Structure optimization of BODIPY photosensitizers for enhanced photodynamic antibacterial activities

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Materials and characterization

4-(Bromomethyl)phenylboronic acid and N-iodosuccinimide (NIS) was bought from HEOWNS (Tianjin, China). Benzyl bromide was bought from Shanghai Aladdin Biochemical Technology Co., Ltd.. Caesium carbonate was bought from Energy Chemical. 1,3-Diphenylisobenzofuran (DPBF) was bought from TCI (Shanghai) Development Co., Ltd.. Living cell nucleic acid dye (SYTO green) and propidium iodide (PI) were purchased from Jiangsu KeyGEN Biotechnology Co., Ltd.. Streptomycin sulfate, broth media and (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime Biotechnology Co., Ltd.. Cell culture dish was purchased from Guangzhou Jet Bio-Filtration Co., Ltd.. 96-Well plates were purchased from Wuxi NEST Biotechnology Co., Ltd.. ^{1}H NMR spectra were measured in $CDCl_3$ or DMSO by spectrometers from Bruker. Analytical balance (XS105DU) and Rainin Pipettes from METTLER TOLEDO were used to quantify solid and liquid respectively. Confocal laser scanning microscopy (CLSM) images were taken using a Zeiss LSM 700 (Zurich, Switzerland). The energy levels were calculated via density functional theory (DFT) at the B3LYP/6-31 G* level by Gaussian 09 package.

Bacterial culture

A single colony of *S. aureus* or *E. coli* on an agar plate was transferred to 3 mL of LB broth medium followed by shaking at 37 ℃ for 8 h. The bacteria were harvested and washed twice with phosphate buffer solution (PBS). Then, the bacteria were diluted with PBS to 10^9 CFU mL^{-1} (optical density at 600 nm (OD_{600}) of 1.0).

In vitro antibacterial performance

Bacteria (*S. aureus* or *E. coli*) were diluted in LB broth medium to 2×10^5 CFU mL^{-1} as the working suspension. The IBDPPe-Ph solution was diluted to desired concentration (containing two-fold serial dilutions) in LB broth medium. Equal volumes of bacterial

suspension and IBDPPe-Ph solution were mixed and seeded into 96-well plates resulting in a final bacteria concentration of approximately 1×10^5 CFU mL^{-1}. LB broth medium without BODIPYs was used as a control. Then the bacteria were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min after being co-cultured at 37 ℃ for 30 min. After the bacterial suspensions were cultured at 37 ℃ for 24 h, the OD values were measured by a microplate reader. The treatment concentration of BODIPYs at which no microbial growth was observed were determined as the minimal inhibitory concentrations (MICs), and the viabilities were calculated using following formula:

$$\text{Viability (\%)} = \frac{OD_{BODIPYs}}{OD_{control}} \times 100\%$$

Antibacterial ability of IBDPPe-Ph was confirmed by bacterial colony counting. The IBDPPe-Ph solution was diluted to desired concentration in LB broth medium and equal volumes of bacterial suspension and IBDPPe-Ph solution were mixed. Then the bacterial suspensions (10^5 CFU mL^{-1}) were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min after being co-cultured at 37 ℃ for 30 min. After that, the bacterial suspensions were diluted 100 fold, and then the diluted bacterial suspensions (200 μL) were inoculated on LB agar plates. After incubation at 37 ℃ for 24 h, the agar plates were photographed. The Antibacterial performance of IBDPPe-PBA, IBDPPy-Ph and IBDPPy-PBA *in vitro* was tested in a similar way.

Inhibition of biofilm formation

Bacteria (*S. aureus* or *E. coli*) were diluted in LB broth medium to 2×10^7 CFU mL^{-1} as the working suspension. The IBDPPy-Ph solution was diluted to desired concentration in LB broth medium and equal volumes of bacterial suspension and IBDPPy-Ph solution were mixed and seeded into 96-well plates. Then the bacterial suspensions (10^7 CFU mL^{-1}) were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min after being co-cultured at

37 ℃ for 30 min. After the bacterial suspensions were cultured at 37 ℃ for 24 h, the origin medium was gently removed and washed twice with sterile water. Then 200 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg mL^{-1}) was added to each well and incubated for 4 h at 37 ℃. After removing MTT solution, 150 μL of dimethyl sulfoxide (DMSO) was added to each well and the OD value was recorded at 490 nm on a microplate reader after shaking for 3 min. The inhibition of biofilms formation performance of IBDPPy-PBA was tested in a similar way.

Detection of ROS generation in biofilms

To prepare biofilms, *S. aureus* (500 μL, 10^7 CFU mL^{-1}) were seeded into a 24-well plate and cultured in a 37 °C incubator for 24 h. Then, the culture media were sucked out and the biofilms were washed with PBS twice. After that, IBDPPy-PBA (500 μL, 10 μM) and DCFH-DA were added, and the biofilms were cultured in the dark for 30 min. Then the biofilms were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min followed by washing with PBS for several times. The ROS generation in biofilms was observed by CLSM.

Activity against mature biofilms

Bacteria (*S. aureus* or *E. coli*) were diluted in LB broth medium to 10^7 CFU mL^{-1} as the working suspension. The bacterial suspension (200 μL) was added to each well (96-well plates), then static incubating for 24 h at 37 °C to formation mature biofilm. The origin medium was gently removed and washed twice with sterile water. The IBDPPy-Ph solution was diluted to the desired concentration and added into each well. After being co-cultured at 37 ℃ for 30 min, the bacterial suspensions were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min. The bacterial suspensions were cultured at 37 ℃ for 30 min, and then the origin medium was gently removed and washed twice with sterile water. Next 200 μL MTT solution (0.5 mg mL^{-1}) was added to each well and incubated for 4 h at 37 ℃. After

removing MTT solution, 150 μL of DMSO was added to each well and the OD value was recorded at 490 nm on a microplate reader after shaking for 3 min. The activity against mature biofilms of IBDPPy-PBA was tested in a similar way.

