Supporting Information

J-aggregates of ethene-bridged aza-BODIPY dimers for bright NIR-II bioimaging and efficient photothermal therapy

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Materials and Method

All chemical agents were purchased from commercial sources and used directly unless otherwise noted. 2-Amino-3-hydroxypyridine**,** 7-(Bromomethyl)pentadecane, 3,6-Di(thiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione, triethylamine, N-bromosuccinimide, ultra-dry 1,4-dioxane and thiazolyl blue (MTT) were purchased form Innochem (Beijing, China). Potassium carbonate, ultra-dry titanium tetrachloride and boron trifluoride diethyl etherate were obtained from J&K Scientific Co., Ltd. Trans-1,2-Bis(tributylstannyl)ethylene was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Pluronic F-127, calcein AM and Propidium Iodide (PI) were obtained from Sigma-Aldrich. N, N-Dimethylformamide, 1,4-dioxane, toluene, petroleum ether, dichloromethane and methanol were purchased from Beijing chemical works. Except that toluene needs to be redistilled with metallic sodium to remove H_2O before use, other reagents were used directly without purification. The 4T1 cells used in the in vitro experiments were purchased from the National Experimental Cell Resource Sharing Service Platform (Headquarters, Beijing). The fetal bovine serum (FBS), streptomycin, penicillin, and RPMI-1640 medium utilized for cell culture were procured from Thermo Fisher Scientific.

^{1}H-NMR spectra were obtained from Bruker Avance 400 or 600 MHz spectrometers. Electrospray ionization mass spectra (ESI-MS) were determined by APEXII FT-ICR mass spectrometer. Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF-MS) were obtained by APEX IV FTMS mass spectrometer. High-

Performance Liquid Chromatography (HPLC) analysis was performed using a Shimadzu LC-20 system. Absorption spectra were acquired from Agilent Cary 7000 UV-VIS-NIR spectrophotometer. Fluorescence (FL) spectra were determined by Edinburgh FLS1000 steady-state/transient FL spectrometer. Dynamic light scattering (DLS) and Zeta potential were performed on Malvern Nano ZEN3600 nanoparticle size and zeta potential analyzer. Cell staining images were captured by Nikon C1 Si full-spectrum laser scanning confocal imaging microscope. Cell MTT absorbance was measured on Thermo Fisher Scientific Microplate Reader.

J-aggregation state test of Bis-PPAB. The J-aggregation state test of **Bis-PPAB** molecules was conducted in a tetrahydrofuran (THF)/ H_2O system. The FL spectra of **Bis-PPAB** (10 μM) in different systems ($f_{w,\ water}$ = 0-90%) were measured using an Edinburgh FLS1000 steady-state/transient FL spectrometer. The excitation light source was fixed at 808 nm, the excitation light source voltage was fixed at 2 V, and the emission slit was fixed at 5 nm.

Preparation of Bis-PPAB NPs. To prepare **Bis-PPAB NPs**, we initially ultrasonically mixed 1 mL of **Bis-PPAB** (1 mg mL^{-1}, THF) with 1 mL of an amphiphilic triblock copolymer, F-127 (50 mg mL^{-1}, THF). Subsequently, the resulting mixture was added dropwise to 10 mL of ultrapure H_2O while being sonicated using a cell disruptor probe (12 W, with cycles of 3 seconds on and 2 seconds off) for a duration of 3 min. The organic solvent, THF, was then removed via rotary evaporation. Subsequently, the solution was filtered through a 0.22 μm H_2O filter and concentrated using a high-speed centrifugation method with a 30K ultrafiltration tube. The final product was designated as **Bis-PPAB NPs** and stored at 4°C for future applications.

Encapsulation efficiency test. Take the nano-assembly utilizing 3 mg **Bis-PPAB** (m_1) and 150 mg of F-127 as a case study. Following the nano-preparation process, the resulting **Bis-PPAB NPs** were subjected to freeze-drying using a freeze dryer. Subsequently, 2 mL of chloroform were added to the obtained solid and thoroughly dissolved via ultrasonic treatment. The concentration of **Bis-PPAB** in the final solution was then quantified by UV-vis absorption spectroscopy to be 1.22 mg (m_2) (Fig. S7). Then, the value of the encapsulation efficiency (EE) was calculated using the formula EE% = (m_2/m_1) x 100%.

Light stability test. The photostability of **Bis-PPAB NPs** was evaluated by comparing it with the FDA-approved cyanine derivative ICG. The aqueous solutions of **Bis-PPAB NPs** and ICG were continuously irradiated with an 808 nm (1 W cm^{-2}) laser for 60 min. The absorption spectra at different irradiation times were recorded using a Hitachi U-3900 spectrophotometer.

