

## Supporting Information

### Single Molecular Nanomedicine with NIR Light-Initiated Superoxide Radical, Singlet Oxygen and Thermal Generation for Hypoxia-Overcoming Cancer Therapy

*Jipsa Chelora, Yuchao Liang, Wei-Chih Wei, Xiao Cui,\* Yafang Xiao, Yingpeng Wan, Zhongming Huang, Shuang Tian, Shengliang Li, Yi-Hsuan Huang, Ken-Tsung Wong,\* and Chun-Sing Lee\**

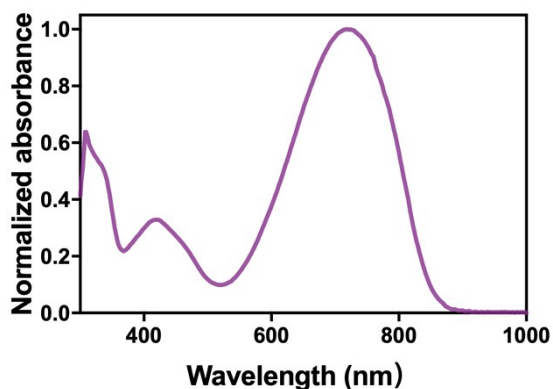


Figure S1. Absorption spectrum of BthB molecule in THF.

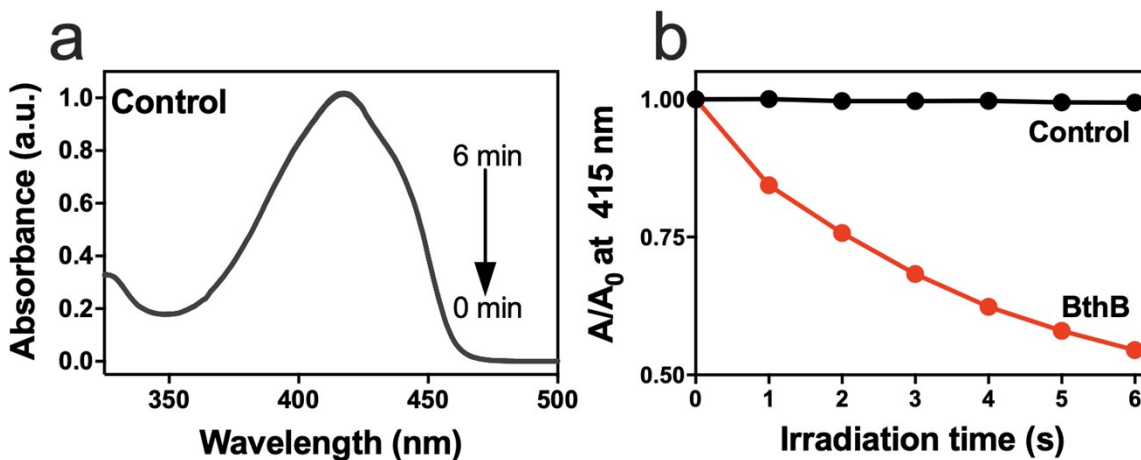
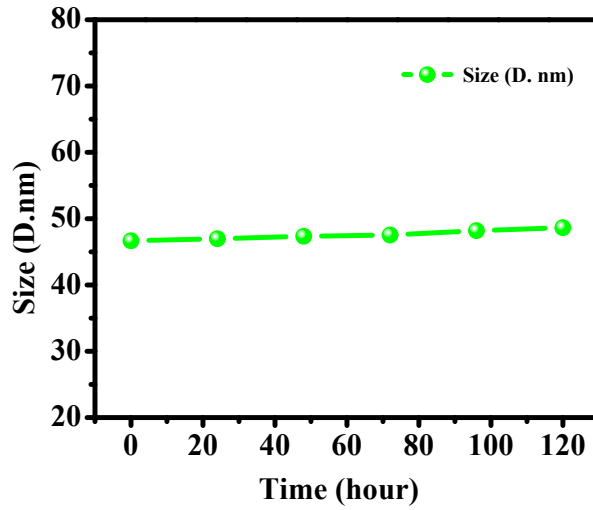
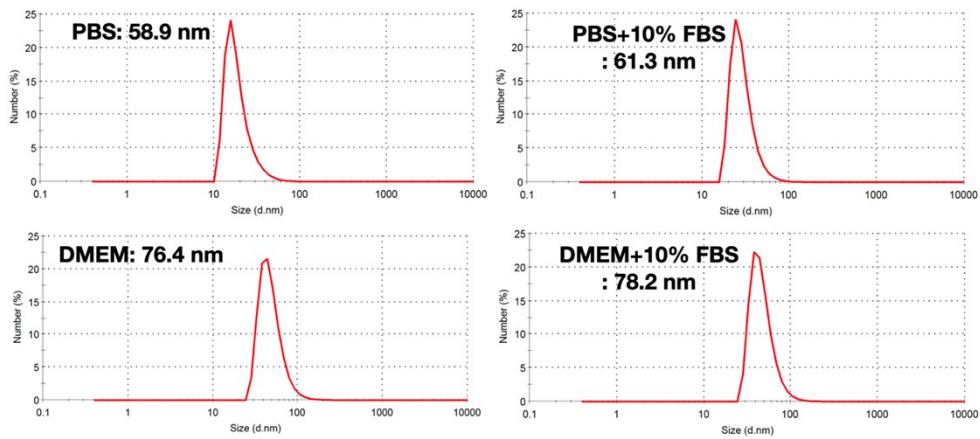