Biofilm imaging

Bacteria (*S. aureus* or *E. coli*) were diluted in LB broth medium to 10^7 CFU mL^{-1} as the working suspension. The bacterial suspension (500 μL) was added to each well (24-well plates), then static incubating for 24 h at 37 °C to formation mature biofilm. The origin medium was gently removed and washed twice with sterile water. The IBDPPy-Ph or IBDPPy-PBA solution was diluted to the desired concentration and added into each well. Then the bacterial suspensions were irradiated with/without green LED light (18 mW cm^{-2}) for 10 min after being co-cultured at 37 ℃ for 30 min. The bacterial suspensions were cultured at 37 ℃ for 30 min, and then the origin medium was gently removed and washed twice with sterile water. The biofilm were stained with SYTO and PI and were imaged by CLSM.

Treatment of biofilm infected wounds

The antibacterial ability of IBDPPy-PBA and IBDPPy-Ph was evaluated via a mouse wound infection model. In brief, a circular cut (1.2 mm in diameter) were created on the back by excision and incubated with *S. aureus* (50 μL, 1.0×10^7 CFU) for 24 h to form *S. aureus* biofilm infected wounds. Then the rats with *S. aureus* biofilm infected wounds were randomly divided into five groups, and IBDPPy-PBA or IBDPPy-Ph (50 μL, 30 μM) were sprayed on the wounds and PBS (50 μL) were considered as the control. The wounds with IBDPPy-Ph or IBDPPy-PBA were irradiated with/without green LED light (18 mW cm^{-2}) (IBDPPy-PBA+L /IBDPPy-PBA, IBDPPy-Ph+L /IBDPPy-Ph) for 10 min after being co-cultured for 30 min.

Cytotoxicity test

The cytotoxicity of IBDPPy-Ph and IBDPPy-PBA was investigated by the classical MTT assay. In brief, NIH 3T3 or L929 cell were seeded into 96-well plates and cultured for 24 h at 37 °C. Next, IBDPPy-Ph or IBDPPy-PBA at a series of concentrations (0-30 μM) were added, and the cells were incubated at 37 °C for 24 h. 20 μL of MTT solution (5 mg mL^{-1}) was added to each well. After 4 h, the media were removed and 150 μL of DMSO was added into each well to dissolve the formazan crystals. Finally, the absorbance of each well at 490 nm was measured by a microplate reader.

Hemolysis assay

Erythrocytes were acquired by centrifugation at 3500 rpm for 5 min and suspended in PBS. Afterward, 1 mL of IBDPPy-Ph or IBDPPy-PBA solution (10 μM) was mixed with 0.2 mL of the erythrocyte suspension. Positive or negative controls were conducted by mixing 1.2 mL of Triton-X 100 or PBS with 0.2 mL of the erythrocyte suspension, respectively. All mixtures were maintained at 37 °C for 4 h and then centrifuged at 3500 rpm for 5 min. A microplate reader was used to measure the OD value of the samples at 405 nm. The calculating equation of the hemolysis rate was as follows:

$$\text{Hemolysis rate (\%)} = [(OD_{sample} - OD_{negative})/(OD_{positive} - OD_{negative})] \times 100\%.$$

Statistical analysis

All statistical analyses were performed with one-way ANOVA test. The results were represented as mean ± standard deviation (SD). Statistical significance is indicated as *P< 0.05, **P< 0.01, and ***P< 0.001.

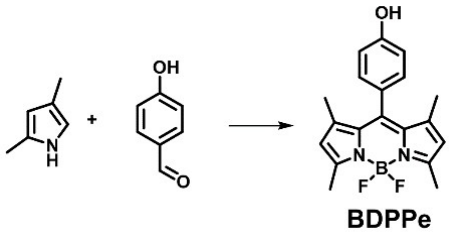

Fig. S1 The synthetic route of BDPPe.

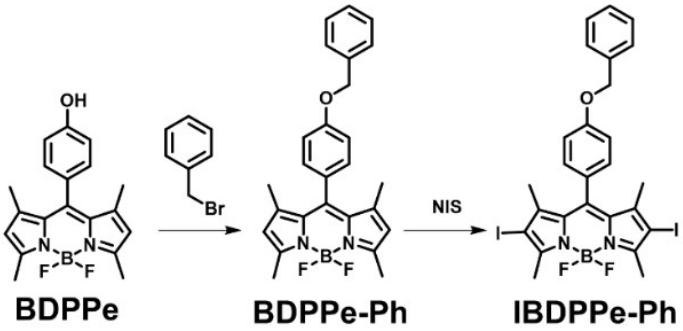

Fig. S2 The synthetic route of IBDPPe-Ph.

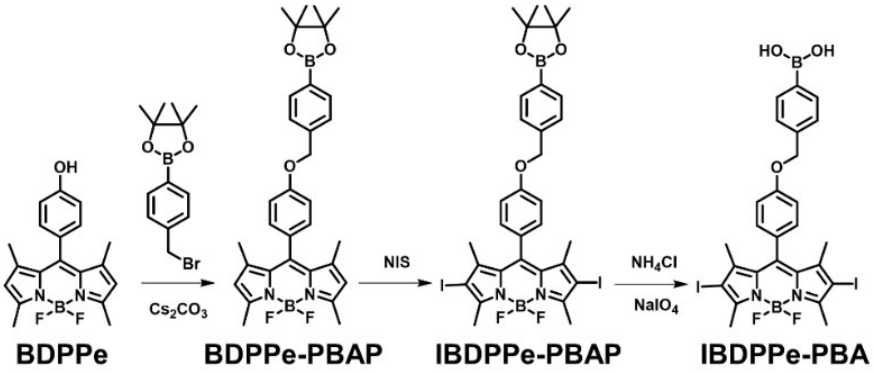

Fig. S3 The synthetic route of IBDPPe-PBA.

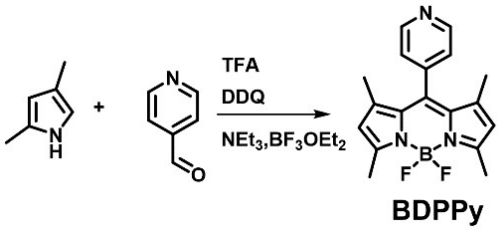

Fig. S4 The synthetic route of BDPPy.