FL quantum yield test. The relative FL quantum yield test method of **Bis-PPAB** refers to the previously published article,[1] with IR-1061 as the reference fluorophore and dichloromethane (DCM, Φ_{ref} = 1.7%) as the solvent.[2]

Measurement of photothermal properties in solutions. Different concentration gradients of **Bis-PPAB NPs** (0-10 μM) solutions were irradiated with an 808 nm laser,

with the laser power density gradient set between 0.2 to 1 W cm^{-2}. The temperature rise curves of the solutions were recorded in real time using a thermocouple within 10 min of laser irradiation. For the determination of photothermal conversion efficiency, the concentration of **Bis-PPAB** was fixed at 10 μM, while the laser power density at 808 nm was maintained at 0.8 W cm^{-2}. Following a duration of 15 min under irradiation, the laser was turned off and allowed to cool naturally; during this process, temperature rise and fall curves were continuously monitored by a thermocouple. In assessing the photothermal stability of **Bis-PPAB**, the solution underwent irradiation with an 808 nm laser at a power density of 0.8 W cm^{-2} for a period of 10 min. Subsequently, the laser was switched off and permitted to cool naturally. This procedure was repeated five times, with real-time recording of temperature rise and fall curves conducted via thermocouple throughout each cycle.

Cell culture and MTT cytotoxicity test. 4T1 (mouse breast cancer cell line) were obtained from the Center of Cells, Peking Union Medical College. 4T1 cells were cultured and passaged in RPMI-1640 medium supplemented with 10% FBS, 1% streptomycin (100 μg mL^{-1}) and penicillin (100 μg mL^{-1}). The cultures were maintained in a 37°C incubator with a humidified atmosphere containing 5% CO_2. 4T1 cells were incubated with **Bis-PPAB NPs** at varying concentrations (0, 1, 2, 5, 10 μM) for 24 hours. After that, the culture medium was removed, and 100 μL of fresh RPMI-1640 medium was added. Subsequently, 20 μL of MTT solution (5 mg mL^{-1}) was introduced, and the cells were further incubated for an additional 4 hours before discarding the solution. Afterward, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed during the MTT assay, and absorbance at 570 nm was measured using a microplate reader. The group treated with a **Bis-PPAB** concentration of zero served as the control group, establishing its cell survival rate at 100%. The cell survival rates corresponding to different sample concentrations were calculated accordingly. Each concentration group underwent parallel testing six times. Phototoxicity analysis was performed by incubating 4T1 cells with **Bis-PPAB NPs** at varying concentrations (0, 1, 2, 5, 10 μM) for a duration of 12 h, followed by the replacement of the culture medium. Subsequently, the cells were irradiated with an 808 nm laser (0.8 W cm^{-2}) for 10 min and then further incubated for an additional 12 h. The cell viability was assessed using the same MTT method employed in the dark toxicity test, with each group of cells being tested in parallel six times.

Calcein AM and Propidium Iodide (PI) cell staining experiment. The 4T1 cells were randomly allocated into three distinct groups: the laser only group, the **Bis-PPAB NPs** group, and the **Bis-PPAB NPs** combined with laser treatment group. In the experimental groups, **Bis-PPAB NPs** (10 μM) were added for a 12 h incubation period, after which the medium was replaced. The **Bis-PPAB NPs** + laser group underwent exposure to an 808 nm laser (0.8 W cm^{-2}) for 10 min followed by an additional 12 h of incubation. Conversely, the **Bis-PPAB NPs** group was incubated solely for 12 h without further treatment. Subsequent to discarding the medium, cells were washed three times with PBS. Following this step, 1 mL of PBS solution was added along with Calcein AM (1 mM, 2 μL) and PI (1 mM, 4 μL) for cell staining purposes. Cell imaging was

conducted using a full-spectrum laser scanning confocal microscope (Nikon C1-Si), and FL images of the cells were processed utilizing NIS-Elements Viewer version 3.20 software

Animal experiment. All animal experiments strictly comply with the Chinese guidelines for animal experiments (Laboratory animal—Guideline for ethical review of animal welfare, GB/T 35892-2018) and received approval from the Experimental Animal Welfare Ethics Committee of the Institute of Process Engineering, Chinese Academy of Sciences (Approval ID: IPEAECA2024047).

Whole-body vascular imaging in NIR-II FL window. Female Balb/c nude mice (6-8 weeks) bearing 4T1 tumors were administered an intravenous injection of 150 μL **Bis-PPAB NPs** (2 mg mL^{-1}). After the injection, imaging was performed with a NIR-II FL system using long-pass filters at 1000 nm and 1100 nm, simultaneously excited by an 808 nm laser.