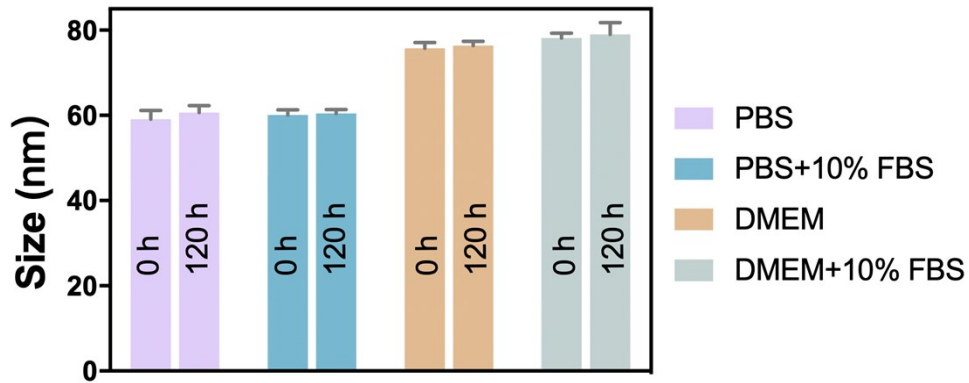
Figure S2. (a) Absorption of only DPBF in THF at different time interval from 0 to 6 min. (b) Comparison of differences in absorption of DPBF between BthB molecule and control (only DPBF) for the singlet oxygen detection of production (irradiated with 808 nm, 1 W/cm<sup>2</sup>).



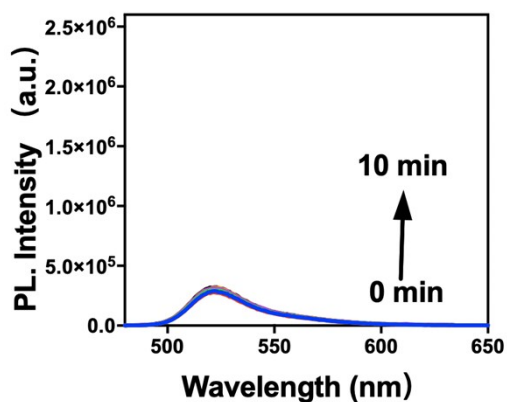
**Figure S3.** Size stability of BthB NPs aqueous solution for 120 hours.



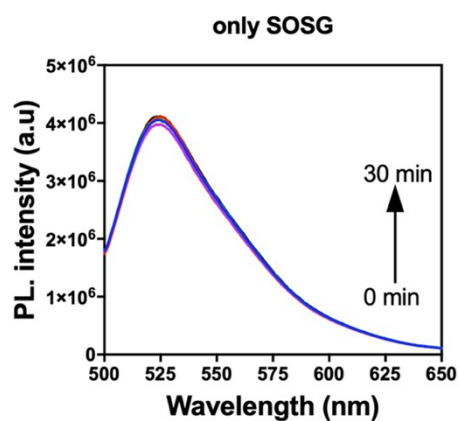
**Figure S4.** Hydrodynamic size of BthB NPs in different media.



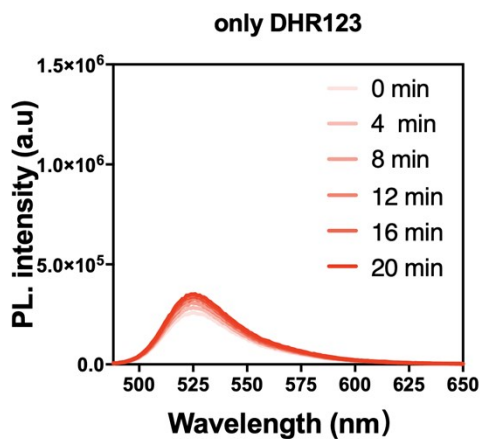
**Figure S5.** Size stability of NPs in different mediums after storage for 120 hours.



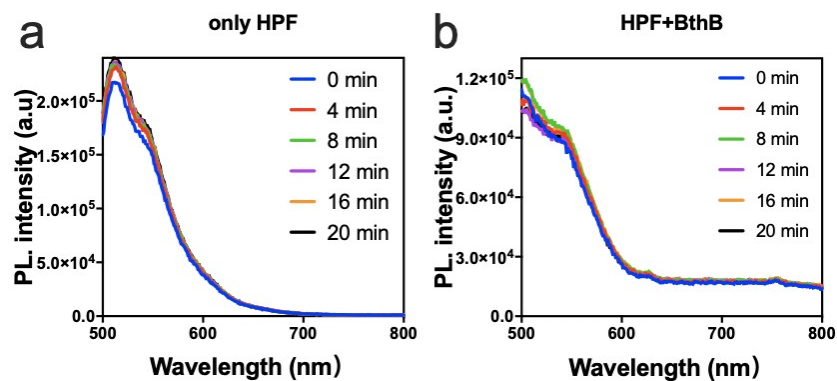
**Figure S6.** Time evolution of only DCFH fluorescence spectra upon irradiation.



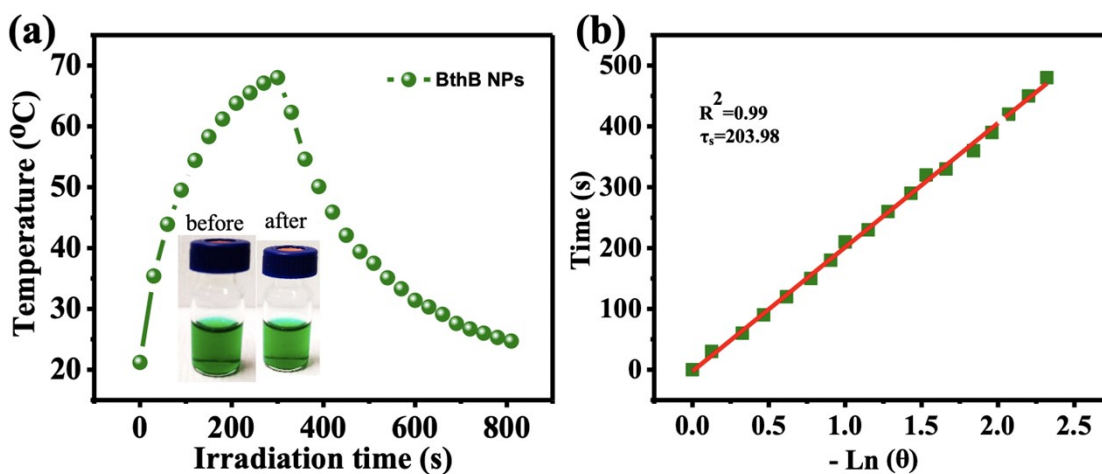
**Figure S7.** Time evolution of only SOSG fluorescence spectra upon irradiation.



