyde (1.12 g, 10 mmol) and 2-methyl-1H-pyrrole (1.78 g, 22 mmol) in 500 mL dichloromethane (CH_2Cl_2) were stirred under N_2 followed by added with trifluoroacetic acid (TFA, 5 drops). The mixture was stirred overnight then added with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 2.27 g, 10 mmol) and stirred for 2 h. Et_3N (10 mL) and $BF_3 \cdot Et_2O$ (12 mL) were added under ice-cold conditions. The mixture was stirred for 8 h and extracted by water. The organic phase was treated with Mg_2SO_4, then filtered and evaporated. Compound 1 with a yield of

14% was obtained by silica gel column chromatograph (gradient elution: CH_2Cl_2: n-hexane = 1:1).

Compound 1 (302 mg, 1 mmol) and 4-(diethylamino) benzaldehyde (708 mg, 4 mmol) in 30 mL toluene were stirred followed by added with piperidine (2.7 mL) and acetic acid (0.7 mL). The mixture was refluxed (120 °C) for 3 h in presence of Dean-Stark apparatus. The solvent was removed and the crude product was purified by silica gel column chromatograph (gradient elution: CH_2Cl_2). At last, the product NIR-BODIPY (compound 2) with a yield of 32% was obtained by settling in n-hexane and filtration.

Synthesis of DPCP. The synthesis of DPCP follows the previously reported method.[2, 3]

Preparation of DBNPs. BODIPY (1 mg) and DPCP (9 mg) were completely dissolved in tetrahydrofuran (THF, 4 mL) and then added dropwise to deionized water (10 mL) with vigorous stirring at ambient temperature. The organic solvent was removed by volatilization and subsequent dialysis with a dialysis bag (MWCO 3500) for 24 h. After centrifugation (3500 r/min, 5 min) to remove big aggregates, DBNPs were obtained. The concentration of BODIPY in DBNPs was determined by UV-vis spectrophotometer. The BODIPY loading capacity was calculated as the equation:

$$DLC\ (wt\%) = \frac{free\ BODIPY\ weight\ in\ the\ nanoparticles}{weight\ of\ nanoparicles} \times 100\%$$

Intermolecular interactions between BODIPY and DPCP. DBNPs were incubated with Triton X-100, sodium dodecyl sulfate (SDS), and urea, respectively, at 37 °C for 12 h. The corresponding size and PDI were recorded by dynamic light scattering (DLS).

***In vitro* stability of DBNPs.** To investigate the *in vitro* stability, DBNPs were cultured with water and Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C for different time. The real-time variation of hydrodynamic particle sizes was recorded by DLS.

***In vitro* photothermal effects**. The photothermal performance of DBNPs in water (200

μL) was recorded with laser irradiation. The temperature changes of DBNPs at different concentrations (BODIPY concentration: 0-50 μg mL^{-1}) at the power intensity of 0.8 W cm^{-2}, and at BODIPY concentration of 12.5 μg mL^{-1} upon 808 nm laser irradiation with different power intensities (0.2-0.8 W cm^{-2}) were recorded. The irradiation time continues for 5 min. The photothermal conversion efficiency (PCE) was calculated using the reported methods by recording the temperature of DBNPs (BODIPY concentration: 12.5 μg mL^{-1}) after 5 min of 808 nm laser irradiation (0.8 W cm^{-2}) during the cooling period.[4] In addition, we tested the photothermal stability of DBNPs (BODIPY concentration: 12.5 μg mL^{-1}) after 4 circles of heating and natural cooling by 808 laser irradiation (0.8 W cm^{-2}). All temperatures were recorded every 10 s. Finally, we compared the absorption and color changes of DBNPs and clinically approved indocyanine green (ICG) before and after irradiation with 808 nm laser (0.8 W cm^{-2}, 5 min).

Singlet oxygen detection. The ability of singlet oxygen ($^1O_2$) generation of nanoparticles was tested using 1,3-diphenylisobenzofuran (DPBF) as the indicator. DBNPs (BODIPY concentration: 0.75 μg mL^{-1}, 2 mL) were mixed with DPBF (1 mg mL^{-1}, 35 μL), and then the above mixture solutions or simple DPBF solution were irradiated by 808 nm laser (0.8 W cm^{-2}). The change of absorption spectra was recorded every 10 s intervals.