Research on the in vivo effects of photothermal therapy. One week after the inoculation of 4T1 tumor cells, the mice were randomly allocated into four groups: the blank group (only PBS), the **Bis-PPAB NPs** group, the Laser control group, and the **Bis-PPAB** NPs + Laser group. 12 h after tail vein injection of PBS or **Bis-PPAB NPs**, the Laser control group and the **Bis-PPAB NPs** + Laser group were treated with 808 nm (1 W cm^{-2}) laser irradiation (10 min). Subsequently, the body weight and tumor volume of the mice were monitored every two days for the following two weeks

Syntheses and characterization

The precursor molecule PPAB was synthesized using the literature procedure without modification.[3,4]

Synthesis of compound PPAB-Br_1: In a double-necked reaction flask, 50 mg of **PPAB** (0.0475 mmol) and 9.7 mg (0.0550 mmol) of N-bromosuccinimide (NBS) were accurately weighed, followed by the addition of 5 mL of chloroform. The resulting mixture was stirred at room temperature for a duration of 2 hours, after which the reaction was terminated. Subsequently, the mixture was extracted using saturated brine and dichloromethane, with the organic phase being collected. The solvent was then removed employing a rotary evaporator. The target product was isolated through column chromatography (petroleum ether/dichloromethane = 2/1, v/v), yielding a green solid (27 mg) with an overall yield of 48%.^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.58 (s, 1H), 7.85 (s, 1H), 7.62 (s, 2H), 7.17 (s, 1H), 7.00 (d, *J* = 7.7 Hz, 1H), 6.89 (t, *J* = 6.6 Hz, 1H), 6.72 (t, *J* = 7.1 Hz, 1H), 6.53 (d, *J* = 8.2 Hz, 3H), 4.08 (t, *J* = 6.7 Hz, 2H), 3.66 (s, 2H), 2.06 (t, *J* = 7.4 Hz, 2H), 1.25 (q, *J* = 16.8, 16.3 Hz, 48H), 0.95 – 0.71 (m, 12H). MALDI-TOF-MS m/z calcd for $C_{56}H_{77}B_2BrF_4N_6O_2S_2$: 1108.4834; found: 1108.093.

Synthesis of compound Bis-PPAB: Under N_2 atmosphere, compound PPAB-Br_1 (25 mg, 0.0226 mmol), trans-1,2-bis(trimethylstannyl)ethene (7.0 mg, 0.0115 mmol), and

$Pd(PPh_3)_4$ (2.6 mg, 0.003 mmol) were dissolved in 4 mL anhydrous toluene. Then heat the mixture to reflux and allow it to react for 8 hours. After cooling the reaction mixture to room temperature, perform extraction using saturated brine followed by dichloromethane. Collect the organic phase and remove the organic solvent utilizing a rotary evaporator. Finally, purify the product through column chromatography (petroleum ether/dichloromethane = 1/2, v/v) to obtain a brownish solid (purity, 98.034%). ^{1}H NMR (600 MHz, Methylene Chloride-*d*2) δ 10.00 (d, *J* = 4.1 Hz, 2H), 9.50 – 9.44 (m, 2H), 7.99 (d, *J* = 6.3 Hz, 2H), 7.95 (d, *J* = 6.0 Hz, 2H), 7.76 (s, 2H), 7.47 (s, 2H), 7.42 – 7.24 (m, 8H), 7.13 (dt, *J* = 13.2, 7.1 Hz, 4H), 4.07 (dd, *J* = 22.7, 6.0 Hz, 8H), 2.08 – 2.01 (m, 4H), 1.56 (d, *J* = 44.2 Hz, 20H), 1.27 (s, 76H), 0.97 – 0.67 (m, 24H). MALDI-TOF-MS m/z calcd for $C_{114}H_{155}B_4F_8N_{12}O_4S_4$: 2082.1533; found: 2082.1583.