**Figure S8.** Time evolution of only DHR123 fluorescence spectra upon irradiation.



**Figure S9.** (a) Time evolution fluorescence spectra of (a) only HPF and (b) HPF with BthB upon irradiation.



**Figure S10.** (a) The temperature change of BthB NPs with a heating curves time (808 nm irradiation,  $1\text{W}/\text{cm}^2$ , 5 min) and cooling curves at room temperature, inset is the photographs of BthB NPs dispersed in water before and after irradiation. (b) Plot of time against the negative natural logarithm of temperature variation during the cooling process.

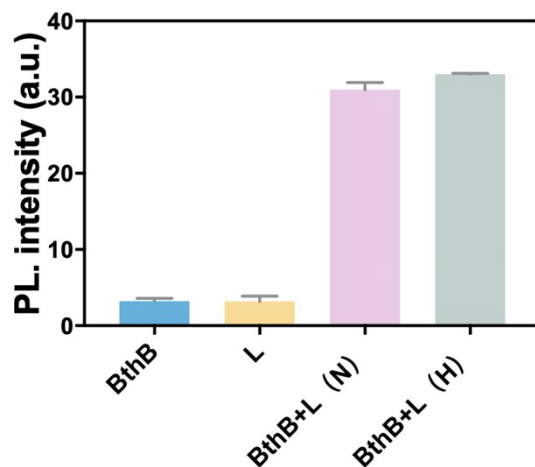


Figure S11. Fluorescence intensities from Figure 3c.

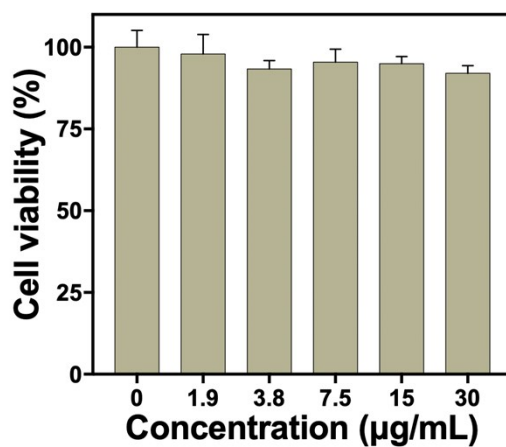


Figure S12. Normal cells (3T3) viability incubated with BthB NPs.

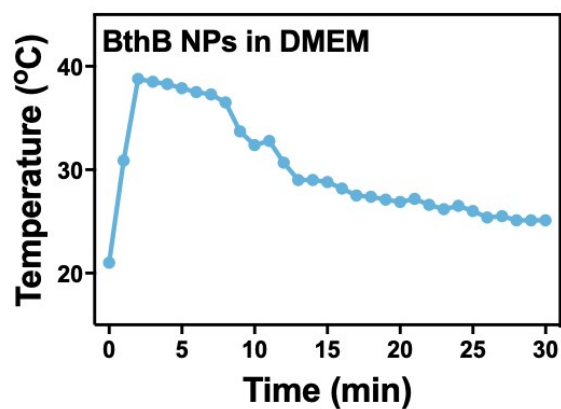
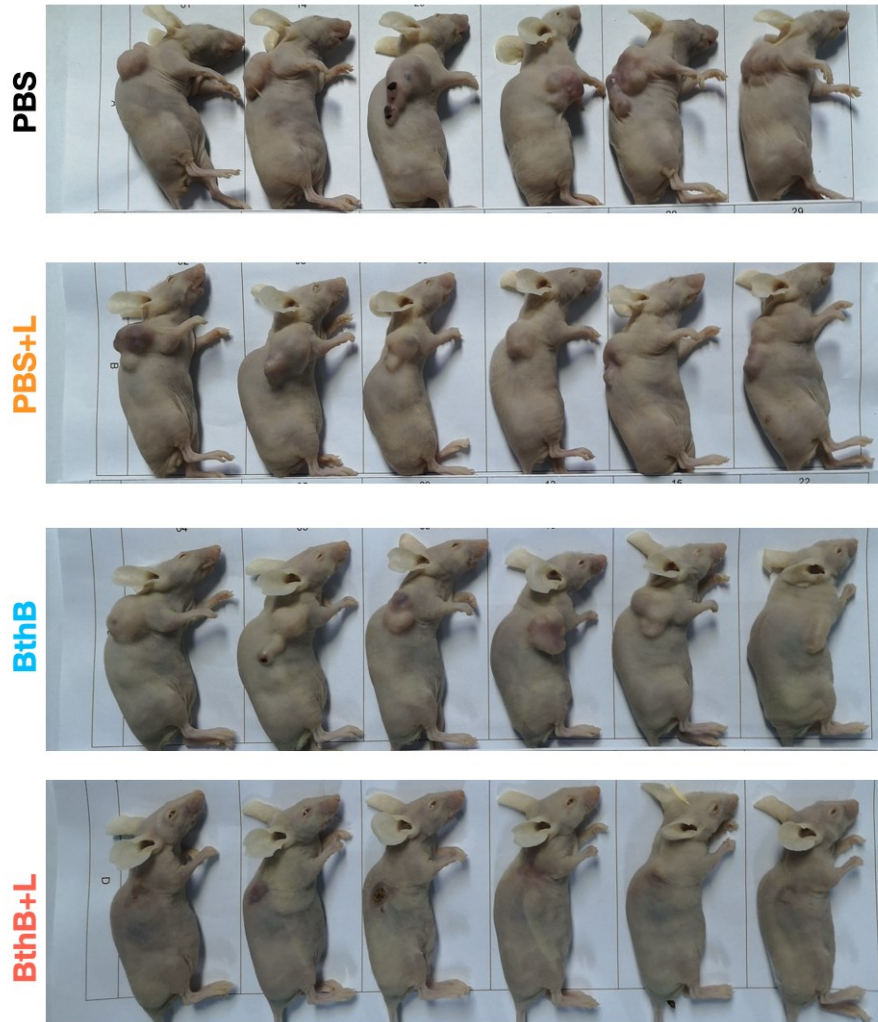
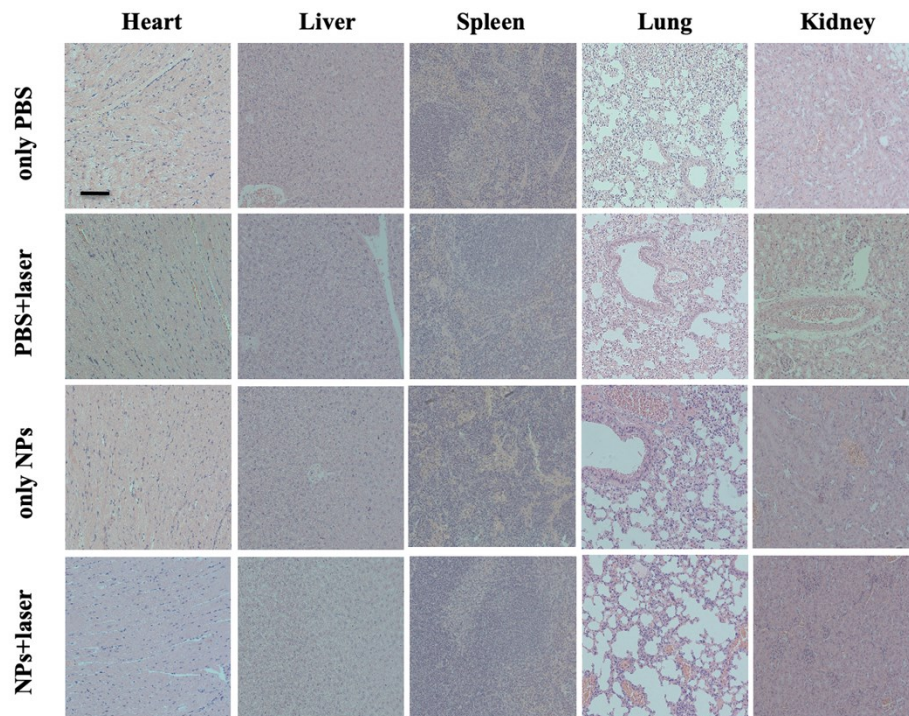