Cell culture. HeLa (human cervical carcinoma) and 4T1 (mouse breast cancer) cells were grown in DMEM (Cytiva) and PRMI-1640 medium, respectively, supplemented with 10% FBS (Kangyuan). All cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Cellular uptake. The cellular uptake of DBNPs was examined by using CLSM. HeLa/4T1 cells were seeded in 6-well plates (a clean cover slip was put in each well) at the density of 2×10^5 cells per well and allowed to adhere for 24 h. And then the medium was replaced with DBNPs diluted with fresh culture medium to BODIPY concentration of 2 μg mL^{-1}. Thereafter cells were incubated for additional 2 h and 6 h at 37 °C, and 6 h at 4 °C. Subsequently, the supernatant was removed and the cells were

washed gently three times with PBS (pH 7.4), fixed with 4% paraformaldehyde (1 mL/ each well) for 10 min and washed thrice with cold PBS. Hoechst 33258 was employed to stain the cell nuclei. And then the cells were observed by CLSM.

The intracellular ROS generation. The intracellular ROS generation of DBNPs was studied using DCFH-DA as a probe. HeLa/4T1 cells were seeded in 24-well plates (a clean cover slip was put in each well) at the density of 1×10^5 cells per well and allowed to adhere for 24 h. And then the medium was replaced with DBNPs diluted with fresh culture medium to BODIPY concentration of 20 μg mL^{-1}. Thereafter cells were incubated for additional 6 h at 37 ^oC, and the cells was irradiated by the 808 nm laser (0.2 W cm^{-2}, 10 min). Subsequently, the supernatant was removed and the cells were washed gently three times with PBS (pH 7.4), stained with DCFH-DA diluted with serum-free media at a ratio of 1:1000 for 30 min in dark. Then the cells were washed thrice with serum-free media (DMEM). Finally, the cells were imaged by CLSM.

Cytotoxicity assays. The cytotoxicity of DBNPs without or with irradiation was examined via MTT protocols. Briefly, HeLa/4T1 cells harvested in a logarithmic growth phase were seeded in 96-well plates at an initial density of 5×10^3 cells/well and incubated in 100 μL DMEM and PRMI-1640 medium at 37 ^oC in 5% CO_2 atmosphere for overnight, respectively. After removing incubation medium, DBNPs (100 μL) dispersions diluted with cell culture media to the desired concentration (BODIPY concentration: 5 to 15 μg mL^{-1}) were added to cell wells. Cells treated with media free of drug were used as control. After 6 h of incubation, the cells were irradiated by 808 nm laser (0.8 W cm^{-2}, 5 min) for light group. After additional incubation for 24 h, 20 μL of MTT in PBS solution with the concentration of 5 mg mL^{-1} was added and the plates were incubated at 37 ^oC for another 4 h. After careful removal of the culture medium supernatant, 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed violet formazan crystals. Finally, the plates were shaken for 3 min, and the absorbance of violet product was quantified at 490 nm by a microplate reader.

Calcein-AM/PI staining tests. To further demonstrate the phototherapeutic effect of DBNPs, HeLa/4T1 cells were stained with the calceinAM/propidium iodide (PI) to

identify dead (red) and live (green) cells. Shortly, HeLa/4T1 cells were incubated with DBNPs with equivalent BODIPY concentration of 12.5 μg mL^{-1}. PBS was set as negative control. Next, the light group was incubated for 6 h and then irradiated by 808 nm laser (0.8 W cm^{-2}) for 5 min. After additional incubation for 24 h, the medium was removed and cells were washed gently. Then cells were incubated with Calcein-AM/PI for 30 min at room temperature, subsequently imaged by the fluorescence microscope.

Cell apoptosis and necrosis detection assays. The cell early and late apoptosis induced by phototherapy of DBNPs were quantified by flow cytometry (FCM). Briefly, HeLa cells were cultured with DBNPs with equivalent BODIPY concentration of 12.5 μg mL^{-1}. PBS was set as negative control. Next, the light group was incubated for 6 h and then irradiated by 808 nm laser (0.8 W cm^{-2}) for 5 min. After additional incubation for 24 h, cells were washed, harvested and collected, and stained with Annexin V-FITC and PI detection kit for about 15 min. Finally, the quantitative analysis of apoptosis and necrosis was determined by FCM.

***In vivo* fluorescence imaging of DBNPs.** All animal experiments have been approved (No. 2022-0006) by the Animal Welfare and Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, and carried out according to the NIH guidelines for the care and use of laboratory animals (NIH publication No. 85-23 Rev. 1985). Female Balb/c mice were obtained from Liaoning Changsheng Biotechnology Co., Ltd. and raised under the required conditions. Mice with subcutaneous 4T1 xenograft tumors were utilized as an animal modal. 4T1 cells were subcutaneously inoculated into the lateral aspect of the anterior left limb of mice (2×10^6 cells in 0.1 mL PBS). The tumor-bearing mice were intravenously injected with DBNPs at BODIPY dose of 3.5 mg kg^{-1} (drug weight/body weight). *In vivo* near infrared fluorescence (NIRF) imaging of the mice was conducted 0, 2, 6, 12, 24, 36, 48, 72 and 96 h post injection. Fluorescence images of mice and excised major organs at different injection times were obtained by *in vivo* imaging system.