Supplementary Figures

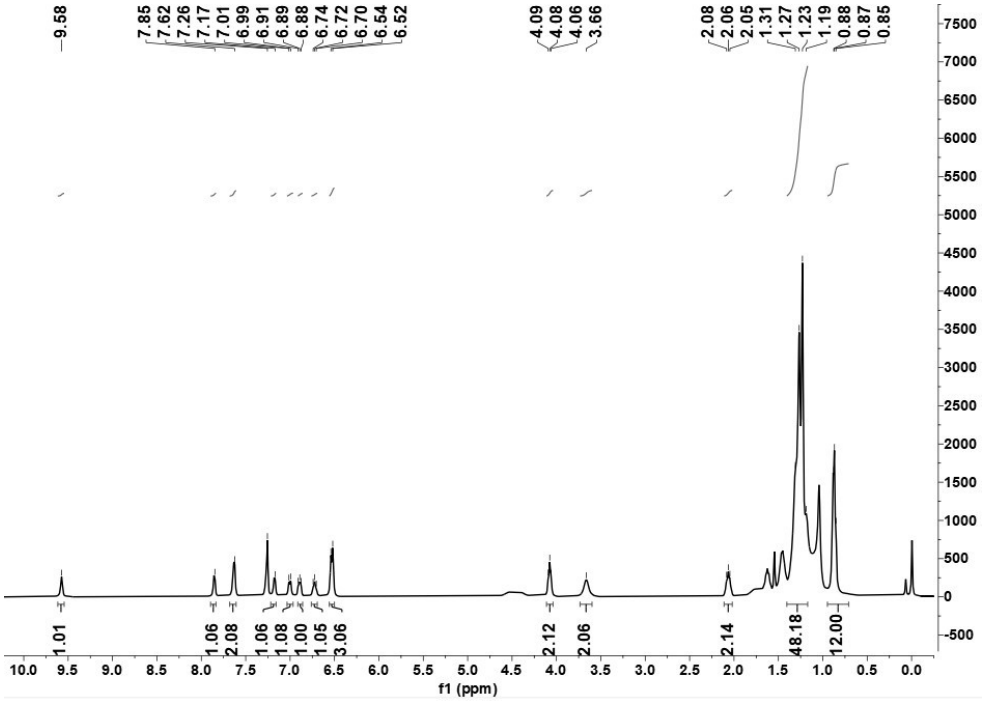


Figure S1. ^{1}H NMR spectrum of **PPAB-Br** in $CDCl_3$.

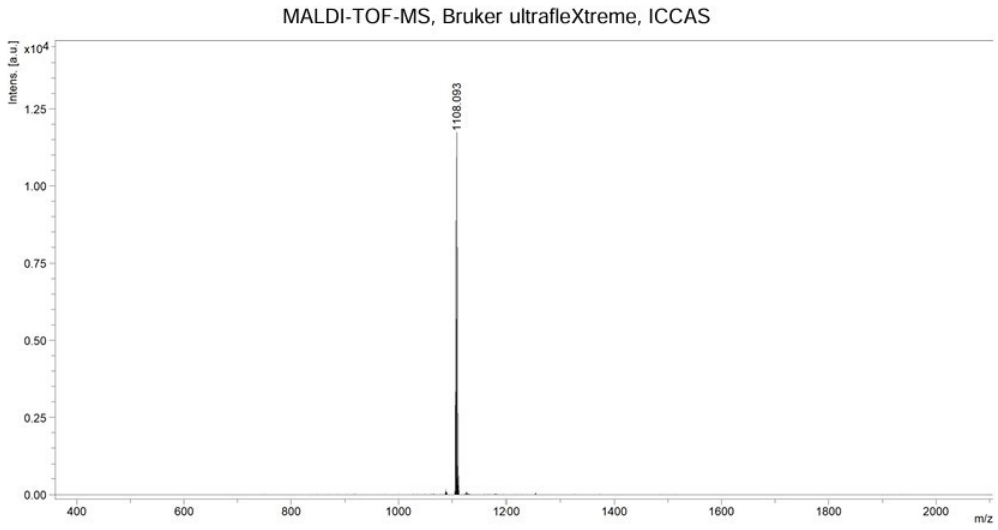


Figure S2. MALDI-TOF-MS spectrum of **PPAB-Br**.

