# **Supporting Information**

# Multifunctional Protein-Based Self-Assembled Nanoplatform: Overcoming Hypoxic Tumor Microenvironment for Enhanced Imaging-Guided Photodynamic Therapy

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# **Experimental Section**

## Synthesis of compound BODIPY 1 (B1).

Compound B1 was synthesized using the method reported in literature<sup>1</sup>. In a 200 mL dried dichloromethane (DCM) solution, 2,4-dimethylpyrrole (760 mg, 8 mmol) and 4-hydroxy benzaldehyde (488 mg, 4 mmol) were dissolved under argon protection. Four drops of trifluoroacetic acid were added, and the reaction mixture was wrapped with aluminum foil and stirred slowly at room temperature for 4 h. Then, 2,3-dichloro-5,6-dicyano-p-benzoquinone (884 mg, 4 mmol) was added to the solution, followed by stirring for 20 min. The reaction mixture was then treated with triethylamine (6 mL) for 5 min, followed by slow addition of boron trifluoride diethyl etherate (6.4 mL) using a syringe. The reaction was continued for 40 min. After completion of the reaction, the resulting dark brown mixture was washed with ultrapure water ( $3 \times 20 \text{ mL}$ ) and saline solution (30 mL), dried with anhydrous sodium sulfate, concentrated under reduced pressure, and purified by silica gel flash column chromatography (ethyl acetate/petroleum ether=1/9, v/v) to obtain red crystals of B1 (yield: 32%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.12 (d, J = 7.8 Hz, 2H), 6.95 (d, J = 7.8 Hz, 2H), 5.98 (s, 2H), 2.55 (s, 6H), 1.44 (s, 6H); HRMS m/z: C<sub>19</sub>H<sub>19</sub>BF<sub>2</sub>N<sub>2</sub>O [M+Na]<sup>+</sup> calcd for 363.1645 found 363.1609.

## Synthesis of compound BODIPY 2 (B2).

Compound B1 (400 mg, 96 mmol) and 3-bromopropyne (205 mg, 140 mmol) were dissolved in acetone (50 mL) containing anhydrous potassium carbonate (132 mg, 77 mmol). The reaction mixture was refluxed for 40 h. After addition of ultrapure water, the product was extracted with DCM, and the extract was dried and the solvent was evaporated. The crude product was purified by silica gel column chromatography using DCM/n-hexane (3:2) as eluent, yielding a red solid B2 (yield: 50%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.19 (d, *J* = 7.7 Hz, 2H), 7.09 (d, *J* = 7.5 Hz, 2H), 6.16 - 5.97 (m, 2H), 4.76 (s, 2H), 2.63 - 2.55 (m, 7H), 1.42 (s, 6H); HRMS m/z: C<sub>22</sub>H<sub>21</sub>BF<sub>2</sub>N<sub>2</sub>O [M+H]<sup>+</sup> calcd for 379.1793 found 379.1777.

#### Synthesis of compound I-BODIPY 4 (B3).

Compound B2 (215 mg, 0.57 mmol) and iodine (335 mg, 1.31 mmol) were added to a solution of ethanol (15 mL), followed by the addition of iodine acid (230 mg, 1.31 mmol) dissolved in 1.5 mL of water. The resulting mixture was stirred at room temperature. After complete consumption of all starting materials, the product was extracted into the organic phase DCM ( $3 \times 30$  mL) upon addition of a saturated solution of sodium thiosulfate solution (10 mL). The extract was dried and the solvent was evaporated using a rotary evaporator. The resulting crude product was purified by silica gel column chromatography using DCM/n-hexane (1:3) as eluent, yielding a red solid B3 (yield: 70%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.16 (d, *J* = 8.2 Hz, 2H), 7.11 (d, *J* = 8.2 Hz, 2H), 4.78 (s, 2H), 2.64 (s, 6H), 2.57 (s, 1H), 1.43 (s, 6H); HRMS m/z: C<sub>22</sub>H<sub>19</sub>BF<sub>2</sub>I<sub>2</sub>N<sub>2</sub>O [M]<sup>+</sup> calcd for 629.9648 found 629.9669.

#### Measurement singlet oxygen quantum yield ( $\Phi_{\Delta}$ )

The quantum yields of singlet oxygen ( $\Phi_{\Delta}$ ) were determined for the photosensitizers B3 and B4 using the following procedure, which is based on the methodology described by Huang et al<sup>2</sup>. Methylene Blue (MB) in dichloromethane was used as the reference, with a known singlet oxygen quantum yield of  $\Phi_{\Delta} = 52\%$ . The absorbance of the singlet oxygen scavenger, 1,3diphenylisobenzofuran (DPBF), was adjusted to approximately 1.0 in air-saturated dimethylformamide (DMF). The photosensitizers were then introduced, and their absorbances were adjusted to approximately 0.2. A cuvette containing the prepared solution was subjected to monochromatic light at a wavelength of 710 nm, emitted from a fluorimeter at the peak absorption wavelength, for a duration of 9 seconds.