In *vivo* phototherapy efficacy and biosafety of DBNPs. Mice with subcutaneous 4T1

xenograft tumors were utilized as an animal modal to evaluate antitumor effects. 4T1 cells were subcutaneously inoculated into the lateral aspect of the anterior left limb of mice (2×10^6 cells in 0.1 mL PBS). The tumor-bearing mice were randomly divided into four groups (n = 5): PBS, PBS + L, DBNPs and DBNPs + L. Then the mice in DBNPs and DBNPs + L groups were intravenously injected with DBNPs at BODIPY dose of 3.5 mg kg^{-1}. At 36 h post-injection, the mice in PBS + L and DBNPs + L groups were irradiated by 808 nm laser at power intensity of 0.8 W cm^{-2} for 10 min. The temperature changes of tumors during the irradiation were recorded by an Infrared thermal imager every two minutes. At the designed time, the body weight and tumor volume of mice were measured. On day 14, mice were sacrificed, and the tumor was excised to intuitionally evaluate the tumor inhibition. Main organs (heart, liver, spleen, lung, and kidneys) and tumor were collected, fixed in 4% paraformaldehyde solution, and then embedded in paraffin, sliced, and stained with hematoxylin and eosin (H&E) to evaluate potential toxicity for main organs and apoptosis degrees for cancer cells.

Supplementary Figures

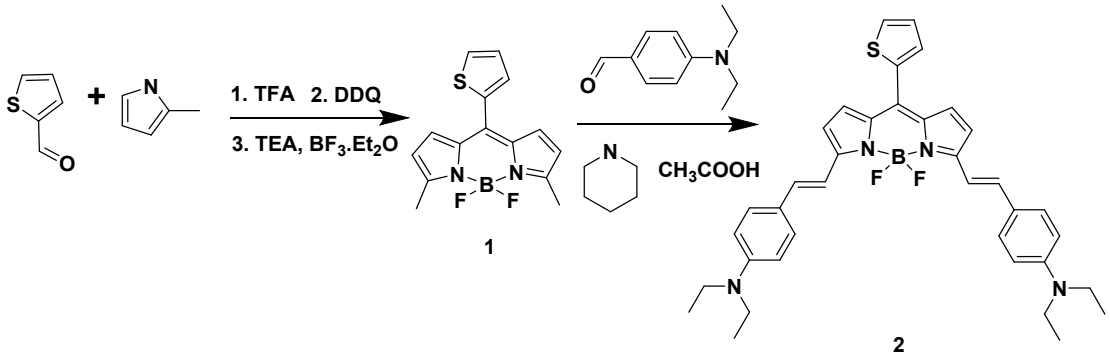

Fig. S1. The synthetic route of NIR-BODIPY (compound 2).

