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Electronic Supplementary Information

Optimizing the mitochondrial-targeting groups of positively-charged BODIPY nanoparticles for enhanced photodynamic therapy

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Experimental Section

Materials: 1,6-Dibromohexane was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). *P*-hydroxybenzaldehyde was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). 2,4-Dimethyl-1H-pyrrole was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Trifluoroacetic acid (TFA) was purchased from Meryer Technologies Co., Ltd. (Shanghai, China). Boron-trifluoride-etherate was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). N-iodosuccinimide was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Triethylamine was purchased from Chinasun Specialty Products Co., Ltd (Jiangsu, China). Triphenylphosphine (TPP) was bought from TitanSCI (Shanghai, China). Trimethylamine (TMA) was purchased from TCI Development Co., Ltd (Shanghai, China). 1-methyl-1H-imidazole was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). 1,3-Diphenylisobenzofuran (DPBF) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). DHR-123 assays, DCFH-DA assays, Mito-Tracker Deep Red 633 assays and thiazolyl blue tetrazolium bromide (MTT) assays were purchased from Beyotime (Nantong, China). Lyso-Tracker Red DND-99 was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). JC-1 assays were purchased from Biosharp (Anhui, China).

Characterizations: ^{1}H NMR spectra were measured in $CDCl_3$ at room temperature by an AVANCE NEO 400 MHz Digital NMR Spectrometer from Bruker. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry was measured at room temperature by an Ultraflextreme MALDI-TOF-MS. The absorption spectra were obtained from a UH-5300 dual-beam UV visible spectrophotometer (Hitachi). The photoluminescence spectra were obtained from a Cary Eclipse Fluorescence Spectrophotometer (Agilent). The TEM and DLS results of the NPs were obtained by Hitachi HT7700 transmission electron microscope (acceleration voltage of 120 kV) and Malvern Zeta-sizer Nano. CLSM images were obtained from a Leica TCS SP5 II (Germany). Cell viability was determined by measuring the absorbance at 490 nm using a Thermo Fisher Scientific MULTISKAN FC USD4600 (USA). The PCCs and fluorescence intensity were calculated by Image J software (version 1.51d). Statistical analysis was performed using GraphPad Prism 9 software (version 9), and P values were calculated by the two-tailed Student's t-test.

Synthesis of 4-(6-bromohexoxy) benzaldehyde: 1,6-Dibromohexane (5.5 mL, 36 mmol), *p*-hydroxybenzaldehyde (1.5 g, 12 mmol) and K_2CO_3 (3 g, 20 mmol) were added to acetone (150 mL), and the mixture was heated to reflux at 70 ℃ for 24 h at N_2 atmosphere. After the reaction mixture was cooled to room temperature, the mixture was filtered and evaporated on a rotary evaporator. Then, the mixture was extracted with CH_2Cl_2 (50 mL) for 3 times. The organic phase was washed with 20 mL of saturated salt water, dried with $NaSO_4$, filtered and evaporated to obtain a white solid

crude product, which was purified on a silica gel column (CH_2Cl_2 : EA = 40 : 1).

Synthesis of BDP-Br: 4-(6-bromohexoxy) benzaldehyde (2.86 g, 10 mmol) and 2,4-dimethyl pyrrole (2.25 mL, 22 mmol) were added in CH_2Cl_2（400 mL), followed by the addition of 7 drops of trifluoroacetic acid. The above mixture was stirred overnight at room temperature with protection from light in the N_2 atmosphere. Then, DDQ (2.3 g, 10 mmol) was slowly added. After reacting for 2 h, triethylamine (10 mL, 73 mmol) was added. 30 min later, boron trifluoride ethyl ether (12 mL, 95 mmol) was slowly added at 0 ℃. After 6 h, the reaction was stopped. The mixture was washed with saturated salt water (50 mL) for 3 times, dried with $MgSO_4$, and the reaction mixture was concentrated after filtration. The oily residue was purified through a silica gel column (CH_2Cl_2 : *n*-hexane = 1 : 1), and the product was subsequently collected and concentrated to give a reddish-brown solid.

Synthesis of BDPI-Br: BDP-Br (0.5 g, 1 mmol) and N-iodosuccinimide (NIS, 0.9 g, 4 mmol) were added to a 100 mL flask. 60 mL CH_2Cl_2 was added and the mixture was stirred at room temperature for 6 h. Silica gel column chromatography was utilized to purify the crude product using *n*-hexane : CH_2Cl_2 (v/v = 30 : 1) as the eluent.

Synthesis of BDP-TPP: BDP-TPP was synthesized according to a reported method.[1] BDP-Br (151 mg, 0.3 mmol) and TPP (105 mg, 0.4 mmol) were added to a dry flask. Then, 15 mL of acetonitrile (CH_3CN) was added, and the mixed solution was stirred at 85 °C for 24 h under nitrogen. The solvent was removed under reduced pressure, and a crude product was obtained. Silica gel column chromatography was utilized to purify the product using CH_2Cl_2 to CH_2Cl_2 : C_2H_5OH (v/v = 10 : 1) as the eluent.

Synthesis of BDPI-TPP: BDP-TPP (38 mg, 0.05 mmol) and N-iodosuccinimide (NIS, 45 mg, 0.4 mmol) were added to a 50 mL flask. 15 mL CH_2Cl_2 was added and the mixture was stirred at room temperature for 6 h. Silica gel column chromatography was utilized to purify the crude product using CH_2Cl_2 : C_2H_5OH (v/v = 10 : 1)

Synthesis of BDPI-TMA: BDPI-TMA was synthesized according to a reported method.[2] BDPI-Br (113 mg, 0.15 mmol) was added into a round bottom flask, then 20 mL C_2H_5OH was added to dissolve BDPI-Br, and $N(CH_3)_3$ (0.1 mL, 3 mmol) was added under stirring at room temperature. After heating and refluxing for 24 h, the solvent was removed under reduced pressure and the product was obtained by precipitation with ether.

Synthesis of BDPI-IMA: BDPI-Br (226.5 mg, 0.3 mmol) and 1-methyl-1H-imidazole (0.24 mL, 3 mmol) were added to a dry flask. Then, 45 mL of acetonitrile (CH_3CN) was added, and the mixed solution was stirred at 85 °C for 12 h under nitrogen. The solvent was removed under reduced pressure, and a crude product was obtained. Silica gel column chromatography was utilized to purify the product using CH_2Cl_2 : MeOH

(v/v = 20 : 1) as the eluent.

Preparation of nanoparticles: BDPI-TPP (1 mg) was fully dissolved in 5 mL of THF, and the solution was added dropwise to 10 mL water under stirring. After stirring at room temperature for 8 h to remove the organic solvent, BDPI-TPP NPs were obtained. The standard curve of BDPI-TPP was developed by UV-vis spectrophotometer to determine the concentration of BDPI-TPP NPs. BDPI-TMA NPs and BDPI-IMA NPs were obtained using the same method.

Generation of $^1O_2$: 1 mg DPBF was dissolved in 1 mL DMSO (1 mg mL^{-1}). BDPI-TPP, BDPI-TMA and BDPI-IMA were diluted to the same concentration. 10 μL DPBF solution was added to 0.6 mL DMSO, BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs respectively, and their absorption spectra were measured after green light irradiation (530 nm, 10 mW cm^{-2}) for different times. The detection interval was 10 s. The generation of $^1O_2$ of nanoparticles was measured using similar methods in deionized water.