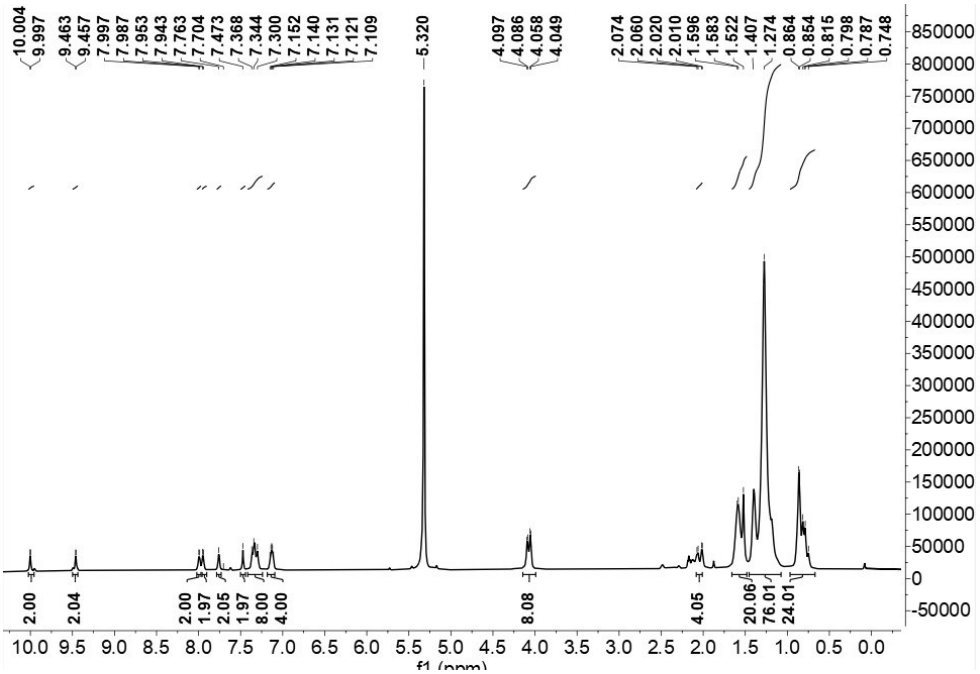


Figure S3. ^{1}H NMR spectrum of **Bis-PPAB** in CD_2Cl_2.

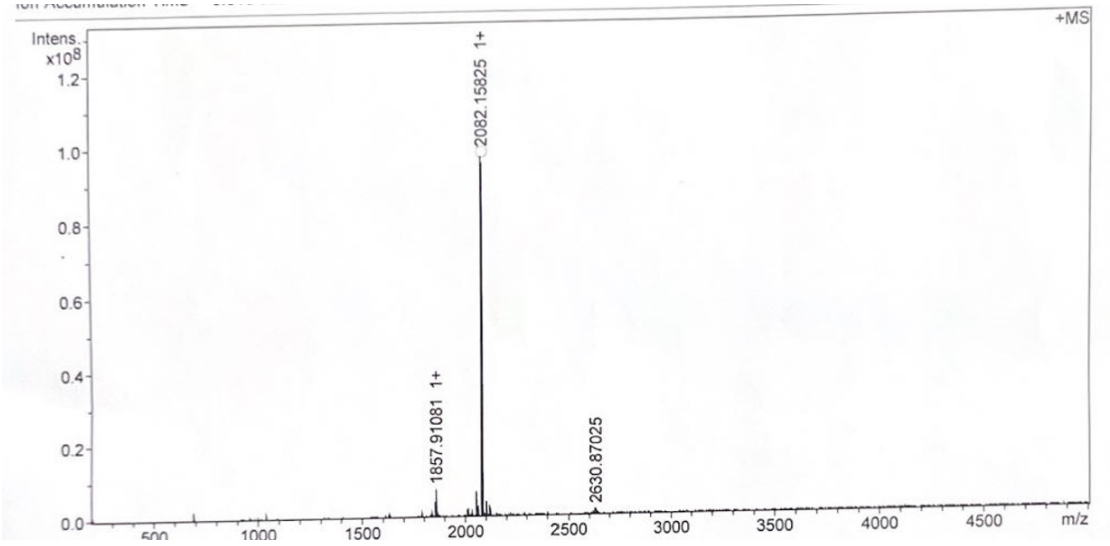
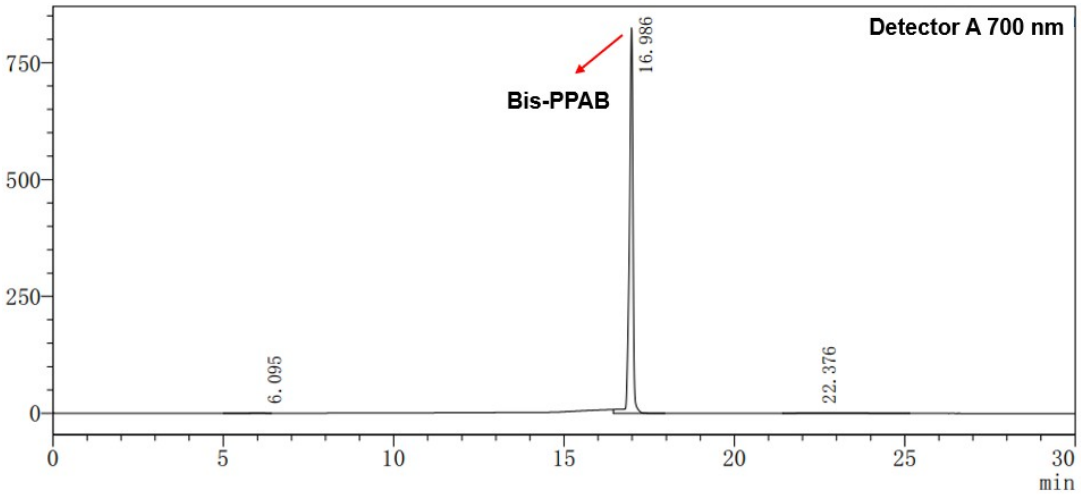


Figure S4. MALDI-TOF-MS spectrum of **Bis-PPAB**.