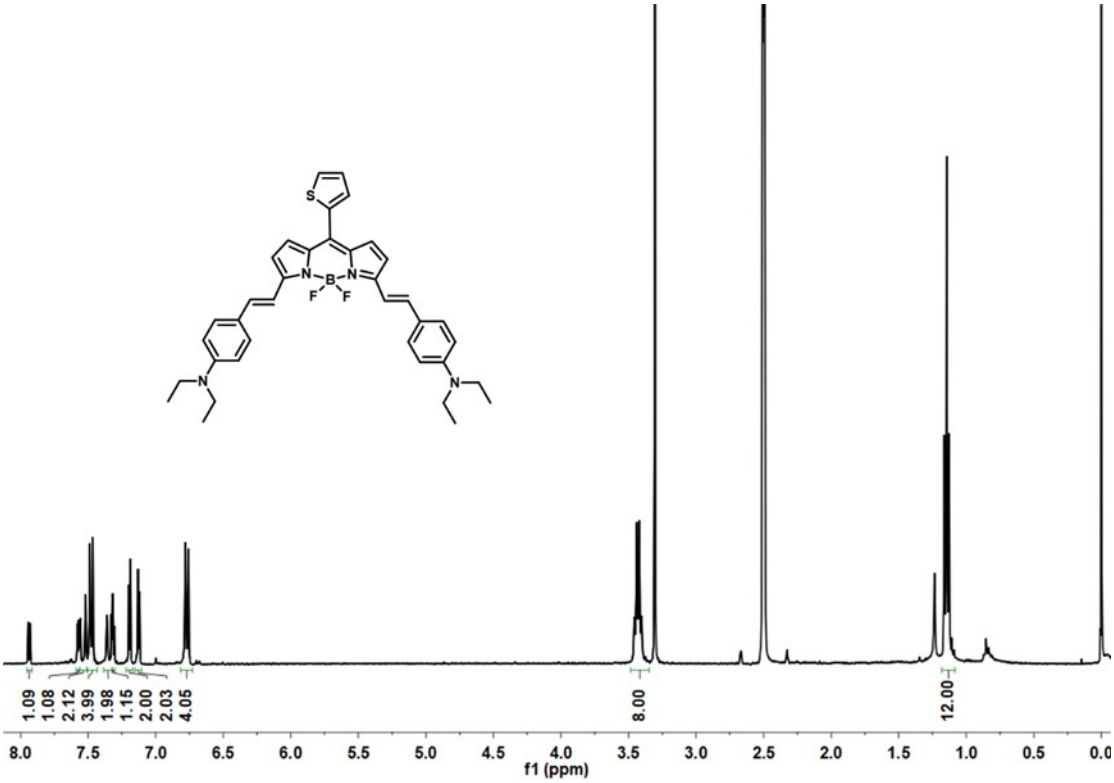

Fig. S2. ^{1}H NMR spectrum of NIR BODIPY in DMSO-d6 (compound 2).

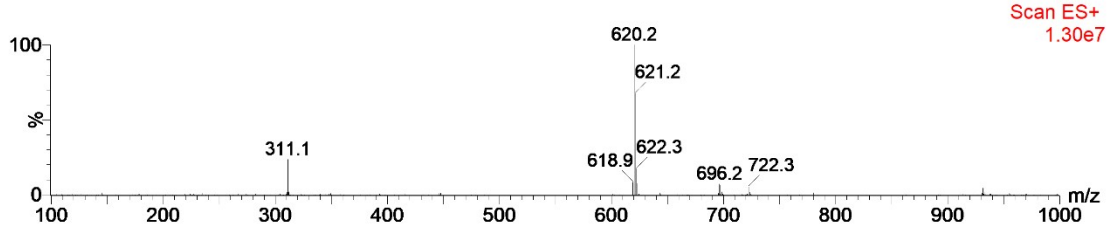

Fig. S3. ESI-mass spectrum of NIR BODIPY (compound 2).

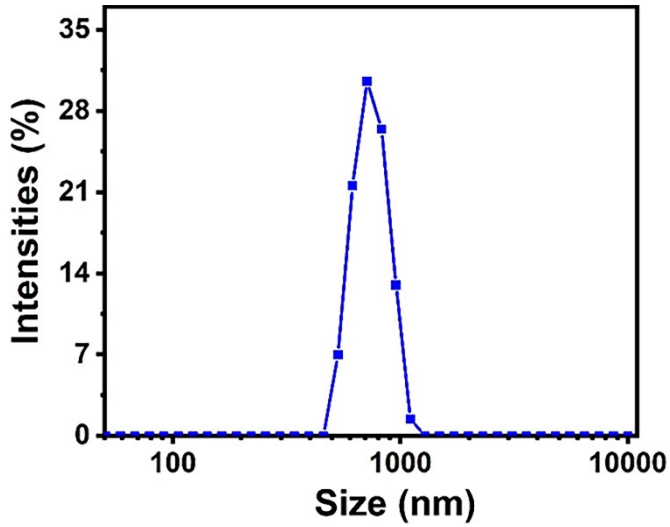

Fig. S4. The size distribution of the aggregates prepared from the assembly of BODIPY in the absence of carriers.

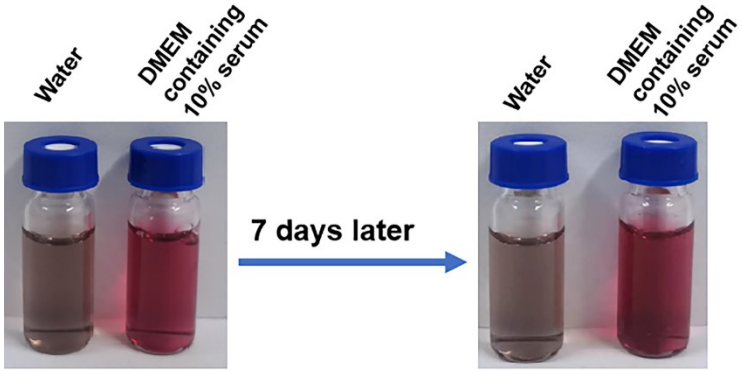

Fig. S5. The photos of DBNPs cultured in water and cell culture medium with 10% FBS for 1 and 7 d.