The slope of the absorbance maxima of DPBF at 415 nm versus time was determined for each photosensitizer. The singlet oxygen quantum yields ( $\Phi_{\Delta}$ ) were calculated using the following equation:

$$\Phi_{\Delta} = \Phi(MB) \times k (B) \times F (MB) / k (MB) \times F (B)$$

Here, k represents the slope of the difference in the change in the absorbance of DPBF at 414 nm with respect to irradiation time, and F denotes the absorption correction factor. The absorption correction factor, F, is calculated as  $1 - 10^{\text{OD}}$ , where OD represents the optical density at the

irradiation wavelength.

For B3, the absorbance was A = 0.134, and the slope was k = -0.08393, resulting in a calculated  $\Phi_{\Delta}$  of approximately 73% (Figure 1b, c and Figure S1). For B4, the absorbance was A = 0.095, and the slope was k = -0.10419, resulting in a calculated  $\Phi_{\Delta}$  of approximately 67% (Figure 1d, e and Figure S1). These findings suggest significant singlet oxygen production by both photosensitizers under the experimental conditions.

### TEM characterization of CIB NPs.

A certain amount of CIB NPs solution was sonicated for 1 min. Next, 10  $\mu$ L of the CIB NPs solution was dropped onto a copper mesh with a micropipette tip and allowed to stand for 15 min. The excess solution on the copper mesh was removed with the micropipette tip, and the mesh was washed with ultrapure water (this step was repeated 3 times). The copper mesh with the CIB NPs sample was then placed in a drying oven until completely dry, and the morphology and size of the CIB NPs were observed using transmission electron microscopy (TEM).

#### DLS characterization of CIB NPs.

1 mL of pre-prepared mixed solution with different assembly conditions was taken. Slowly add 2 mL of pH 7.4 PBS into the solution. Then, the solution was sonicated in the dark for 30 min with a water temperature of 0 °C. Next, 3 mL of the sample to be tested was added to the specialized dish of dynamic light scattering (DLS) instrument for the measurement of the hydrated particle size of nanoparticles.

## Fluorescence spectra characterization of CIB NPs.

Firstly, the prepared assembly solutions under four different assembly conditions were placed in designated colorimetric vessels. Subsequently, fluorescence spectra of each sample were analyzed using a fluorescence spectrometer. Specifically, the excitation wavelength was set at  $\lambda_{max, Abs}$  of B4, and the corresponding  $\lambda_{max, Em}$  was recorded for each sample.

## Cell culture.

The A549 and 4T1 cells were cultured in Ham's F-12 nutrient medium (F-12K) and RPMI Medium 1640 medium, respectively, supplemented with 10% fetal bovine serum (FBS) and a triple antibiotic mixture (penicillin-streptomycin-gentamicin), and maintained using standard protocols.

Both cell types were grown in a humidified incubator at 37 °C and 5% CO<sub>2</sub> concentration for optimal growth.

## **Tumor model establishment**

Female nude mice weighing  $19\pm1g$  were used in the experiment. The nude mice were given sterilized tap water and sterilized food. A549 cells were used to establish the tumor model. A suspension of  $2\times10^7$  cells/mL PBS was prepared and washed three times by centrifugation. Then,  $2\times10^6$  cells were subcutaneously implanted into the right thigh of the nude mice to establish a solid tumor model. The nude mice were regularly examined, and the tumor long and short diameters as well as the nude mice's body weight were recorded. The formula for calculating tumor volume is: tumor volume (V) =  $(a \times b^2)/2$ , where a and b represent the long and short diameters of the tumor, respectively. All animal investigations were performed in accordance with the institute guidelines of the Laboratory Animal Ethics Committee of Hainan Medical University and Chinese regulations on laboratory animal regulations.

#### Synthesis routes of B1, B2, B3, and B4.

Under acidic conditions in trifluoroacetic acid, both α-hydrogens of two 2,4-dimethylpyrroles underwent condensation with 4-hydroxy benzaldehyde, followed by elimination of one pyrrole nitrogen hydrogen upon treatment with 2,3-Dichloro-5,6-dicyano-p-benzoquinone. The resulting dipyrroles then underwent complexation with boron trifluoride in triethylamine under basic conditions, yielding product B1. Subsequent condensation with 3-bromopropyne led to product B2. The introduction of iodine and iodic acid then facilitated substitution reactions, leading to the formation of product B3, the iodinated derivative of B2. Finally, B3 was subjected to Knoevenagel condensation with anisic aldehyde to produce B4.