Figure S13. Temperature changes of BthB NPs in DMEM under 808 nm irradiation (0.5W/cm<sup>2</sup>).



**Figure S14.** Photos of mice after various treatments.





**Figure S15.** H&E staining of main organs after various treatments, scale bar is 50  $\mu$ m.

## Experimental Section

**Materials:** DSPE-PEG2000, 2',7'-Dichlorofluorescin (DCFH), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT), deferoxamine (DFO), Dihydrorhodamine 123 (DHR 123), 1,3-Diphenylisobenzofuran (DPBF), and Hoechst 33342 were bought from Sigma-Aldrich. Singlet oxygen sensor green (SOSG), Calcein-AM-propidium iodide (PI) kit, Hoechst 33342 (for staining cell nuclei) and Image-iT™ Green Hypoxia Reagent were purchased from Invitrogen Thermo Fisher. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin/ethylene diamine tetra acetic acid (0.5% trypsin, 5.3 mM EDTA tetra-sodium), Dulbecco's phosphate buffered saline (PBS), antibiotic agents such as penicillin and streptomycin (100 U/mL) were purchased from Gibco Life Technologies. Hydroxyphenyl fluorescein (HPF) was purchased from ENZO Life Science. Tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were ordered from J&K Scientific Ltd.

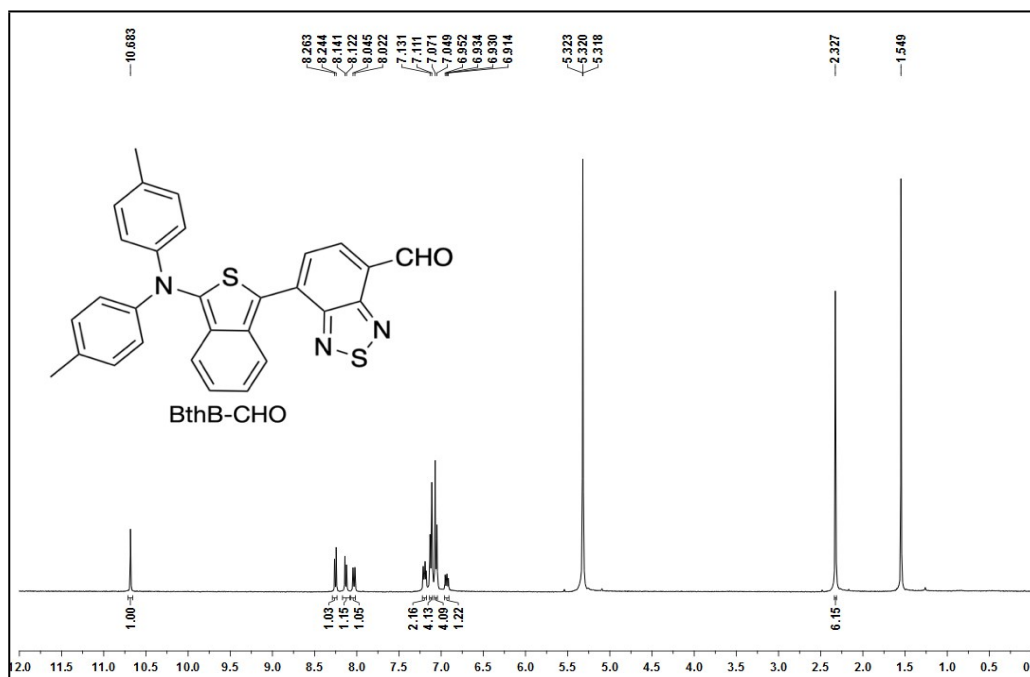
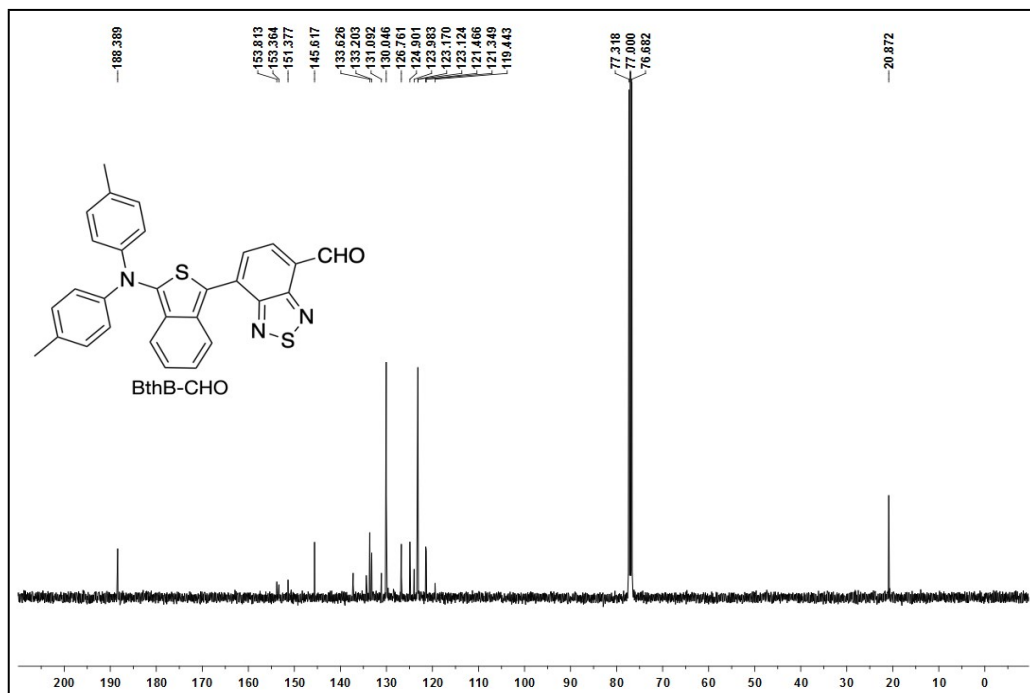
All chemicals were used without further purification. All chemicals were collected from commercial suppliers and used as received without further purification.