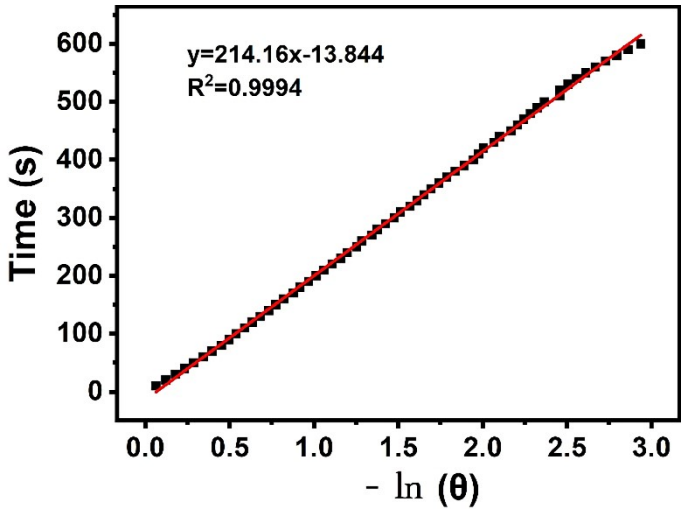

Fig. S6. Linear plot of the cooling time versus -Lnθ calculated from the cooling stage.

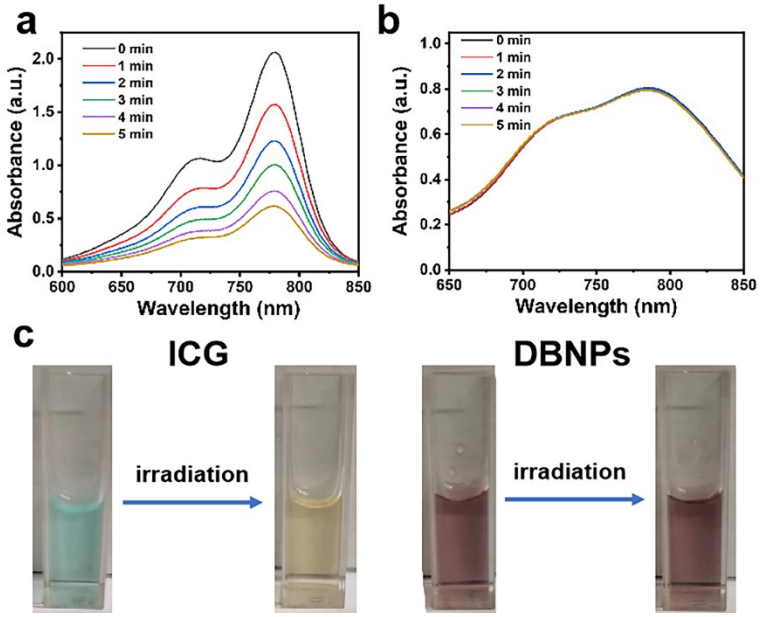

Fig. S7. The change of absorption spectra of ICG (a) and DBNPs (b) after the 808 nm laser irradiation at a power intensity of 0.8 W cm^{-2}. (c) The corresponding color changes of ICG and DBNPs after 5 min of irradiation.

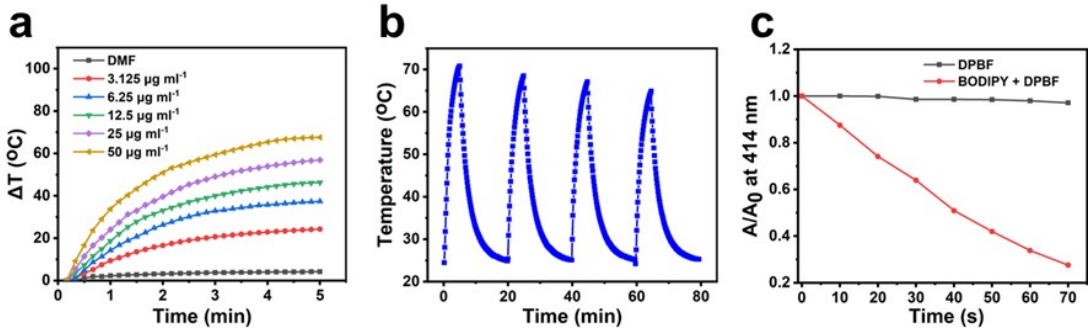

Fig. S8. (a) Photothermal properties of free BODIPY in DMF at different concentrations (0-50 μg mL^{-1}) under laser irradiation (808 nm, 0.8 W cm^{-2}). (b) The temperature change curves of BODIPY in DMF undergoing four cycles of irradiation/cooling. (c) The absorbance decay rates of DPBF in DMF at 414 nm with/without BODIPY in DMF (BODIPY concentration: 0.75 μg mL^{-1}) upon irradiation (808 nm laser, 0.8 W cm^{-2}) for 70 s.