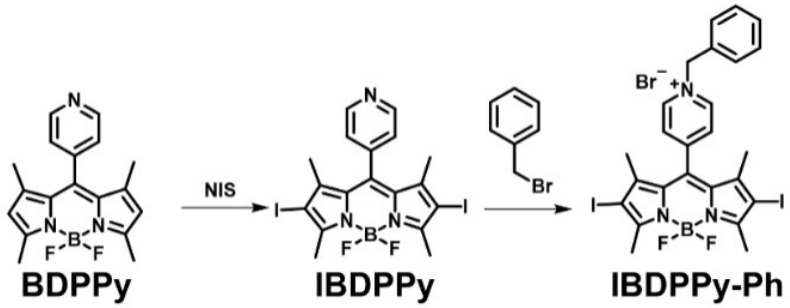

Fig. S5 The synthetic route of IBDPPy-Ph.

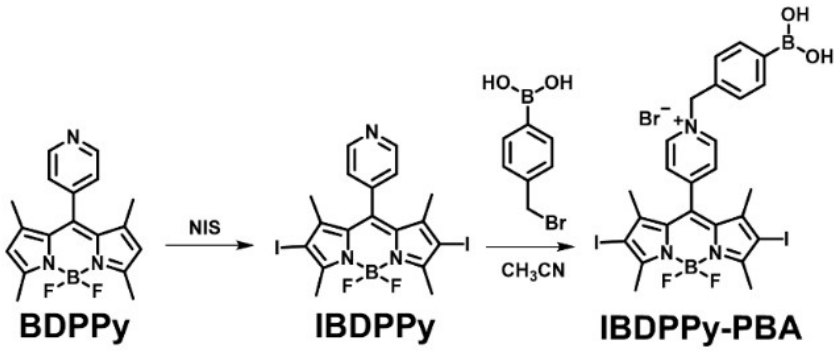

Fig. S6 The synthetic route of IBDPPy-PBA.

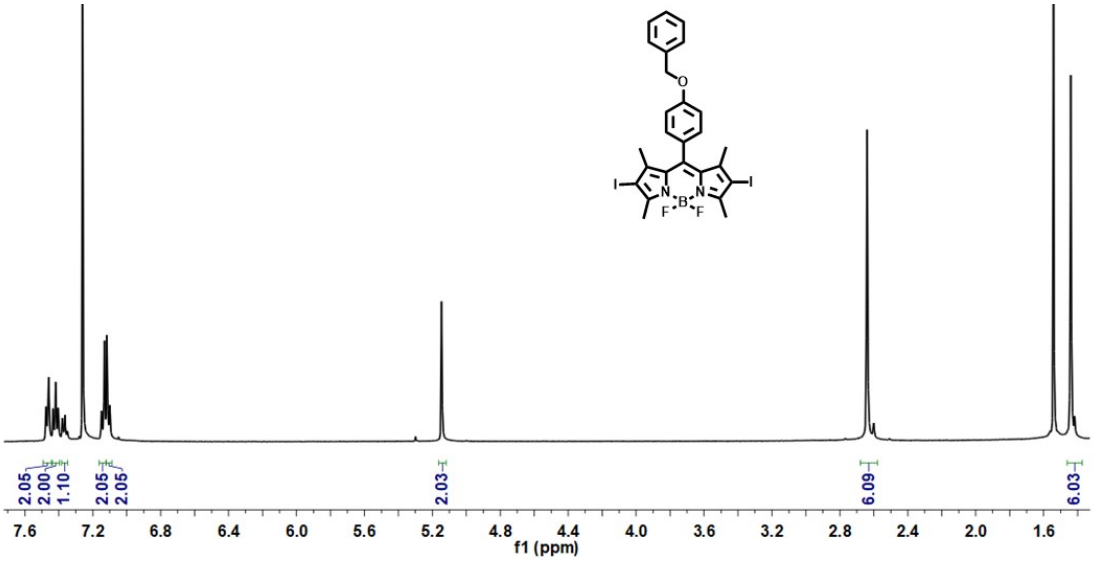

Fig. S7 The ^{1}H NMR spectrum of IBDPPe-Ph. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (d, J = 7.3 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.38 – 7.35 (m, 1H), 7.14 (d, J = 8.8 Hz, 2H), 7.11 (d, J = 8.7 Hz, 2H), 5.15 (s, 2H), 2.62 (d, J = 19.6 Hz, 6H), 1.43 (d, J = 11.2 Hz, 6H).

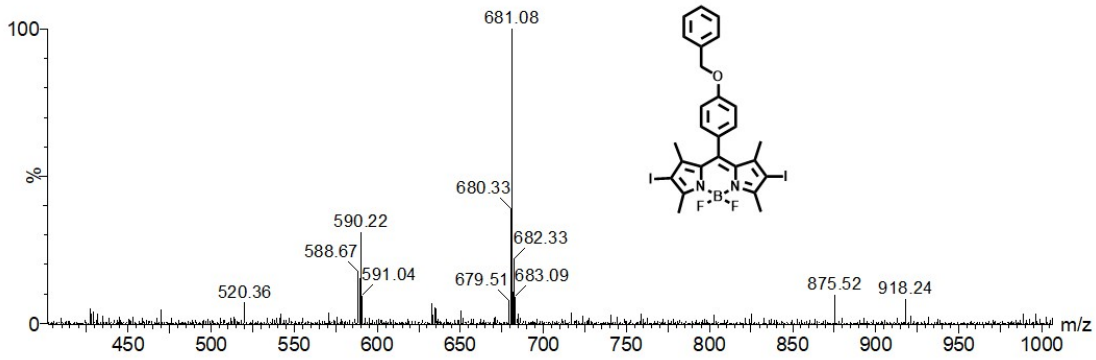

Fig. S8 The ESI-MS spectrum of IBDPPe-Ph.