Singlet oxygen quantum yields

The Φ values of BDPI-TPP, BDPI-TMA, BDPI-IMA and their NPs were determined by using DPBF as the singlet oxygen sensor. The value was calculated from the following equation:

$$\Phi=\Phi_{ref}\,(W / W_{ref})\,(A_{ref} / A)\,(\eta_s / \eta_r)^2$$

where, Φ_{ref} is the standard singlet oxygen quantum yield of Methylene Blue (MB, Φ_{ref} = 0.52 in CH_2Cl_2).[3] R and R_{ref} are the slope of the absorbance over time of DPBF in the presence of BDPI-TPP / BDPI-TMA / BDPI-IMA and MB, respectively. A and A_{ref} are the absorption correction factors for BDPI-TPP / BDPI-TMA / BDPI-IMA, which were calculated by $A = 1 - 10^{-OD}$ (OD at the irradiation wavelength).

Generation of $O_2^{-}\cdot$: 1 mg DHR123 was dissolved in DMF (5mM). BDPI-TPP, BDPI-TMA and BDPI-IMA were diluted to the same concentration in a mixed solution of water and DMF (v / v = 9 :1). 1 μL DHR123 solution was added to 1 mL mixed solution, BDPI-TPP NPs, BDPI-TMA NPs or BDPI-IMA NPs, and their photoluminescence spectra were measured after green light irradiation (530 nm, 10 mW cm^{-2}) for different times. The detection interval was 20 s.

ROS detection in cells: The experiment was done according to the specification of ROS detection kit (DCFH-DA) which was purchased from Beyotime.

Colocalization analysis of lysosome: HeLa cells, 4T1 cells and B16-F10 cells were seeded in glass-bottom Petri dishes at a density of 1×10^5 cells per well and incubated for 12 h. The lysosomes were stained with Lyso-Tracker (50 nM) for 30 min. Subsequently, the cells were incubated with 5 μg mL^{-1} of BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs. At designated time intervals, the cells were imaged using CLSM. The excitation wavelengths were 488 nm for BDPI NPs and 633 nm for Lyso-

Tracker. Imaging parameters remained consistent across each group.

Cell uptake: HeLa cells, 4T1 cells and B16-F10 cells were seeded in glass-bottom Petri dishes at a density of 1×10^5 cells per well and incubated for 12 h. Then, the cells were incubated with 5 μg mL^{-1} of BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs for 30 min. Subsequently, the nuclei were stained with Hoechst (2 μM) for 10 min. The cells were washed with PBS for 3 times and imaged using CLSM. The excitation wavelengths were 405 nm for Hoechst and 488 nm for BDPI NPs. Imaging parameters remained consistent across each group.

Mitochondrial targeting ability: HeLa cells, 4T1 cells and B16-F10 cells were seeded in glass-bottom Petri dishes at a density of 1×10^5 cells per well and incubated for 12 h. Then, the cells were incubated with 5 μg mL^{-1} of BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs for 30 min. Subsequently, the mitochondria were stained with Mito-Tracker Deep Red (100 nM) for 25 min. After staining, remove the staining solution and add fresh culture medium for imaging. The excitation wavelengths were 488 nm for BDPI NPs and 633 nm for Mito-Tracker Deep Red. Imaging parameters remained consistent across each group. Colocalization analysis completed by Image J software.

JC-1 assays: The experiment was done according to the specification of the MMP detection kit (JC-1) which was purchased from Biosharp. The experimental group was pre-incubated with BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs (5 μg mL^{-1}) for 30 min and irradiated with green light (530 nm, 10 mW cm^{-2}) for 7 min. Then use JC-1 assays to detect MMP.

Cytotoxicity assays: HeLa cells, 4T1 cells and B16-F10 cells were inoculated in a 96-well plate, which was placed in a 37 ℃ incubator. After incubation for 12 h, different concentrations of BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs were added to the well plate with cells. The dark group was incubated under dark conditions for 24 hours. The light group was irradiated with green light (530 nm, 10 mW cm^{-2}) for 25 minutes after incubation for 18 hours and incubated for another 4 hours. After incubation, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well. After 4 h, the supernatant was aspirated and 150 μL of DMSO was added. The well plate was placed in a microplate reader to detect their OD values at 490 nm.

References

1 H Wen, Q Wu, C Li, T Sun and Z Xie, 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BDPI)-Triphenylphosphine Nanoparticles as a Photodynamic Antibacterial Agent, *ACS Appl. Nano Mater.*, 2022, **5**, 1500–1507.

2 H. Wang, C. Li, Q. Wu, H. Wen, T. Sun and Z. Xie, A cationic BODIPY photosensitizer decorated with quaternary ammonium for high-efficiency photodynamic inhibition of bacterial growth, *J. Mater. Chem. B*, 2022, **10**, 4967-4973.

3 Z Li, Y Chen, X Lv and W Fu, A tetraphenylethene-decorated BODIPY monomer/dimer with intense fluorescence in various matrices, *NewJ. Chem.*, 2013, **37**, 3755.

Table S1. Spectroscopic and photophysical data of the compounds.

Reagent	λ_{abs}/nm [a]	λ_{em}/nm [b]	Stokes shift	Φ/% [d]
BDPI-TPP	536	557	21	78.63
BDPI-TPP NPs	538	/	/	1.44
BDPI-TMA	535	556	21	86.01
BDPI-TMA NPs	533	/	/	1.67
BDPI-IMA	535	561	26	100
BDPI-IMA NPs	536	/	/	4.39

[a] Maximum absorption wavelength. [b] Maximum emission wavelength. [c] Singlet oxygen quantum yield, Methylene Blue (MB) in DCM as a reference.

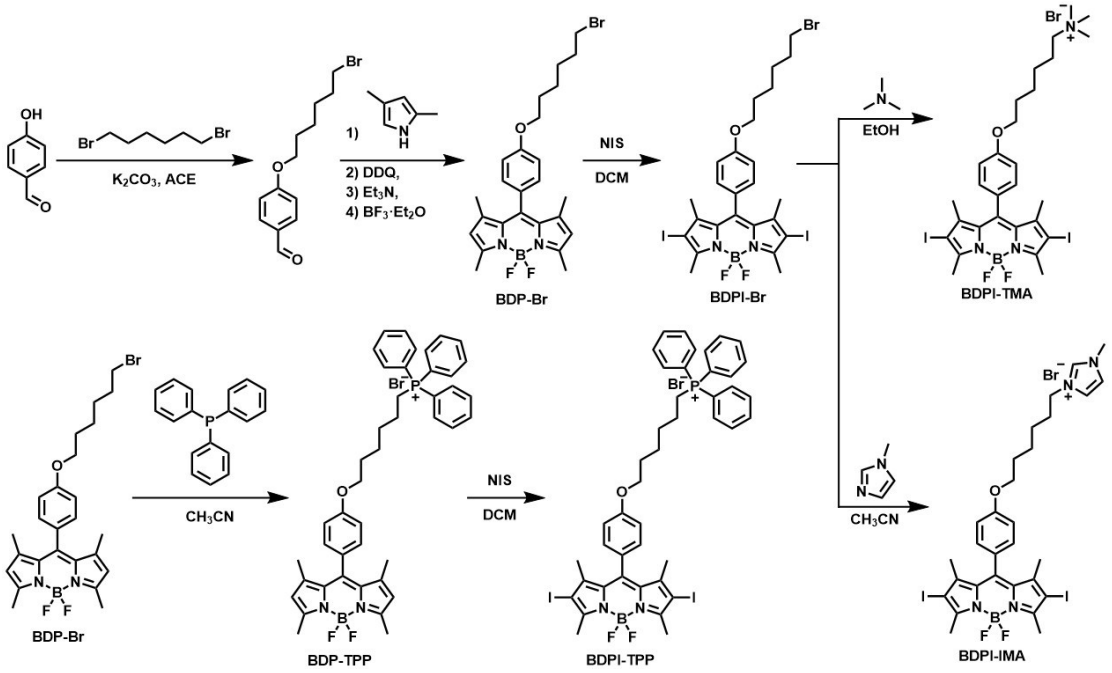

Fig. S1 Synthetic routes of BDPI-TPP, BDPI-TMA, and BDPI-IMA.