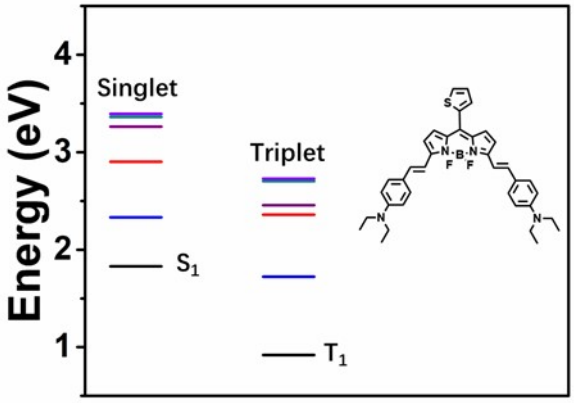

Fig. S9. TD-DFT calculated energy-level diagrams of excited singlet (left) and triplet (right) states of NIR-BODIPY.

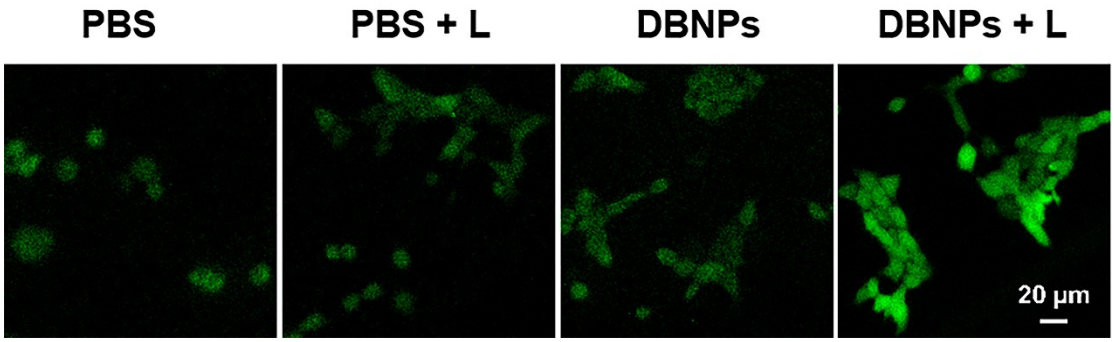

Fig. S10. Intracellular $^1O_2$ generation in 4T1 cells mediated by DBNPs (BODIPY concentration: 20 μg mL^{-1}) upon 808 nm laser irradiation at the power intensity of 0.2 W cm^{-2} for 10 min using DCFH-DA as the indicator. Scale bar, 20 μm.

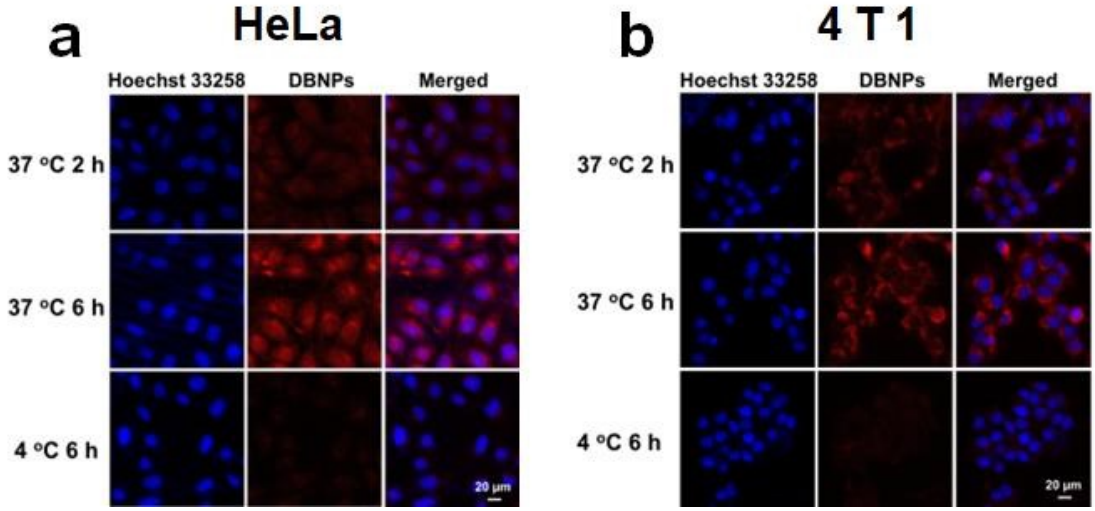

Fig. S11. CLSM images of HeLa (a) and 4T1 cells (b) incubated with DBNPs

(BODIPY concentration: 2 μg mL^{-1}) at 37 °C for 2 and 6 h, and 4 °C for 6 h. Scale bars, 20 μm.