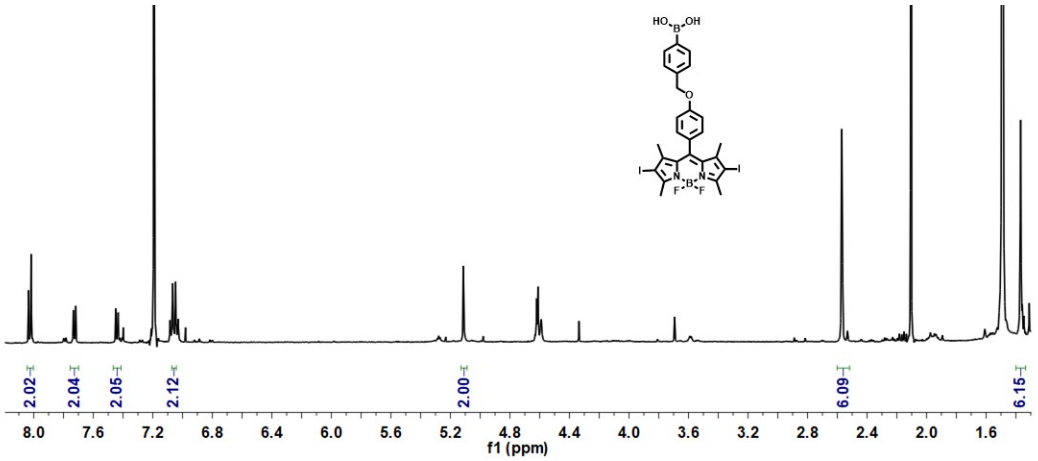

Fig. S9 The ^{1}H NMR spectrum of IBDPPe-PBA. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (s, 2H), 7.73 (d, J = 7.9 Hz, 2H), 7.47 – 7.41 (m, 2H), 7.05 (dd, J = 10.1, 2.3 Hz, 2H), 5.11 (s, 2H), 2.57 (s, 6H), 1.37 (s, 6H).

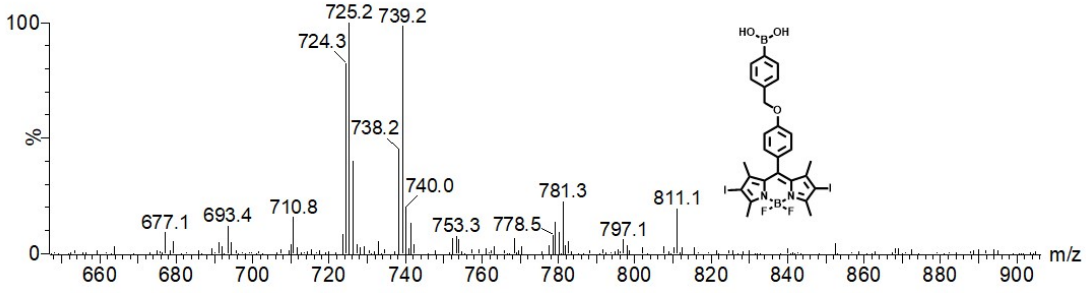

Fig. S10 The ESI-MS spectrum of IBDPPe-PBA.

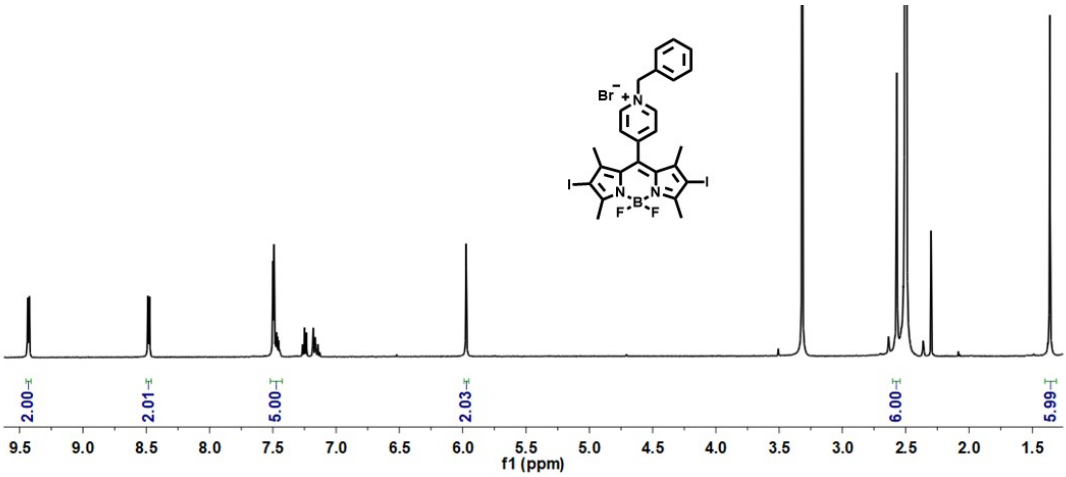

Fig. S11 The ^{1}H NMR spectrum of IBDPPy-Ph. ^{1}H NMR (500 MHz, DMSO) δ 9.43 (d, J = 6.7 Hz, 2H), 8.48 (d, J = 6.8 Hz, 2H), 7.52 – 7.43 (m, 5H), 5.97 (s, 2H), 2.57 (s, 6H), 1.36 (s, 6H).

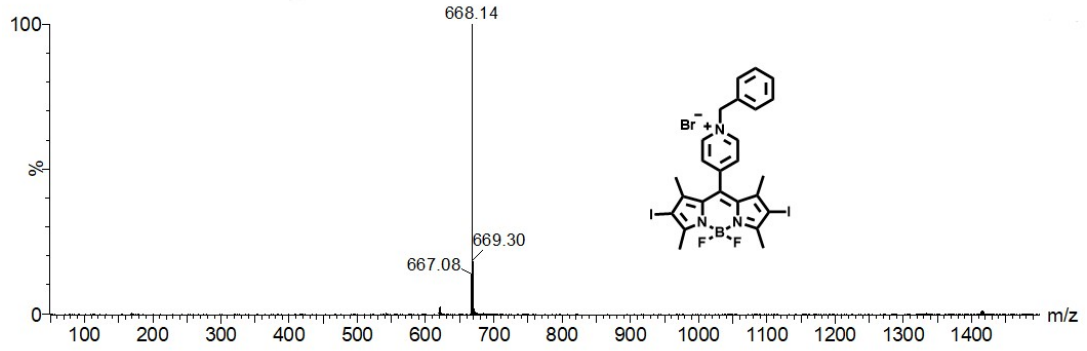

Fig. S12 The ESI-MS spectrum of IBDPPy-Ph.