**Figure S1.** a) The absorbance degradation of DPBF in the appearance of MB as PSs under irradiation of Xe lamp. b) Linear fitting of absorbance degradation in a).



**Figure S2.** Images of B1, B2, B3, and B4 in DCM were recorded under daylight and 365 nm UV lamp irradiation.



Figure S3. <sup>1</sup>H NMR of B1.



Figure S4. Mass spectrometry of B1.



Figure S5. <sup>1</sup>H NMR of B2.



Figure S6. Mass spectrometry of B2.



**Figure S7.** <sup>1</sup>H NMR of B3.



Figure S8. Mass spectrometry of B3.



Figure S9. <sup>1</sup>H NMR of B4.



Figure S10. Mass spectrometry of B4.

Study of optical properties of B1, B2, B3 and B4



Figure S11. Ultraviolet absorption spectra of B1 and B2.



Figure S12. Fluorescence emission spectra of B1 and B2.



Figure S13. Ultraviolet absorption spectra of B3.



Figure S14. Fluorescence emission spectra of B3.



Figure S15. Ultraviolet absorption spectra of B4.



Figure S16. Ultraviolet absorption spectra of B4 in DCM and DMSO.



Figure S17. Fluorescence emission spectra of B4.

TEM and DLS characterization of CAT.



Figure S18. TEM image of CAT in PBS solution. Scale bar: 20 nm.



Figure S19. DLS of CAT in PBS solution.



Figure S20. TEM image at high CAT concentration (CAT: B4=5:1). Scale bar: 40 nm.



Figure S21. DLS at high CAT concentration (CAT: B4=5:1).



Figure S22. TEM image at high B4 concentration (CAT: B4=1:5). Scale bar: 500 nm.



Figure S23. DLS at high B4 concentration (CAT: B4=1:5).



**Figure S24.** a) The green precipitate was observed in the CAT: B4 = 1:10 solution. b) TEM image of the lowest CAT concentration. Scale bar: 300 nm.



**Docking simulation of CAT and B4** 

Figure S25. The docking result of the entire complex based on CAT and B4.



Figure S26. Zoomed-in view of the interaction area in Figure 25.

Stability of CIB NPs at different pH conditions



Figure S27. Size change of the assembly under different pH conditions.



Figure S28. Variation of FL. intensity for CIB NPs under different pH conditions.



Figure S29. Singlet oxygen generation ability of CIB NPs detected by the FL. intensity of SOSG.



Figure S30. Zeta potential of CIB NPs measured by DLS over 72 h.

Oxygen generation ability of CIB NPs.



Figure S31. Oxygen production by CIB NPs in different concentrations of H<sub>2</sub>O<sub>2</sub> solution.

Comparison of free CAT and CIB NPs



Figure S32. Comparison of oxygen production of CAT and CIB NPs in H<sub>2</sub>O<sub>2</sub> solution.

# Dark toxicity assay



**Figure S33.** The effect of varying concentrations of CIB NPs on the viability of A549 cells was tested using the CCK-8 assay.



**Figure S34.** The effect of varying concentrations of CIB NPs on the viability of 4T1 cells was tested using the CCK-8 assay.

Cellular phototoxicity studies.



Figure S35. CIB NPs generate oxygen in H<sub>2</sub>O<sub>2</sub> solution under light irradiation.

In vitro therapeutic effect



**Figure S36.** CLSM images of A549 cells stained with Calcein-AM and PI after conducting different treatments were displayed in normoxic conditions.



Figure S37. The relative fluorescence intensity of A549 cells in Figure S35 stained with Calcein-AM and PI after different treatments.

# Evaluation of in vivo PDT efficacy in subcutaneous tumors



**Figure S38.** Digital pictures of A549 tumor-bearing mice after different treatments during two weeks, including the control group (PBS), control group (PBS)+laser, BSA-I-BODIPY+laser, and CAT-I-BODIPY+laser.



Figure S39. *Ex vivo* fluorescence images of major organs and tumours at 30 h post-injection of CIB NPs.



Figure S40. The hemocompatibility of CIB NPs with different concentrations.

Compounds	Solvent	$\lambda_{\max, Abs}(nm)$	$\lambda_{\max, Em} (nm)$	$ riangle\lambda^a(nm)$
B1	DCM	500	545	45
B2	DCM	500	545	45
В3	DCM	532	557	25
B4	DCM	658	688	30

Table S1. Photophysical data of B1, B2, B3 and B4.

# References

- 1. Q. Wang, H. Lu, L. Gai, W. Chen, G. Lai and Z. Li, Dye. Pigment., 2012, 94, 183–186.
- 2. L. Huang, J. Zhao, S. Guo, C. Zhang and J. Ma, J. Org. Chem., 2013, 78, 5627–5637.