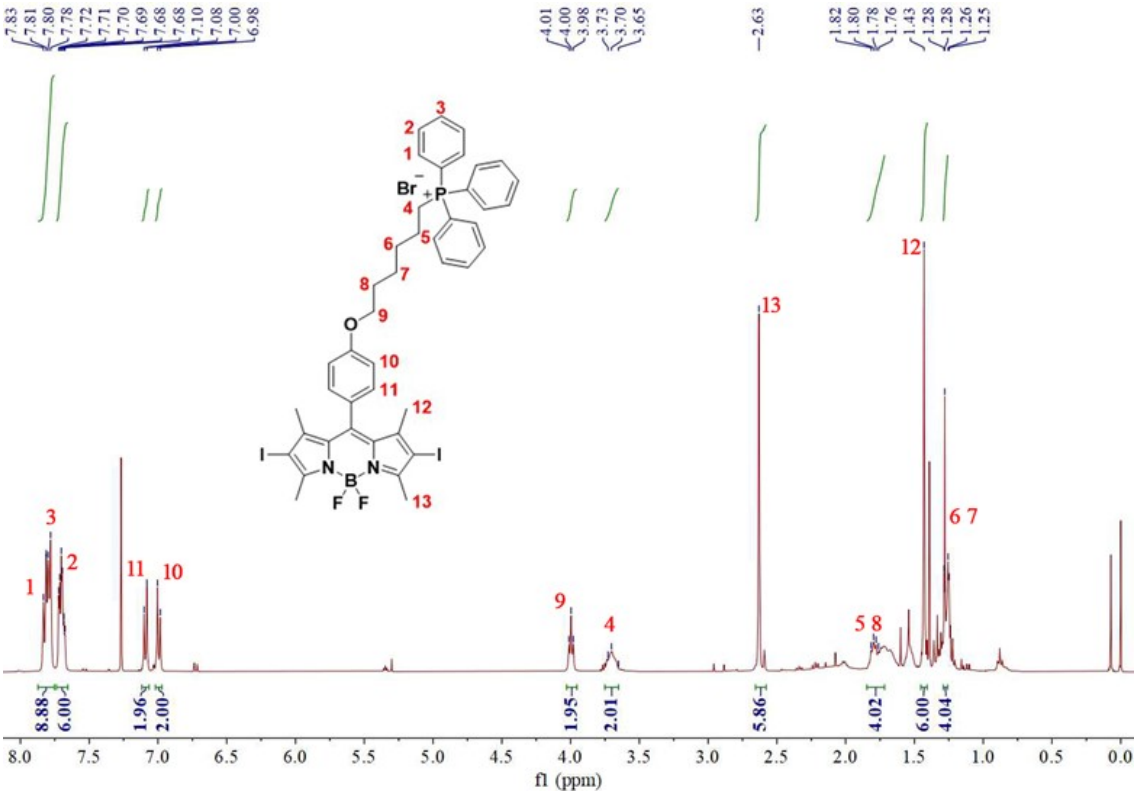

Fig. S2 ^{1}H NMR spectrum of BDPI-TPP.

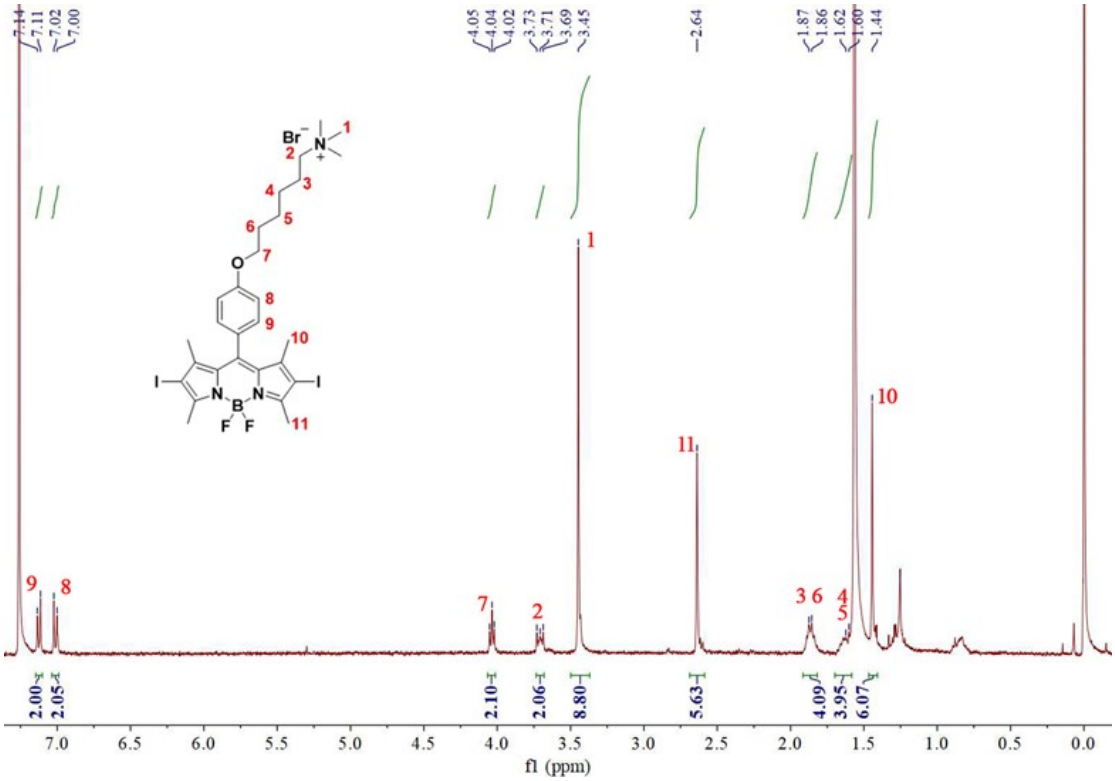

Fig. S3 ^{1}H NMR spectrum of BDPI-TMA.

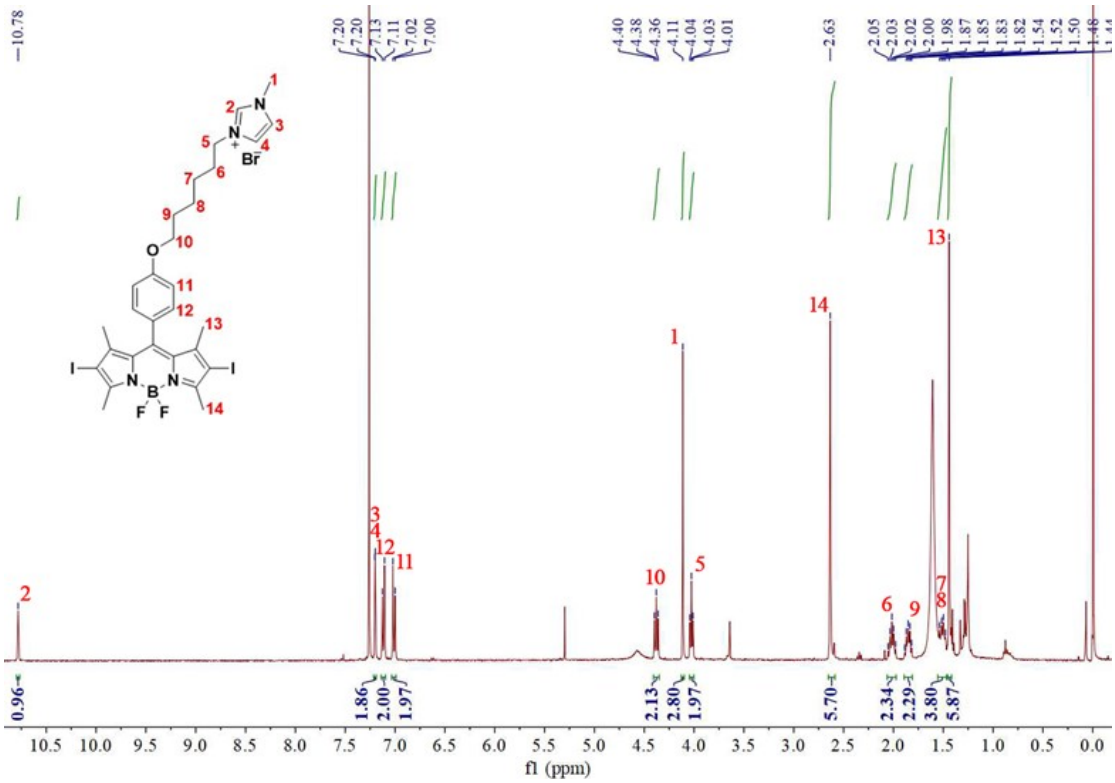

Fig. S4 ^{1}H NMR spectrum of BDPI-IMA.