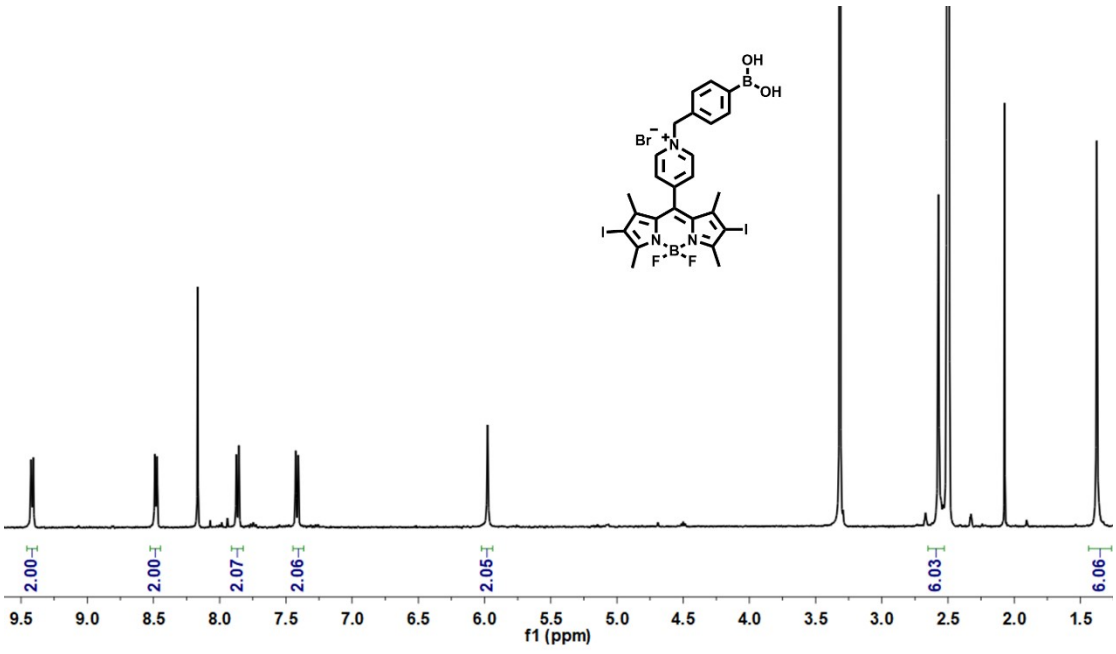

Fig. S13 The ^{1}H NMR spectrum of IBDPPy-PBA. ^{1}H NMR (500 MHz, DMSO) δ 9.42 (d, J = 6.6 Hz, 2H), 8.48 (d, J = 6.7 Hz, 2H), 7.86 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 5.98 (s, 2H), 2.57 (s, 6H), 1.38 (s, 6H).

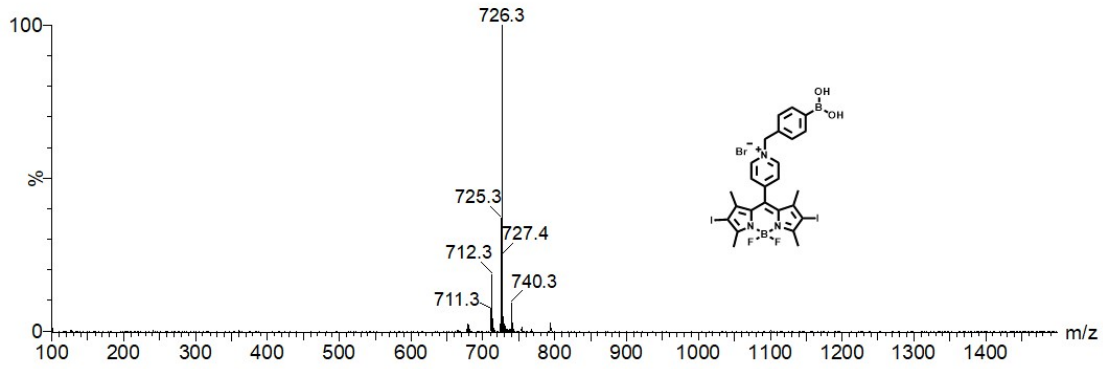

Fig. S14 The ESI-MS spectrum of IBDPPy-PBA.