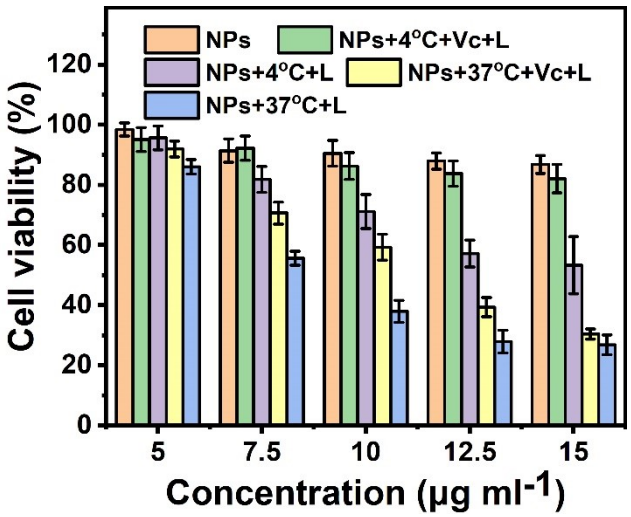

Fig. S12. *In vitro* cytotoxicity against 4T1 cells of DBNPs with or without 808 nm laser irradiation (Vc = Vitamin C, 0.8 Wcm^{-2} for 5 min).

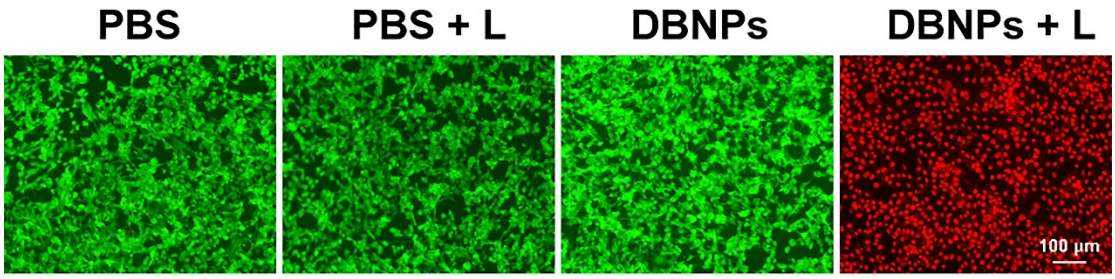

Fig. S13. Live/dead staining images of 4T1 cells incubated with PBS, and DBNPs (BODIPY concentration: 12.5 μg mL^{-1}) without or with the 808 nm laser irradiation (0.8 W cm^{-2}) for 5 min. Scale bar, 100 μm.

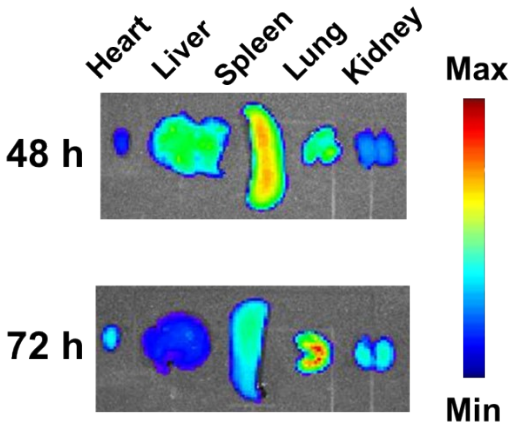

Fig. S14. The fluorescence images of excised major organs (heart, liver, spleen, lung

and kidney) at 48 h and 72 h post-injection.

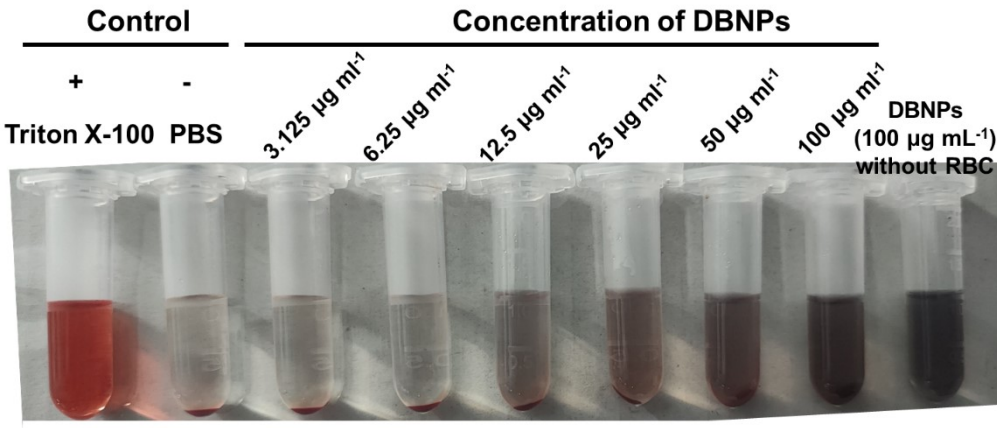

Fig. S15. Photo of hemolysis of DBNPs at different concentration (Triton X-100 as the positive control).