Peak Number	Retention time	Peak Area	Peak height	Concentration
1	6.095	19950	531	0.330
2	16.986	5927357	823831	98.034
3	22.376	98939	902	1.636
Total		6046246	825264	

Figure S5. HPLC analysis of **Bis-PPAB** at 700 nm.

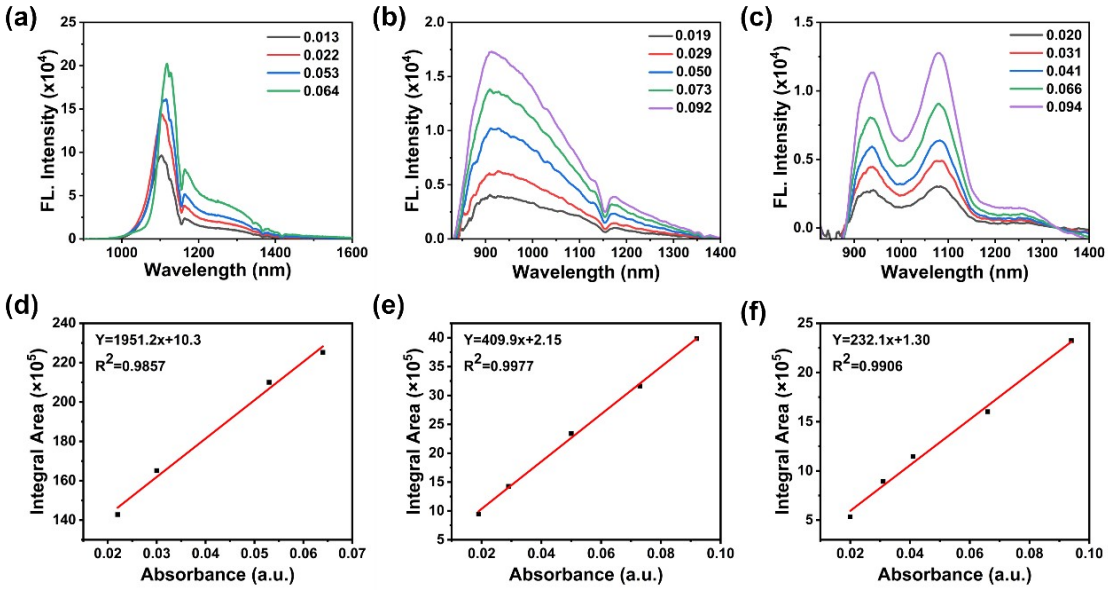


Figure S6. FL intensity of (a) IR-1061 in DCM; (b) **Bis-PPAB** in DCM; (c) **Bis-PPAB NPs** in H_2O at different absorbance. The linear fitting between the FL integrated area and the absorbance of (d) IR-1061 in DCM; (e) **Bis-PPAB** in DCM; (f) **Bis-PPAB NPs** in H_2O.

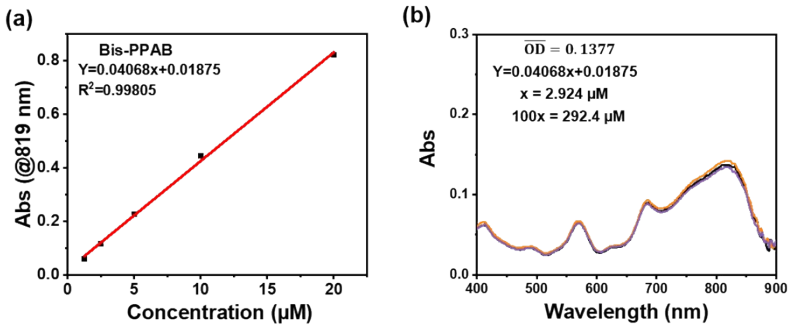


Figure S7. (a) The standard absorption curves of **Bis-PPAB** in $CHCl_3$. (b) The absorption spectrum of the final solution after being diluted one hundred times. n = 3.

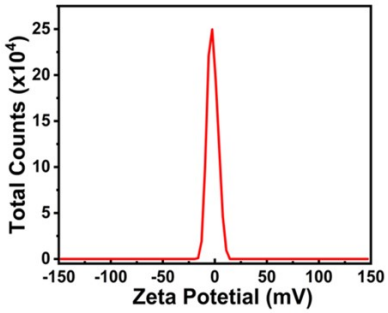


Figure S8. Zeta potential of **Bis-PPAB NPs**.