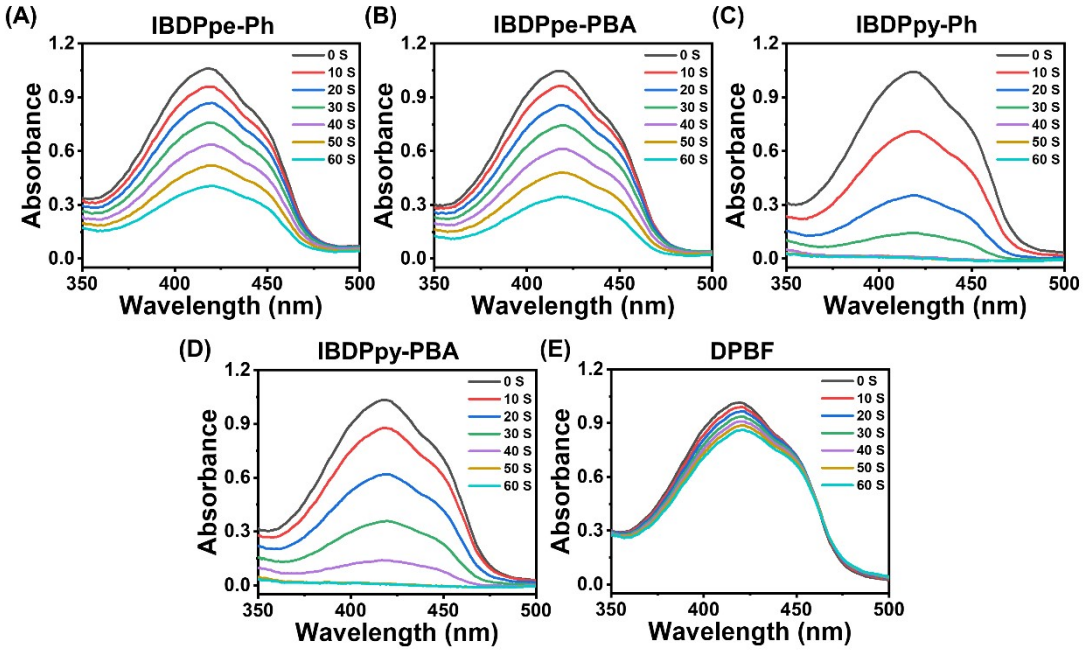

Fig. S15 The singlet oxygen ($^1O_2$) generation of (A) IBDPPe-Ph, (B) IBDPPe-PBA, (C) IBDPPy-Ph and (D) IBDPPy-PBA (2 μM) upon green light irradiation using DPBF as the indicator. (E) The change of the fluorescence of DPBF solution without photosensitizer upon green light irradiation.

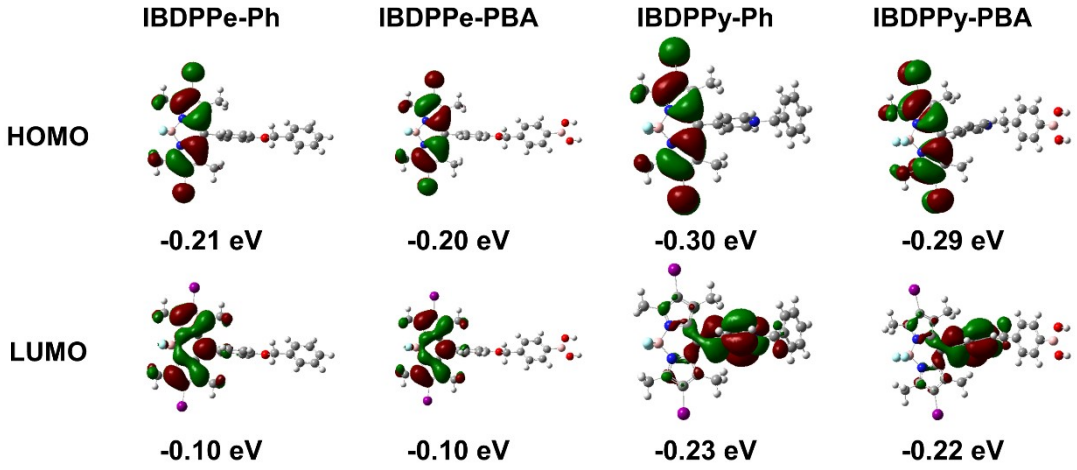

Fig. S16 HOMO and LUMO distributions of 4 BODIPYs.

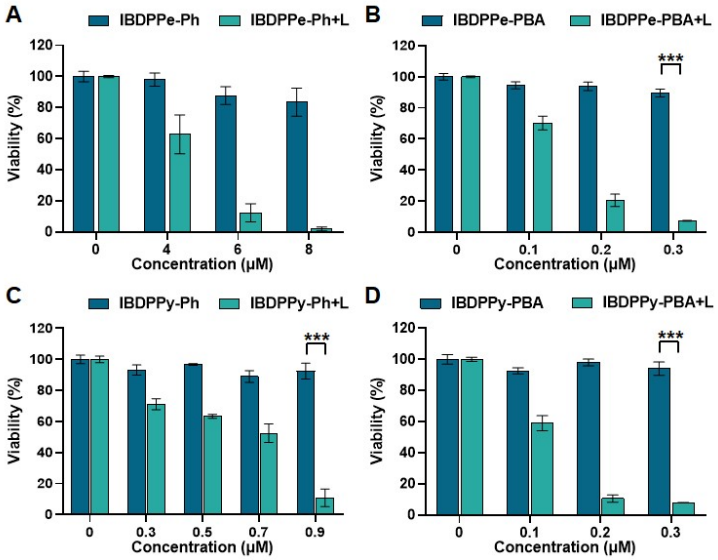

Fig. S17 The bacterial viability of *S. aureus* treated with different concentrations of 4 BODIPYs with or without green light irradiation (18 mW cm^{-2}).

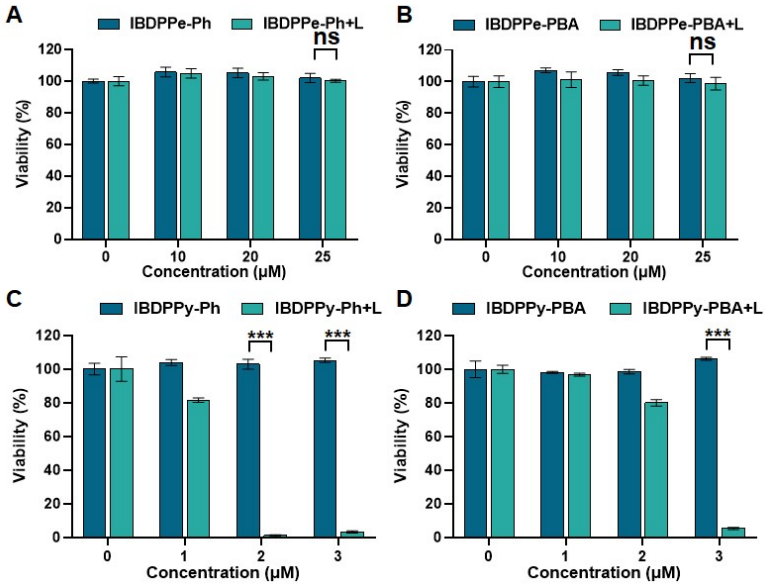

Fig. S18 The bacterial viability of *E. coli* treated with different concentrations of 4 BODIPYs with or without green light irradiation (18 mW cm^{-2}).