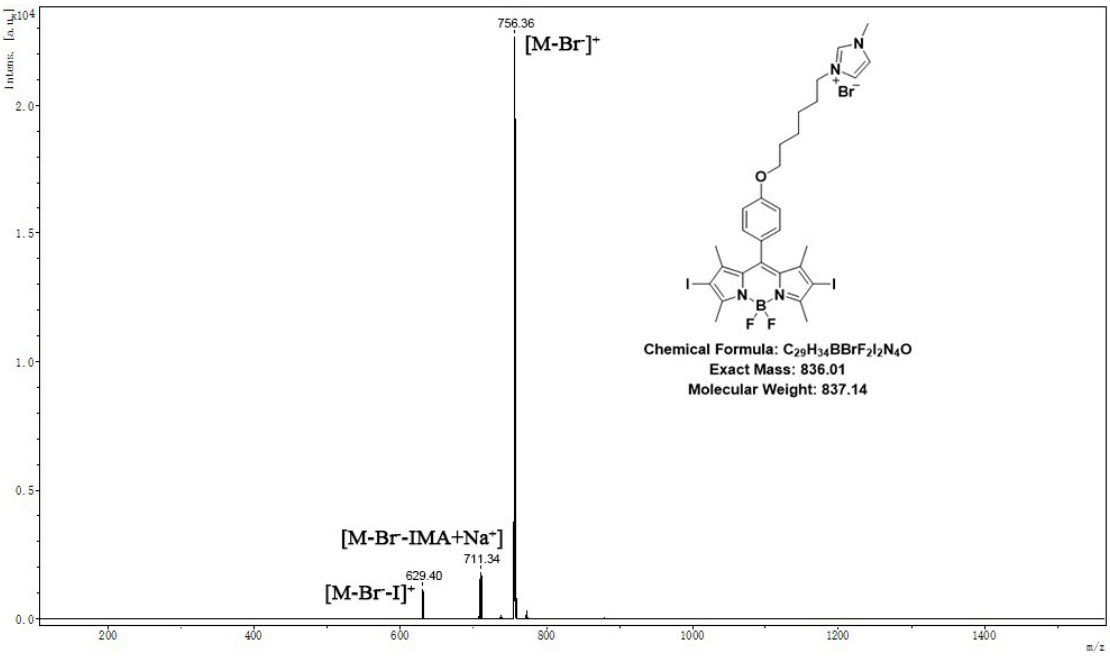

Fig. S7 MALDI-TOF/MS spectrum of BDPI-IMA.

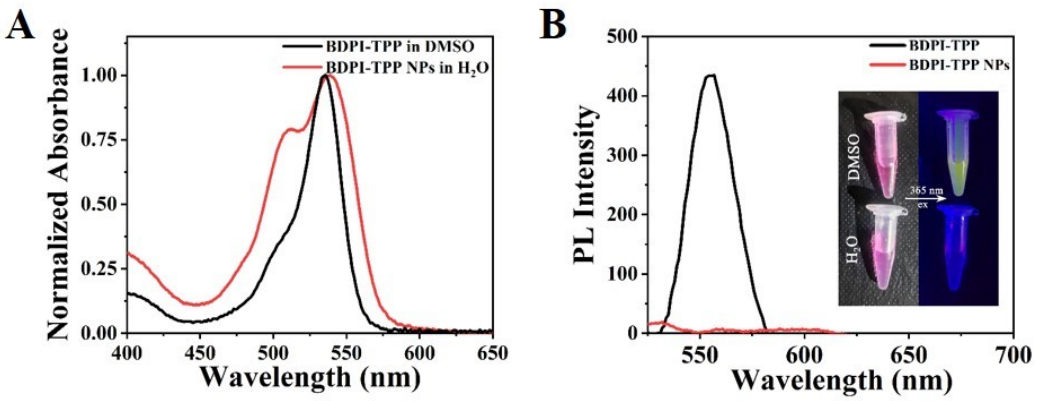

Fig. S6 (A) Normalized absorption spectrum and (B) Emission spectrum of BDPI-TPP in DMSO and BDPI-TPP NPs in water. Inserted image: Photos of BDPI-TPP in DMSO and BDPI-TPP NPs in water before and after 365 nm light irradiation.

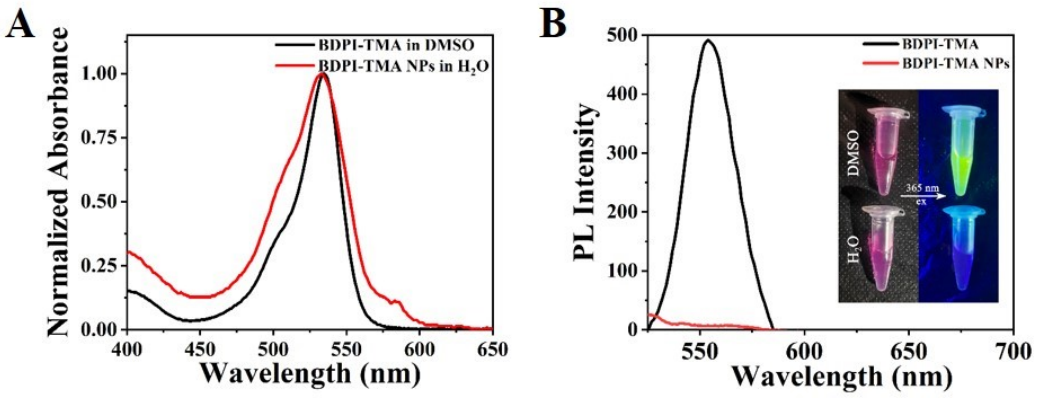

Fig. S5 (A) Normalized absorption spectra and (B) Emission spectra of BDPI-TMA in DMSO and BDPI-TMA NPs in water. Insert image: Photos of BDPI-TMA in DMSO and BDPI-TMA NPs in water before and after 365 nm light irradiation.