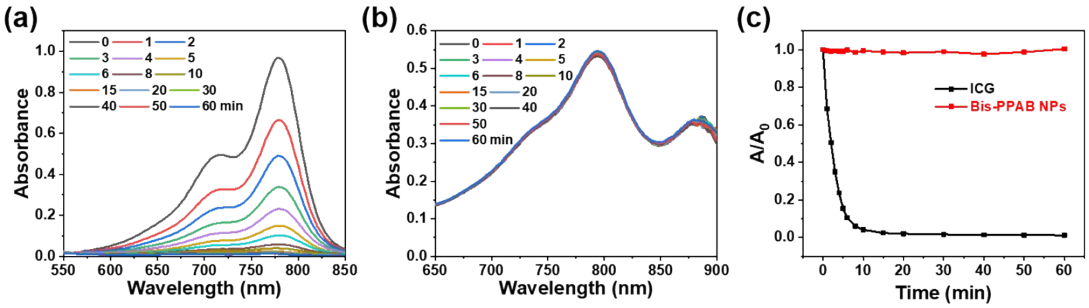


Figure S9. Absorption spectra of (a) ICG and (b) **Bis-PPAB NPs** in H_2O under continuous 808 nm laser irradiation for 60 min (1 W cm^{-2}). (c) The absorbance ratio of ICG and **Bis-PPAB NPs** at different irradiation times.

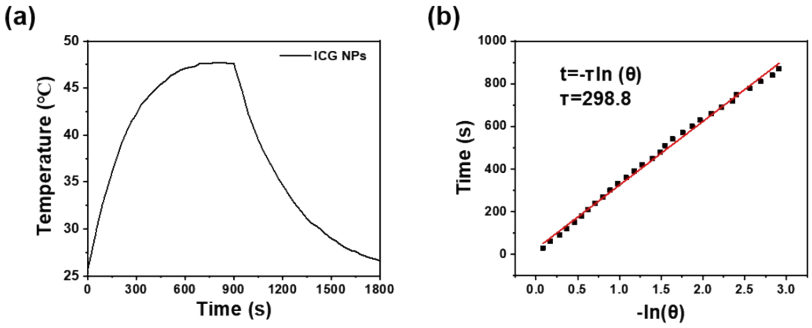


Figure S10. (a) Temperature changes of ICG NPs (10 μM) under 808 nm laser (0.8 W cm^{-2}) irradiation for 15 min. (b) The linear fitting between cooling time and -ln(θ).

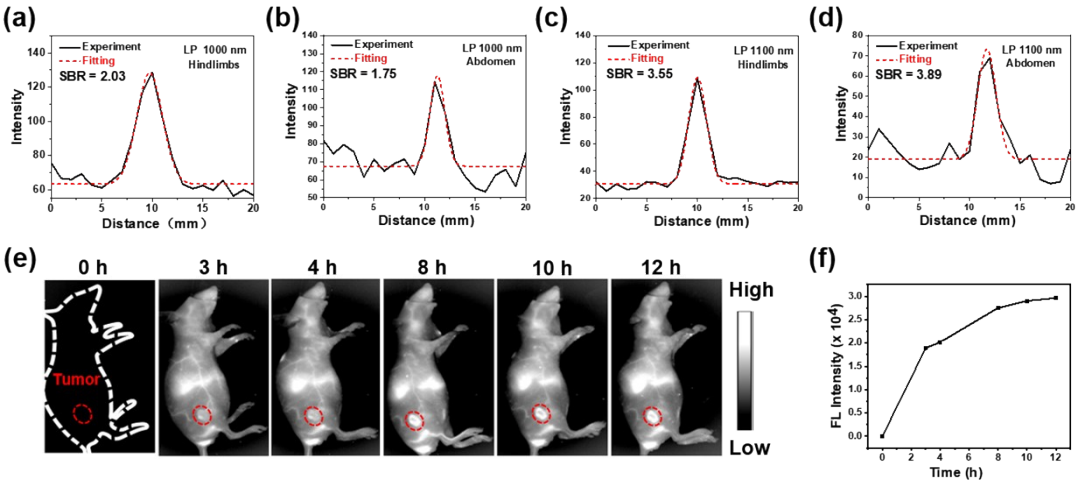


Figure S11. Cross-sectional FL intensity profiles and SBR values of (a) leg and (b) abdomen vessels using a 1000 nm LP filter, and (c) leg and (d) abdomen vessels using a 1100 nm LP filter. (e) NIR-II FL images of **Bis-PPAB NPs** at various time points post-injection, with (f) corresponding FL intensity.