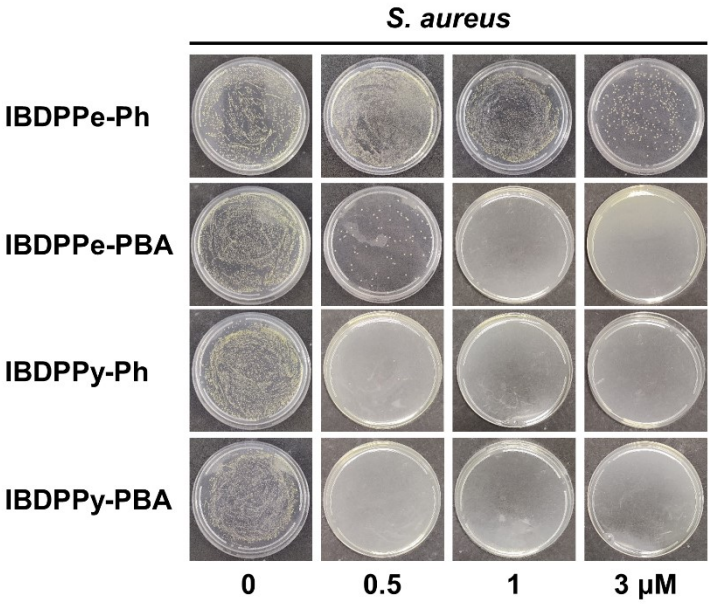

Fig. S19 Photographs of *S. aureus* on LB agar plates after incubation with IBDPPe-Ph, IBDPPe-PBA, IBDPPy-Ph and IBDPPy-PBA under green light irradiation (18 mW cm^{-2}).

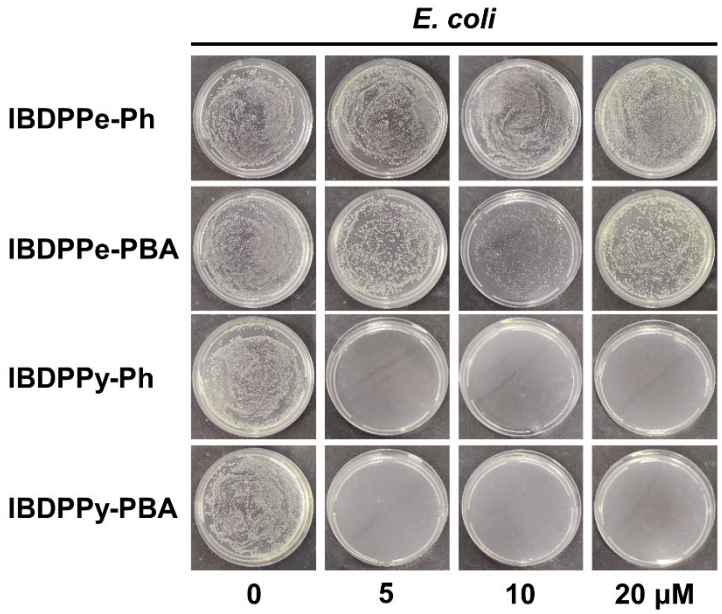

Fig. S20 Plate photographs of *E. coli* on LB agar plates after incubation with IBDPPe-Ph, IBDPPe-PBA, IBDPPy-Ph and IBDPPy-PBA under green light irradiation (18 mW cm^{-2}).

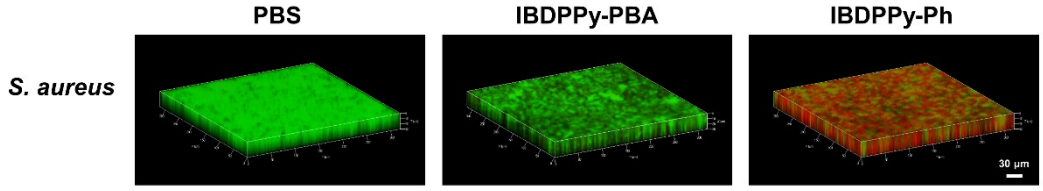

Fig. S21 (A) 3D CLSM images of *S. aureus* biofilms incubated with PBS, IBDPPy-PBA or IBDPPy-Ph (30 μM) under dark condition.

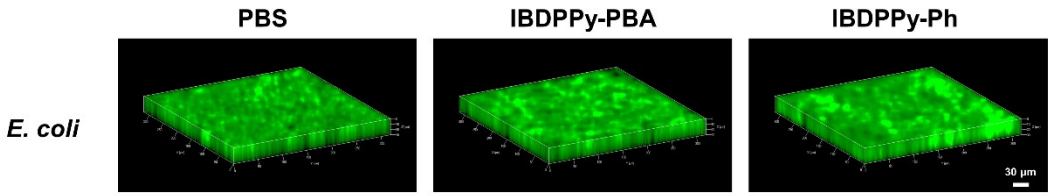

Fig. S22 3D CLSM images of *E. coli* biofilms incubated with PBS, IBDPPy-PBA or IBDPPy-Ph (2 μM) under dark condition.

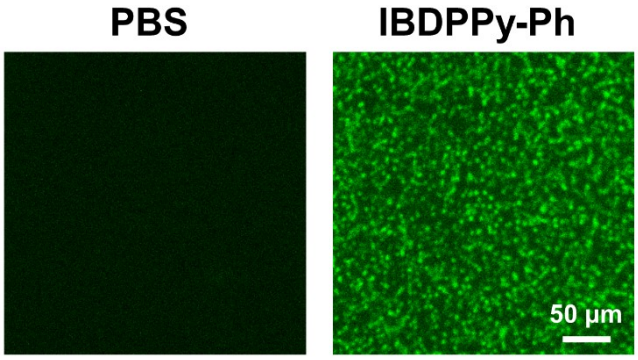

Fig. S23 Detection of ROS generation in *S. aureus* biofilms incubated with PBS and IBDPPy-Ph (10 μM) with DCFH-DA as the probe under green light irradiation (18 mW cm^{-2}).

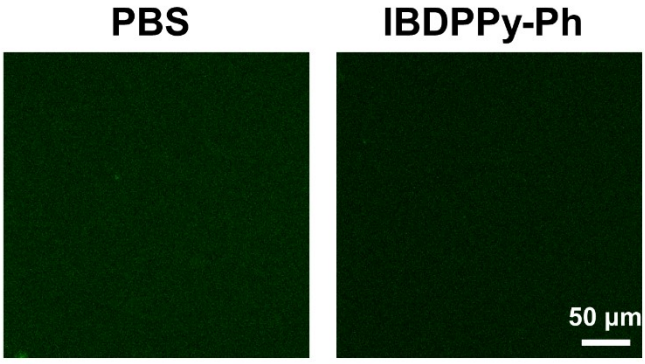

Fig. S24 Detection of ROS generation in *S. aureus* biofilms incubated with PBS and IBDPPy-Ph (10 μM) with DCFH-DA as the probe under dark condition.