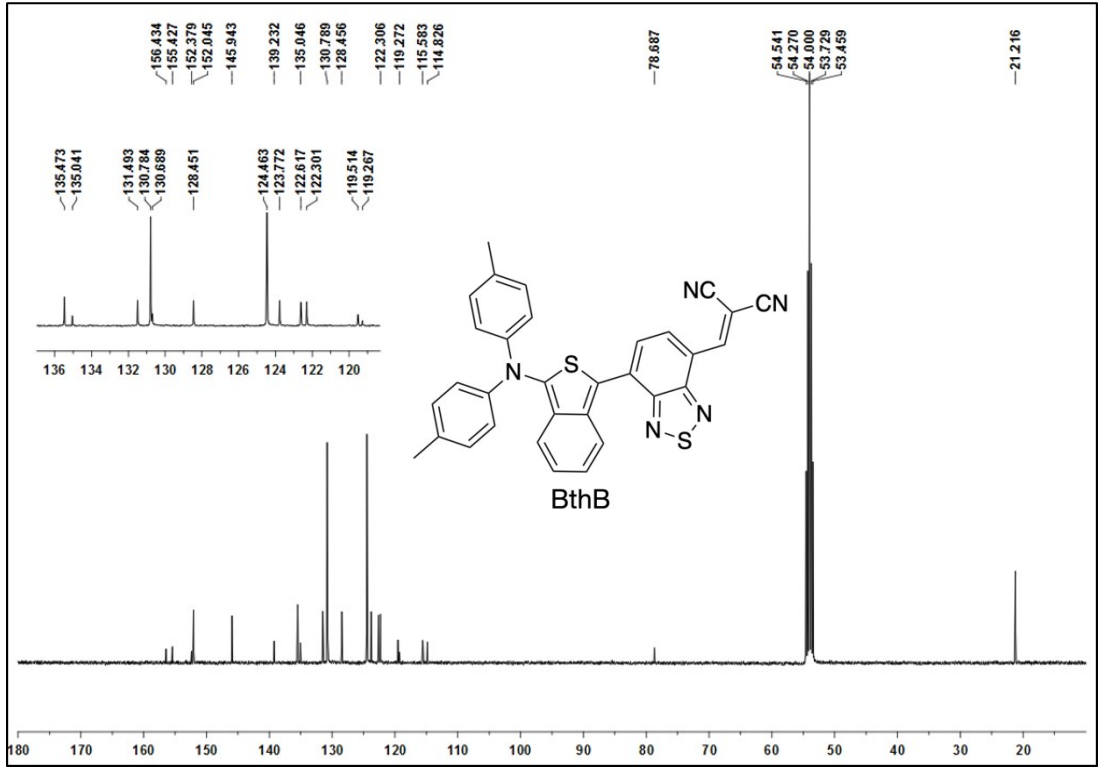
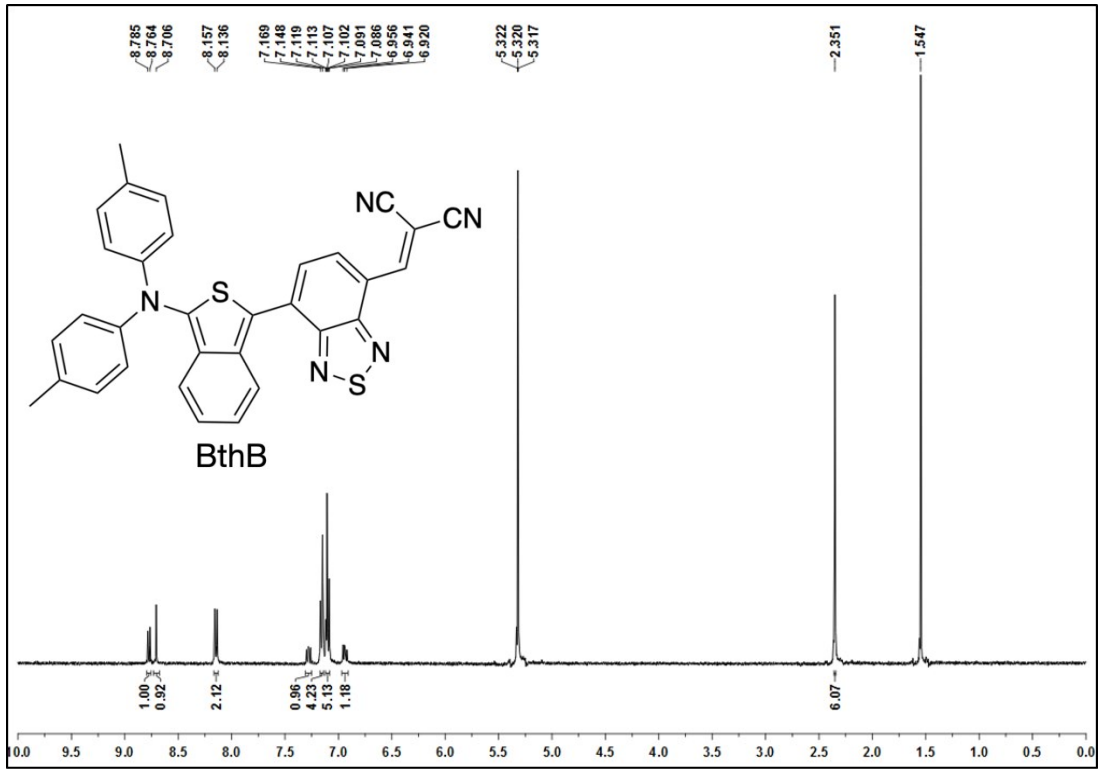
**Synthesis of BthB molecules:** The mixture of 7-bromo-2,1,3-benzothiadiazole-4-carboxaldehyde (0.16 g, 0.67 mmol), ditolylaminobenzo[*c*]thiophene (0.33 g, 1.00 mmol), tris(dibenzylideneacetone)dipalladium (0.03 g, 0.04 mmol), potassium carbonate (0.28 g, 2.00 mmol), pivalic acid (69 mg, 0.67 mmol) was dissolved in N, N-dimethylacetamide (0.28 M, 12 mL) and heated at 80 °C under argon for 16 h. The crude reaction mixture was extracted with EtOAc. The combined organic solution was washed with water and dried over MgSO<sub>4</sub>, and then concentrated to give a crude product, which was purified by column chromatography on silica gel with the eluent of CH<sub>2</sub>Cl<sub>2</sub>: Hexanes (3:2) to afford the intermediate BthB-CHO as a blue solid (0.33 g, 67%). M.p. 140~141 °C, <sup>1</sup>H NMR (400 MHz, CD<sub>2</sub>Cl<sub>2</sub>) δ 10.68 (s, 1H), 8.25 (d, *J* = 7.6 Hz, 1H), 8.13 (d, *J* = 7.6 Hz, 1H), 8.03 (d, *J* = 9.2 Hz, 1H), 7.19 (dd, *J* = 8.5, 5.2 Hz, 2H), 7.12 (d, *J* = 8.3 Hz, 4H), 7.06 (d, *J* = 8.5 Hz, 4H), 6.93 (dd, *J* = 8.4, 6.8 Hz, 1H), 2.33 (s, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 188.3, 153.8, 153.3, 151.3, 