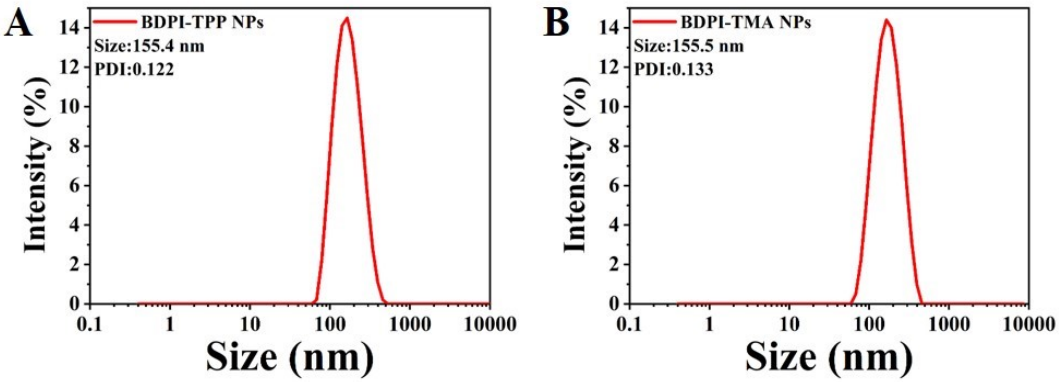

Fig. S8 Size and size distribution of (A) BDPI-TPP NPs and (B) BDPI-TMA NPs characterized by DLS.

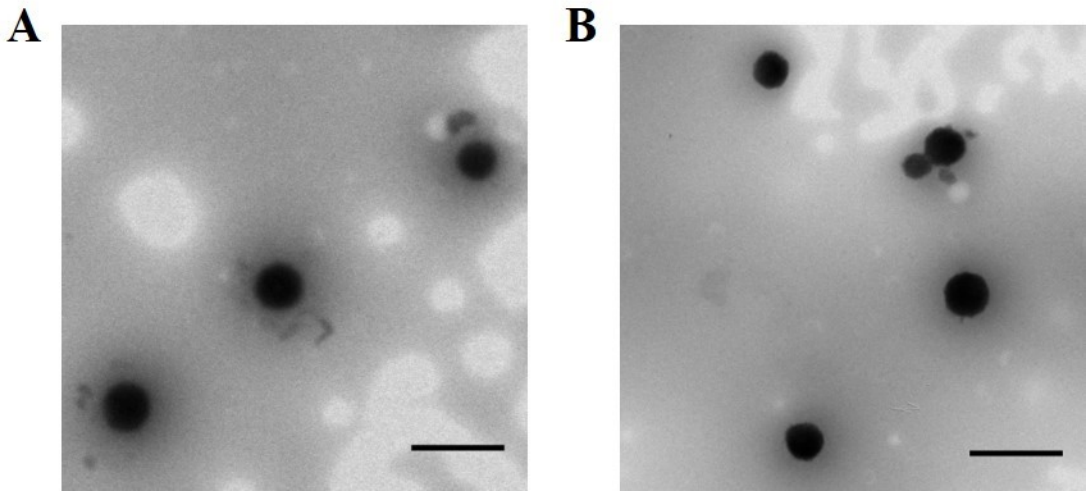

Fig. S9 TEM images of (A)BDPI-TPP NPs and (B) BDPI-TMA NPs. Scale bar, 300nm.

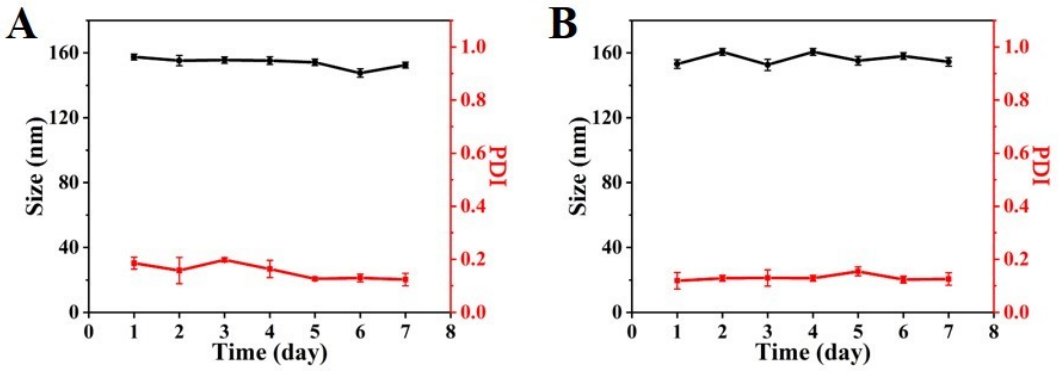

Fig. S10 Size and PDI of (A)BDPI-TPP NPs and (B) BDPI-TMA NPs in water for a week.

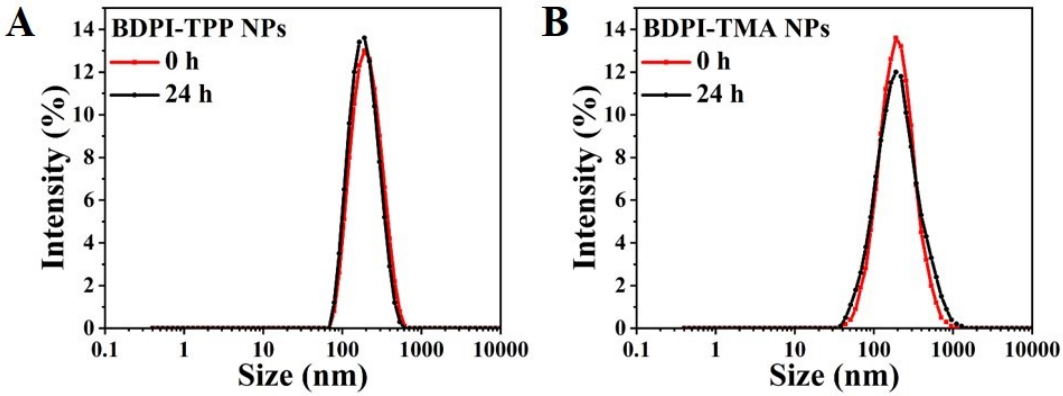

Fig. S11 The colloidal stability of (A)BDPI-TPP NPs and (B)BDPI-TMA NPs in DMEM with 10 % FBS measured by DLS for 24 h.

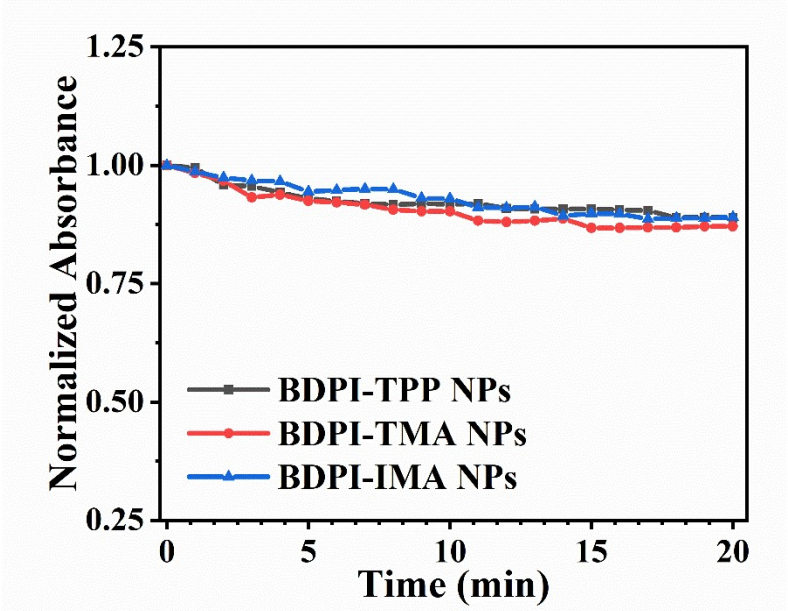

Fig. S12 The bleaching of BDPI NPs in H_2O over time under green light irradiation (530 nm, 10 mW cm^{-2}).