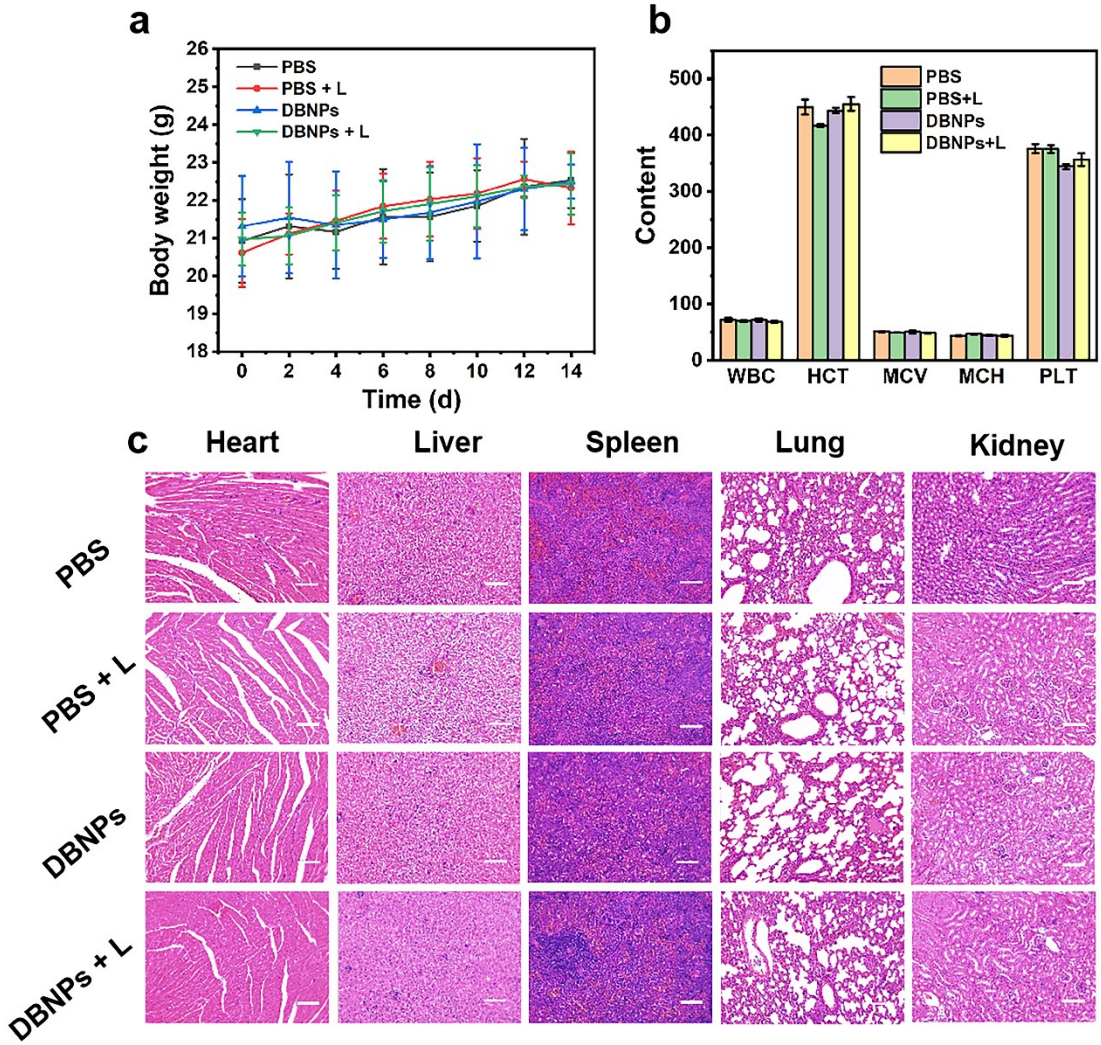

Fig. S16. *In vivo* biosafety of DBNPs. (a) Body weight changes of the mice during the

therapy period. The results are represented as mean ± SD (n = 5). (b) Blood routine examination of the mice with different treatment (WBC, white blood cell count; HCT, Hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin, PLT, platelet). (c) H&E staining images of the main organs of mice in different groups (PBS, PBS + L, DBNPs, and DBNPs + L). Scale bars, 100 μm.

Reference

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4. C. Li, W. Zhang, S. Liu, X. Hu and Z. Xie, *ACS Appl. Mater. Interfaces*, 2020, **12**, 30077-30084.