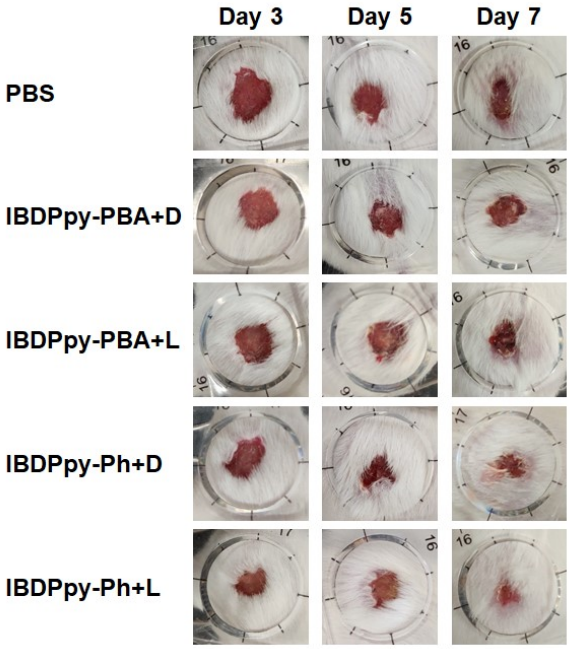

Fig. S25 Photographs of *S. aureus* infected wounds on the 3th, 5th and 7th day after different treatments.

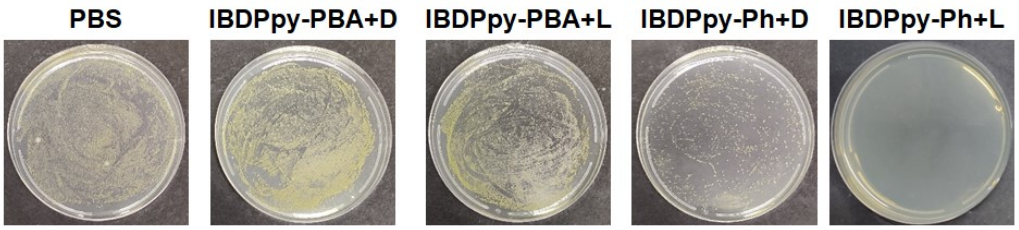

Fig. S26 Photographs of agar plates with bacterial colonies separated from the wound tissues of mice after different treatments on the 8th day.

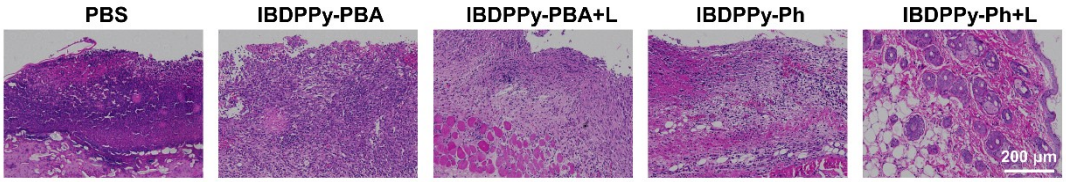

Fig. S27 H&E staining of the infected wounds on the 8th day after different treatments.

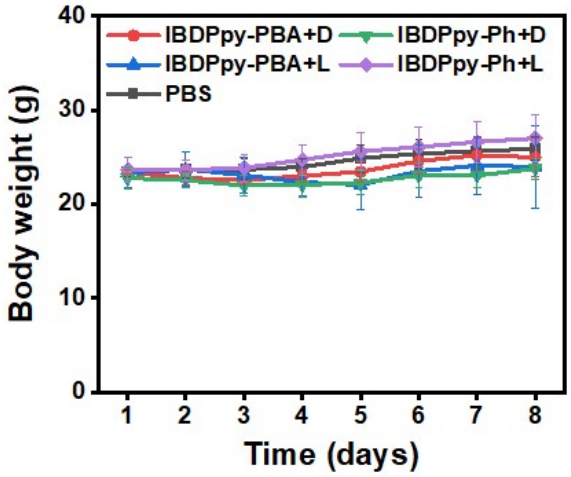

Fig. S28 The body weight changes of the mice in the groups during the treatment period.

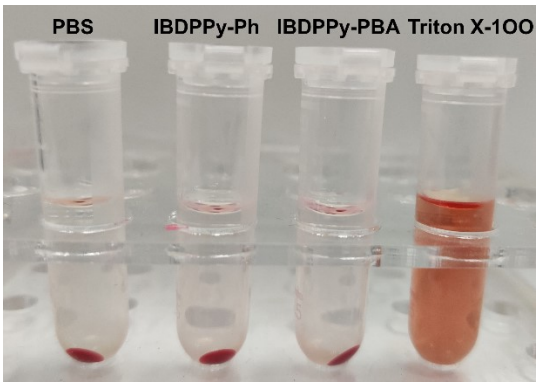

Fig. S29 Photo of hemolysis of IBDPPy-Ph and IBDPPy-PBA (Triton X-100 as the positive control).

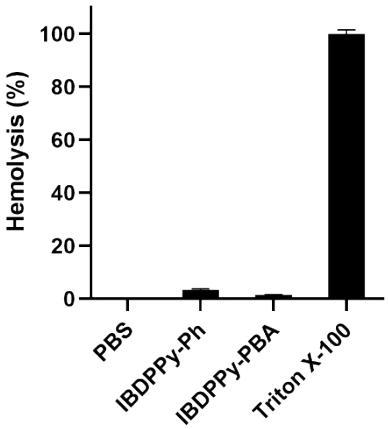

Fig. S30 Hemolysis ratios of IBDPPy-Ph and IBDPPy-PBA (Triton X-100 as the positive control).