145.6, 137.2, 134.3, 133.6, 133.2, 131.0, 130.0, 126.7, 124.9, 123.9, 123.2, 123.1, 121.4, 121.3, 119.4, 20.8; HRMS (*m/z*, FAB<sup>+</sup>) calcd. for C<sub>29</sub>H<sub>21</sub>N<sub>3</sub>OS<sub>2</sub> 491.1126, found 491.1121.

To a solution of compound BthB-CHO (1.25 g, 2.55 mmol) and malononitrile (0.50 g, 7.64 mmol) in DCM (26 mL) was added triethylamine (0.1 mL) dropwise. The reaction mixture was stirred at room temperature for 30 min. The crude product was isolated by precipitation with MeOH and further purified by the reprecipitating from CH<sub>2</sub>Cl<sub>2</sub>/MeOH to afford BthB (0.39 g, 73 %) as a brown solid. M. p. 226 °C (DSC), <sup>1</sup>H NMR (400 MHz, CD<sub>2</sub>Cl<sub>2</sub>) δ 8.77 (d, *J* = 8.3 Hz, 1H), 8.71 (s, 1H), 8.15 (d, *J* = 8.6 Hz, 1H), 7.31–7.25 (m, 1H), 7.16 (d, *J* = 8.2 Hz, 4H), 7.13–7.07 (m, 5H),



6.97– 6.90 (m, 1H), 2.35 (s, 6H) ;  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_2\text{Cl}_2$ )  $\delta$  156.4, 155.4, 152.3, 152.0, 145.9, 139.2, 135.4, 135.0, 131.5, 130.7, 130.6, 128.4, 124.4, 123.7, 122.6, 122.3, 119.5, 119.2, 115.5, 114.8, 53.4, 21.2; HRMS ( $m/z$ , FAB $^+$ ) calcd for  $\text{C}_{32}\text{H}_{21}\text{N}_5\text{S}_2$  539.1238, found 539.1231.