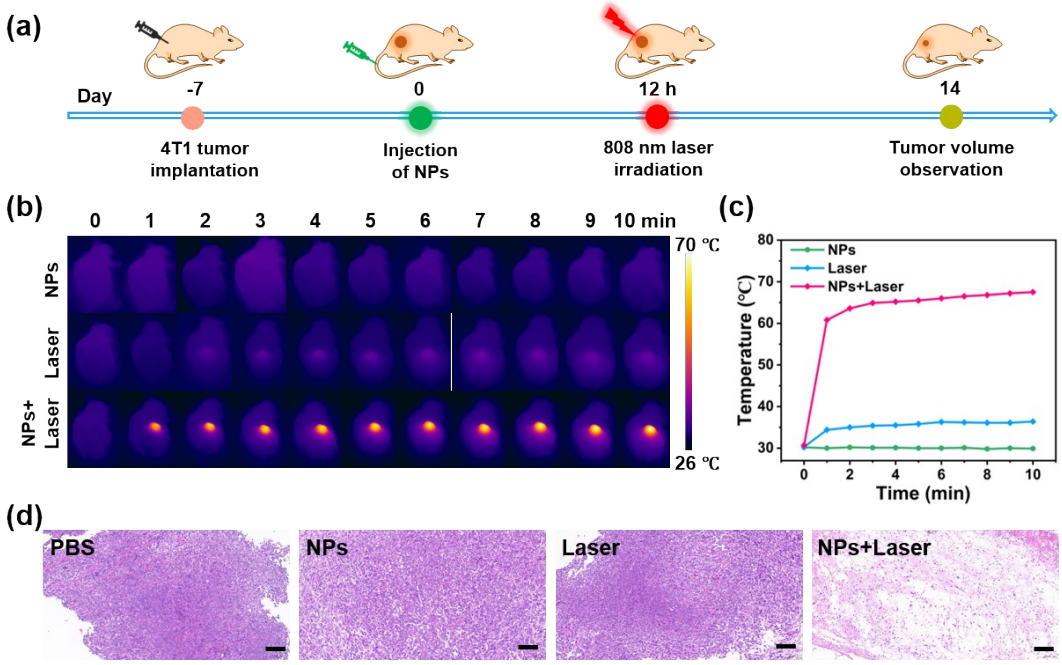


Figure S12. (a) Schematic illustration of establishing 4T1 tumor-bearing mice to evaluate the therapeutic efficacy of **Bis-PPAB NPs**. (b) Thermal imaging of 4T1 tumor-bearing mice under 808 nm laser irradiation (0.8 W cm^{-2}) at 12 hours post-injection of PBS or **Bis-PPAB NPs**, along with (c) the corresponding temperature variation curve in the tumor region. (d) H&E staining pictures of 4T1 tumors after different treatments. Scale bar = 100 μm.

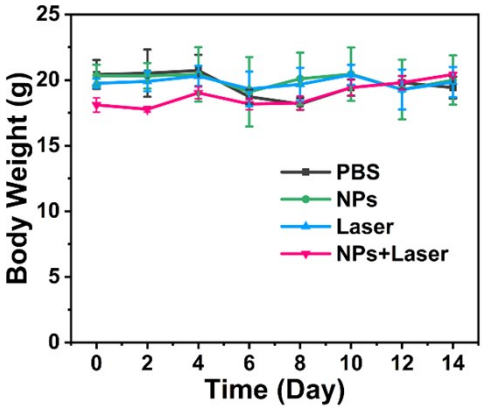


Figure S13. Mice body weight of different treatment groups during the therapy period (n = 3).

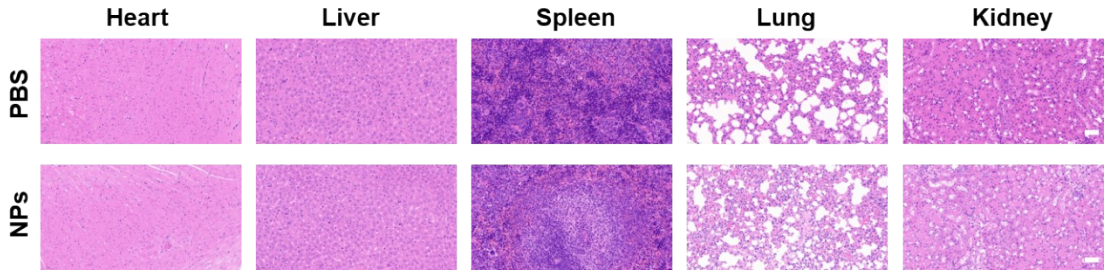


Figure S14. Hematoxylin and eosin (H&E) staining results of major organs (heart, liver, spleen, lung, and kidney) in mice following treatment with either PBS buffer or **Bis-PPAB NPs**, respectively. Scale bar = 100 μm.

Reference

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