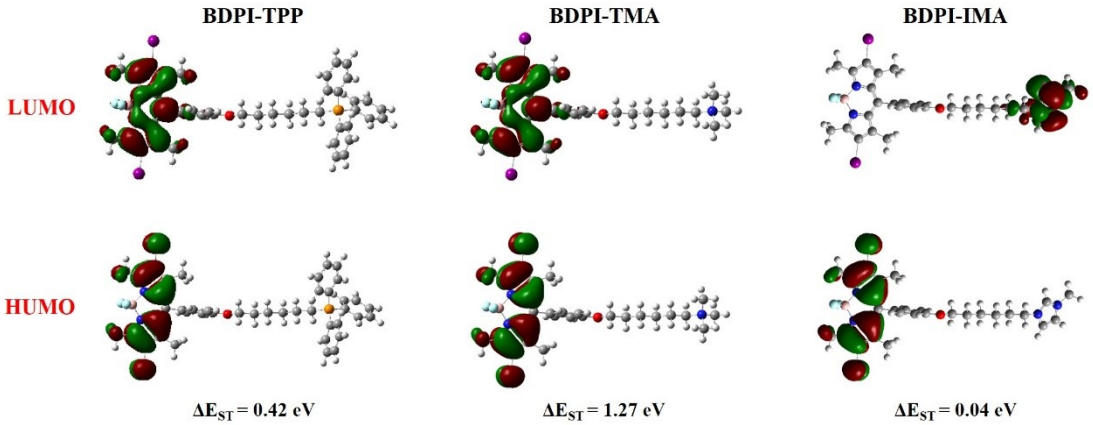

Fig. S13 HOMOs and LUMOs of the geometry structures.

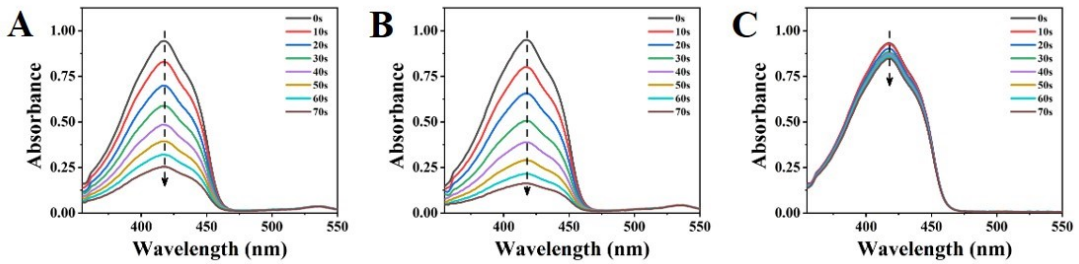

Fig. S14 Absorption spectrum of DPBF in DMSO in presence of (A) BDPI-TPP and (B) BDPI-TMA under green light irradiation (530 nm, 10 mW cm^{-2}) for different times. (C) The bleaching of DPBF in DMSO over time under green light irradiation (530 nm, 10 mW cm^{-2}).

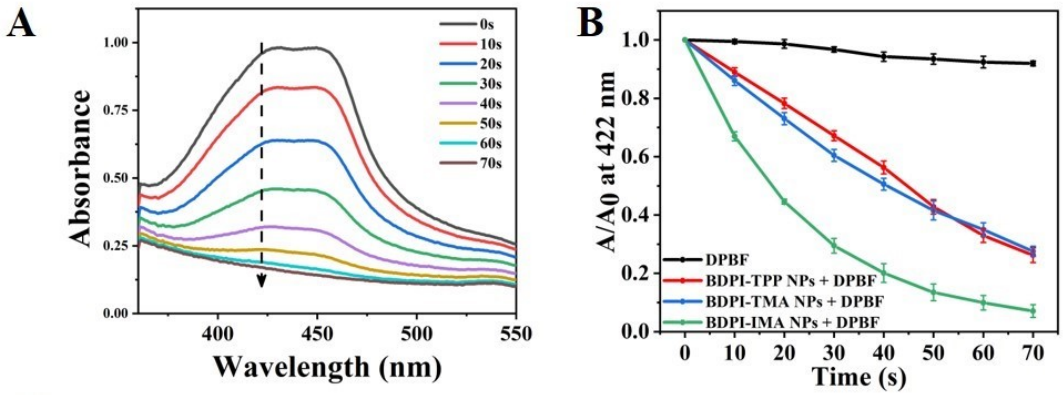

Fig. S15 (A) Absorption spectrum of DPBF in the presence of BDPI-IMA NPs under green light irradiation (530 nm, 10 mW cm^{-2}) for different times. (B) Bleaching of DPBF over time by $^1O_2$ generation in presence of BDPI-TPP NPs, BDPI-TMA NPs or BDPI-IMA NPs under green light irradiation (530 nm, 10 mW cm^{-2}) for different times.

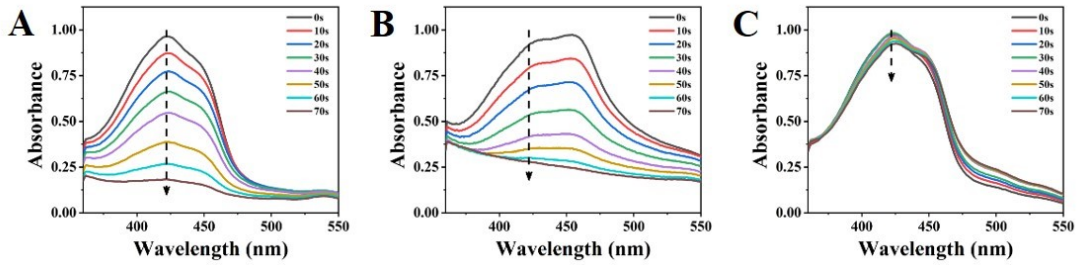

Fig. S16 Absorption spectrum of DPBF in water in presence of (A) BDPI-TPP NPs and (B) BDPI-TMA NPs under green light irradiation (530 nm, 10 mW cm^{-2}) for different times. (C) The bleaching of DPBF in water over time under green light irradiation (530 nm, 10 mW cm^{-2}).

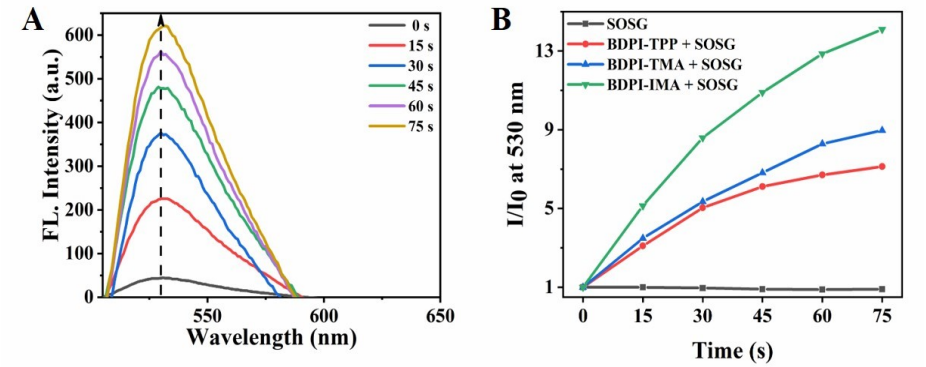

Fig. S17 (A) Fluorescence spectrum of SOSG in presence of BDPI-IMA with green light (530 nm, 10 mW cm^{-2}) irradiation as time went. (D) Plot of the relative fluorescence intensity (I/I_0) of SOSG solution containing photosensitizers versus the irradiation time.