**Preparation of BthB NPs:** BthB NPs were prepared by the reprecipitation method. 0.5 mL BthB molecules (1 mg/mL in THF) and 0.5 mL DSPE-PEG (5 mg/mL in THF) were fully mixed by ultrasound. Then 1 mL of mixture was slowly injected to 9 mL of DI water and stirred for 48 h at room temperature to obtain a nanoparticles solution. The as-prepared solution was filtered with 0.22  $\mu\text{m}$  to obtain uniform nanoparticles. Finally, the sample was collected with a 100,000 kDa ultrafiltration tube and the concentrated solution was stored at 4 °C for future use.

**Characterization:** Size and morphology of the nanoparticles were investigated using TEM (CM200 FEG, Philips). Zeta potential and hydrodynamic size of nanoparticles were assessed with a DLS (Dynamic Light Scattering) instrument (Malvern Zetasizer Nano ZS). Absorption spectra and fluorescence spectra were obtained from Ultra-Violet Visible spectrophotometer (Shimadzu 1700) and a Spectrofluorometer (Fluormax-4). Leica TCS SPE/SP5 laser scanning confocal microscope was used for cell imaging.

**Singlet oxygen detection in BthB molecule:** ROS detection of BthB molecule is measured initially. DPBF was used as a probe for singlet oxygen detection and its absorbance get reduced upon exposure to laser irradiation. DPBF-BthB-THF mixture was irradiated at 60-sec interval with 808 nm laser (1 W/cm<sup>2</sup>) and the absorption was measured.

**Total ROS Detection by DCFH in BthB NPs:** DCFH fluorescence intensity was applied to detect total reactive oxygen species in aqueous solution. Typically, 0.6 mL DCFH (concentration: 10 nM) was mixed with 11.6  $\mu\text{L}$  of BthB NPs dispersed in water (working concentration: 30  $\mu\text{g}/\text{mL}$ ). Additional water was added to the mixture to make the total volume 0.7 mL. Control group were

prepared by adding 0.1 mL water in 0.6 mL DCFH. After that, the as-prepared sample and control group were exposed to 808 nm irradiation ( $0.25 \text{ W/cm}^2$ ) for 10 min, and the photoluminescence (PL) intensities of DCFH from 488 to 700 nm were recorded every 2 min in a spectrofluorometer (460 nm excitation).

**Detection of  $^1\text{O}_2$  Generation in Normoxia by SOSG:**  $^1\text{O}_2$  generation in solution were monitored with SOSG via its fluorescence intensity. Briefly, 2  $\mu\text{L}$  SOSG in methanol (stock concentration: 1.65 mM, working concentration: 5.5  $\mu\text{M}$ ) was mixed with the BthB NPs solution (working concentration: 30  $\mu\text{g/mL}$ ) and irradiated by 808 nm laser ( $0.5 \text{ W/cm}^2$ ). Then the fluorescence intensities of SOSG at 525 nm of different irradiation intervals (0, 5, 10, 15, 20, 25 and 30 min) were recorded in a photoluminescence spectrofluorometer (488 nm excitation).

**Detection of  $\text{O}_2^{\cdot-}$  generation:** DHR 123 probe fluorescence intensity was applied to detect  $\text{O}_2^{\cdot-}$  generation. Typically, 30  $\mu\text{L}$  DHR 123 (stock concentration: 1 mM, working concentration: 10  $\mu\text{M}$ ) was mixed with 120  $\mu\text{L}$  BthB NPs (working concentration: 60  $\mu\text{g/mL}$ ). Additional water was added to the mixture to make the total volume 3.0 mL. Control group were prepared by adding 30  $\mu\text{L}$  DHR 123 in 2970  $\mu\text{L}$  water. In order to extract oxygen for the stimulated hypoxia, the prepared solution in a tightly sealed Schlenk tube was quickly frozen using liquid nitrogen for 5 min, then pull vacuum for 5 min while it is frozen. By pulling vacuum, most of oxygen can be removed. This procedure was repeated for three time, and then the mixtures in a tightly sealed Schlenk tube were directly exposed to 808 nm laser irradiation ( $0.5 \text{ W/cm}^2$ ). The PL intensity of DHR 123 from 488 to 700 nm were recorded every 4 min in a photoluminescence spectrofluorometer (460 nm excitation).

**Detection of Extracellular •OH:** HPF probe fluorescence intensity was applied to detect •OH generation. Typically, 12.7  $\mu\text{L}$  HPF (stock concentration: 2.36 mM, working concentration: 10  $\mu\text{M}$ ) was fully mixed with 120  $\mu\text{L}$  BthB NPs (working concentration: 60  $\mu\text{g}/\text{mL}$ ). Additional water was added to the mixture to make the total volume 3.0 mL. After that, the mixtures were exposed to irradiation from an 808 nm laser (0.5  $\text{W}/\text{cm}^2$ ) for 20 min. Finally, the PL intensity of HPF from 490 to 800 nm were recorded every 4 min in a spectrofluorometer (460 nm excitation). Control group were prepared by adding 12.7  $\mu\text{L}$  HPF in 2987.3  $\mu\text{L}$  water. Oxygen extraction follows the above procedure and then the mixtures were directly exposed to 808 nm laser irradiation (0.5  $\text{W}/\text{cm}^2$ ). The PL intensity of HPF from 500 to 800 nm were recorded every 4 min in a photoluminescence spectrofluorometer (460 nm excitation).