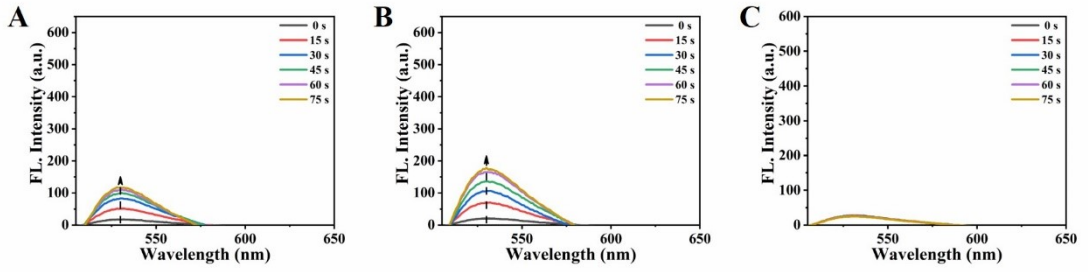

Fig. S18 Fluorescence spectrum of SOSG in presence of (A)BDPI-TPP and (B)BDPI-TMA under green light irradiation (530 nm, 10 mW cm^{-2}) for different times. (C) Fluorescence spectrum of SOSG under green light irradiation (530 nm, 10 mW cm^{-2}) for different times.

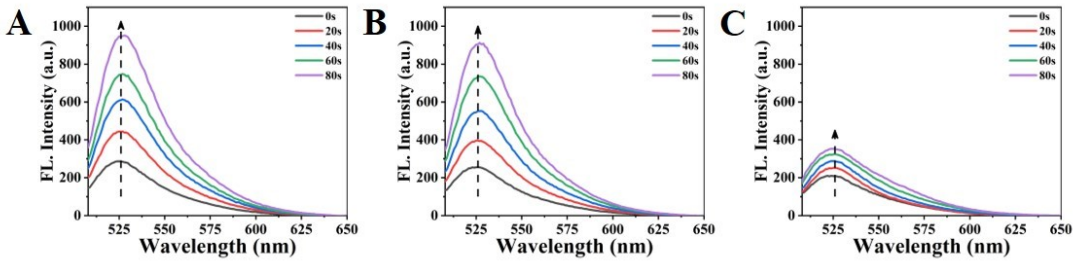

Fig. S19 Fluorescence spectrum of DHR123 in presence of (A)BDPI-TPP and (B)BDPI-TMA under green light irradiation (530 nm, 10 mW cm^{-2}) for different times. (C) Fluorescence spectrum of DHR123 under green light irradiation (530 nm, 10 mW cm^{-2}) for different times.

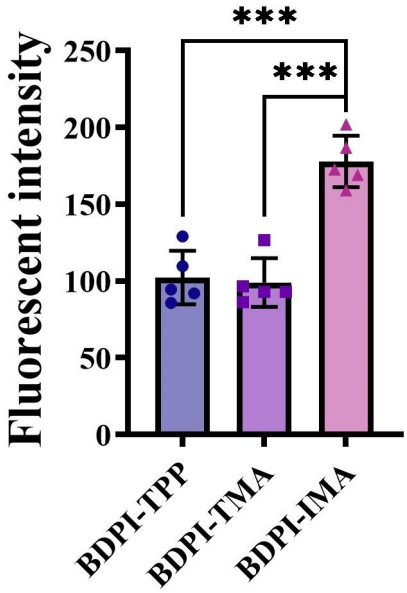

Fig. S20 Fluorescence of intracellular $^1O_2$ production in HeLa cells using the DCFH-DA assay. Error bars represent the mean ± S.D., n = 5. ***p < 0.001.

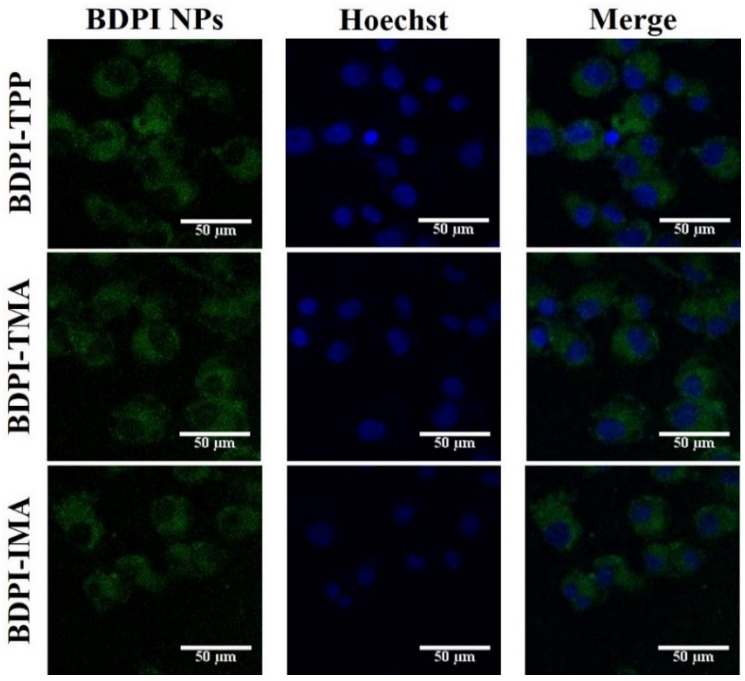

Fig. S21 CLSM images of HeLa cells incubated with BDPI NPs. Cell nuclei stained by Hoechst showed blue fluorescence and BDPI NPs showed green fluorescence in cells. Their images and overlays of both images were shown from left to right. Scale bar, 50 μm. Blue channel: Hoechst (λ_{ex} = 405 nm, λ_{em}: 425~465 nm); Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm).

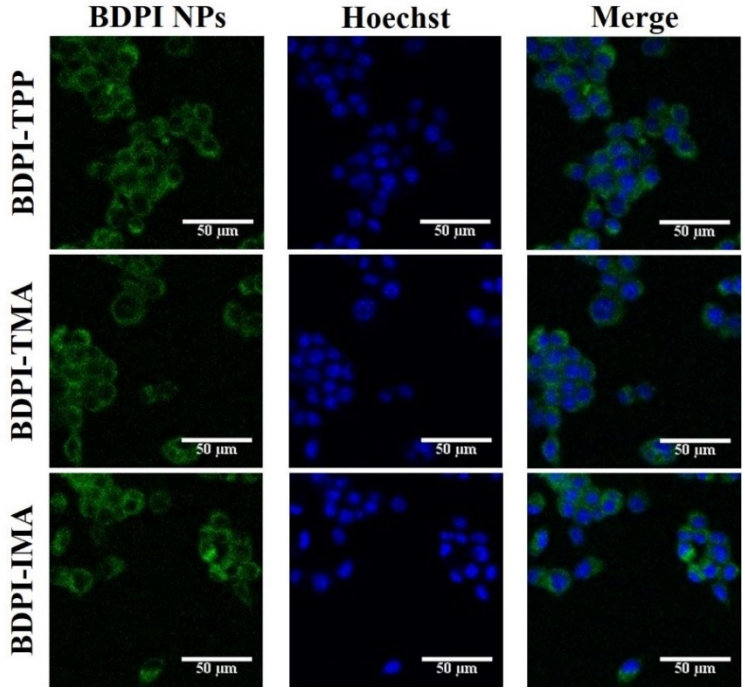

Fig. S22 CLSM images of 4T1 cells incubated with BDPI NPs. Cell nuclei stained by Hoechst showed blue fluorescence and BDPI NPs showed green fluorescence in cells. Their images and overlays of both images were shown from left to right. Scale bar, 50 μm. Blue channel: Hoechst (λ_{ex} = 405 nm, λ_{em}: 425~465 nm); Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm).