**Photothermal effect and photothermal efficiency:** The temperature changes of BthB NPs dispersion at different concentrations (120, 60, 30, 15, 7.5  $\mu\text{g}/\text{mL}$ ) were investigated. To measure photothermal conversion efficiency ( $\eta$ ), 0.5 mL of BthB NPs were irradiated with 808 nm laser at 1  $\text{W}/\text{cm}^2$  for 5 min, and the nanoparticle solution was cooled down to room temperature by switching off the laser and the temperature was noted at a 30-second interval.  $\eta$  was calculated according to the reported papers.<sup>[1]</sup>

**Intracellular Hypoxia Evaluation:** Intracellular hypoxia was confirmed by the fluorescence intensity of Image-iT™ Green Hypoxia Reagent. It is non-fluorescent when live cells are in an environment with normal oxygen concentrations (20%) and becomes fluorescent when oxygen levels are decreased. A549 cells were seeded in 27 mm nunc glass base dish cultured with DMEM

(Invitrogen) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) in 5% CO<sub>2</sub>, 20% O<sub>2</sub> at 37 °C in a humidified incubator. Then Image-iT™ Green Hypoxia Reagent (working concentration: 10 μM) was added to DMEM and incubated for 30 min. Then the original culture medium was replaced by 2 mL fresh medium containing 40 μL DFO (stock concentration: 10 mM, working concentration: 200 μM) and the cells were returned to incubator for 4 h (5% CO<sub>2</sub>, 20% O<sub>2</sub> at 37 °C). DFO induces hypoxia by chelating iron for excretion and DFO stabilizes HIF-1α from proteolysis by inhibiting the activity of iron-dependent prolyl hydroxylases subsequently reducing the potential for oxygen transport. Next, the cells were washed thoroughly with PBS three times. Finally, green fluorescence inside cells were recorded on a Leica TCS SPE Spectral Confocal Microscope from 500 to 600 nm under 488 nm excitation.

**Intracellular ROS Detection in Normoxia and Hypoxia:** A549 cells were seeded in 27 mm nunc glass base dish cultured with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO<sub>2</sub>, 20% O<sub>2</sub> at 37 °C in a humidified incubator. For hypoxia simulation, the original culture medium was replaced by 2 mL fresh medium containing 40 μL DFO (stock concentration: 10 mM, working concentration: 200 μM) and 30 μL of BthB NPs (stock concentration: 2 mg/mL, final concentration: 30 μg/mL). For normoxia, the original culture medium was replaced by 2 mL fresh medium only containing same concentration of nanoparticles. Then the hypoxic and normoxic cells were returned to incubator (5% CO<sub>2</sub>, 20% O<sub>2</sub> at 37 °C) and incubated for 6 h. Next, 2 μL of DCFH-DA (stock concentration: 10 mM, working concentration: 10 μM) and 1 μL Hoechst 33324 (stock concentration: 10 mg/mL, working concentration: 5 μg/mL) was added to medium for 30 min staining. Then A549 cells were irradiated by 808 nm laser (0.5 W/cm<sup>2</sup>, 5 min) and washed with PBS for three times. At last, blue (405 nm EX, 410-500 nm EM) fluorescence from Hoechst

33324 and green (488 nm EX, 500-600 nm EM) fluorescence from DCF were recorded on a Leica TCS SPE Spectral Confocal Microscope.

**Cell Viability:** A549 cell viabilities treated with nanoparticles were measured by MTT assay. Cells were seeded on 96-well plates in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO<sub>2</sub>, 20% O<sub>2</sub>. The cells were grown overnight in the incubator at 37 °C in a humidified incubator. The old medium was removed and 200 µL fresh medium containing aqueous solution of nanoparticles at different concentrations was then added for 4 hours of incubation, untreated set of cells were used as control. Next, the cells in different groups were exposed to 808 nm laser irradiation for 5 min (1 W/cm<sup>2</sup>). For hypoxic group, 200 µL fresh medium contains same concentration of nanoparticles and 4 µL DFO (stock concentration: 10 mM, working concentration: 200 µM) were added. The cells in hypoxia were exposed to 808 nm laser irradiation for 30 min (0.5 W/cm<sup>2</sup>). Then the plate was returned to incubator for 12 h. Next, 200 µL DMEM containing 20 µL MTT stock solution (5 mg/mL in PBS) were mixed together and added into each well by replacing the old medium-nanoparticle mixture. After 4 hours of incubation, the medium was completely removed and 200 µL of DMSO was added to each well. The plate was shaken for 1 min at room temperature to dissolve precipitates. A microplate reader (BioTek Power wave XS microplate reader) was used to determine the absorbance of MTT at 540 nm.

***In Vivo* Antitumor Activity and Biosafety:** Balb/c nude (female) mice, 5 weeks of age, were housed in a specific pathogen-free room. For investigating the therapeutic efficacy of the BthB NPs *in vivo*, the antitumor effects were conducted on A549 tumor-bearing nude mice. Before commencing PDT treatment, A549 cells were subcutaneously injected around 3 weeks. When the



tumors grew to about 60 mm<sup>3</sup> in volume, these mice were divided into four groups randomly with five mice in each group. The mice then received tail intravenous injections at 0 day and the BthB NPs was administered at an equivalent dose (200 μL, 1 mg/mL). Different formulations were administered, including only PBS (PBS), only laser (PBS+L), only NPs (BthB), NPs plus laser (BthB +L), respectively. After injection for 8 h, laser irradiation (808 nm, 1 W/cm<sup>2</sup> for 10 min) at 1 day was administrated to mice. The temperature changes of mice were collected with a thermal imaging camera. Both tumor weights and tumor volumes were recorded every two days during the whole treatments. After 32 days of treatment, the mice were sacrificed, blood samples were collected from mice with various treatments for blood biochemical parameters evaluation, mainly including urine (UA), albumin (ALB), total protein (TP), albumin-globulin ratio (A/G), globulin (GLOB), creatinine (CREA), aspartate transaminase (AST) and total bilirubin (TBIL).

1. a) Y. F. Xiao, C. Xiang, S. Li, C. Mao, H. Chen, J. X. Chen, S. Tian, X. Cui, Y. Wan, Z. Huang, X. Li, X. H. Zhang, W. Guo, C. S. Lee, *Small* **2020**, 16, 2002672; b) S. Li, Q. Deng, Y. Zhang, X. Li, G. Wen, X. Cui, Y. Wan, Y. Huang, J. Chen, Z. Liu, L. Wang, C. S. Lee, *Adv. Mater.* **2020**, 32, 2001146.