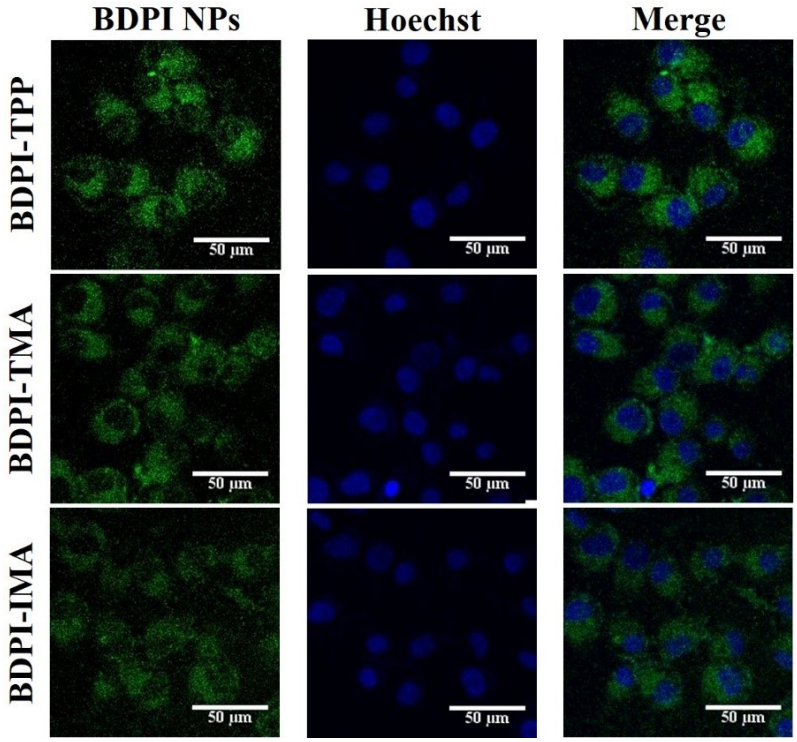

Fig. S23 CLSM images of B16-F10 cells incubated with BDPI NPs. Cell nuclei stained by Hoechst showed blue fluorescence and BDPI NPs showed green fluorescence in cells. Their images and overlays of both images were shown from left to right. Scale bar, 50 μm. Blue channel: Hoechst (λ_{ex} = 405 nm, λ_{em}: 425~465 nm); Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm).

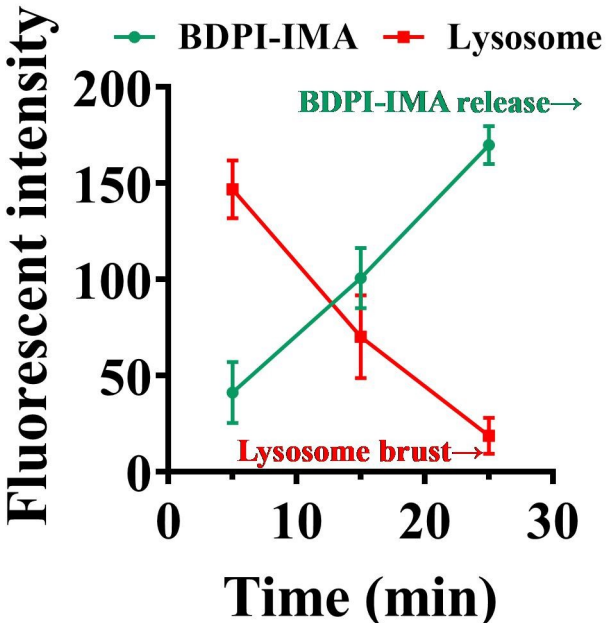

Fig. S25 Fluorescence intensity of HeLa cells incubated with Lyso-Tracker Red and BDPI-IMA NPs for different durations. Error bars represent the mean ± S.D., n = 3.

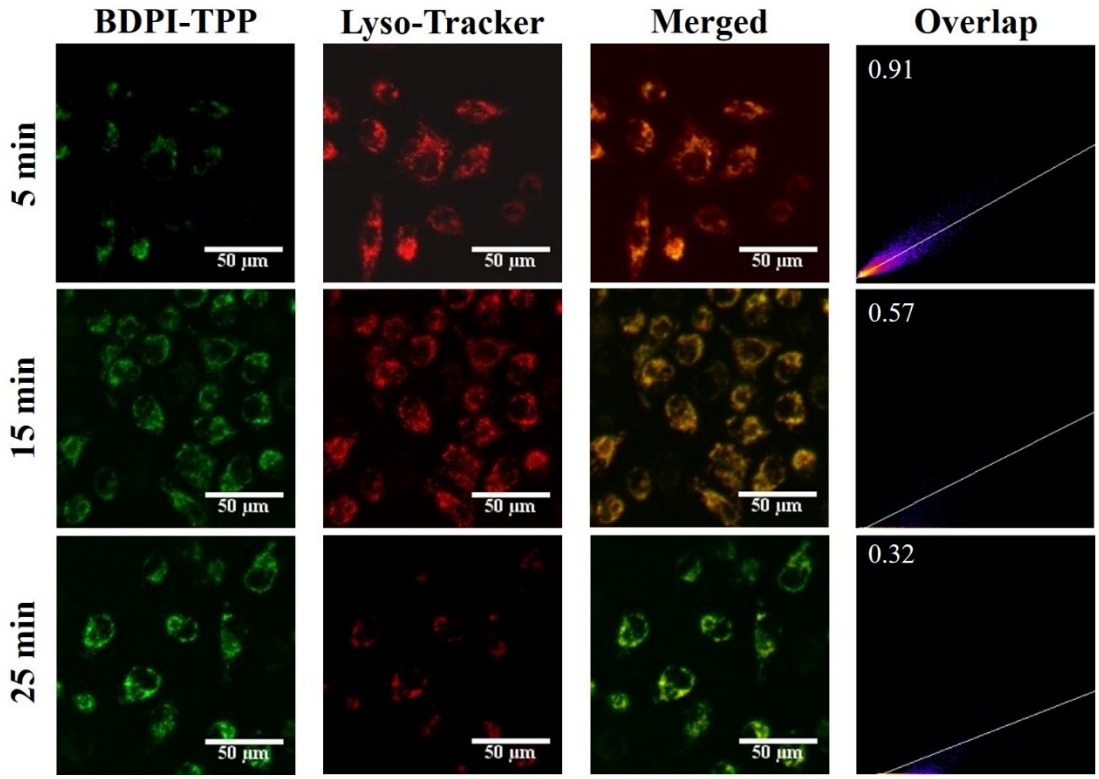

Fig. S25 CLSM images of HeLa cells incubated with Lyso-Tracker Red and BDPI-TPP NPs for different durations and merged images. Scale bar, 50 μm. Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm); Red channel: Lyso-Tracker (λ_{ex} = 633 nm, λ_{em}: 660~730 nm).

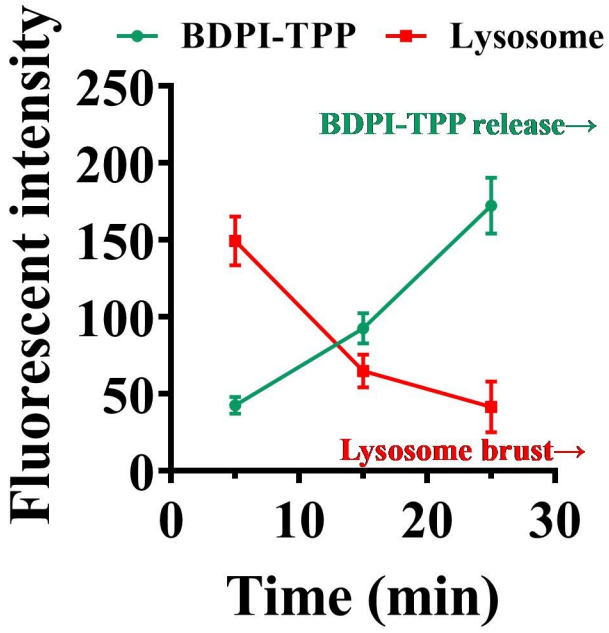

Fig. S26 Fluorescence intensity of HeLa cells incubated with Lyso-Tracker Red and BDPI-TPP NPs for different durations. Error bars represent the mean ± S.D., n = 3.

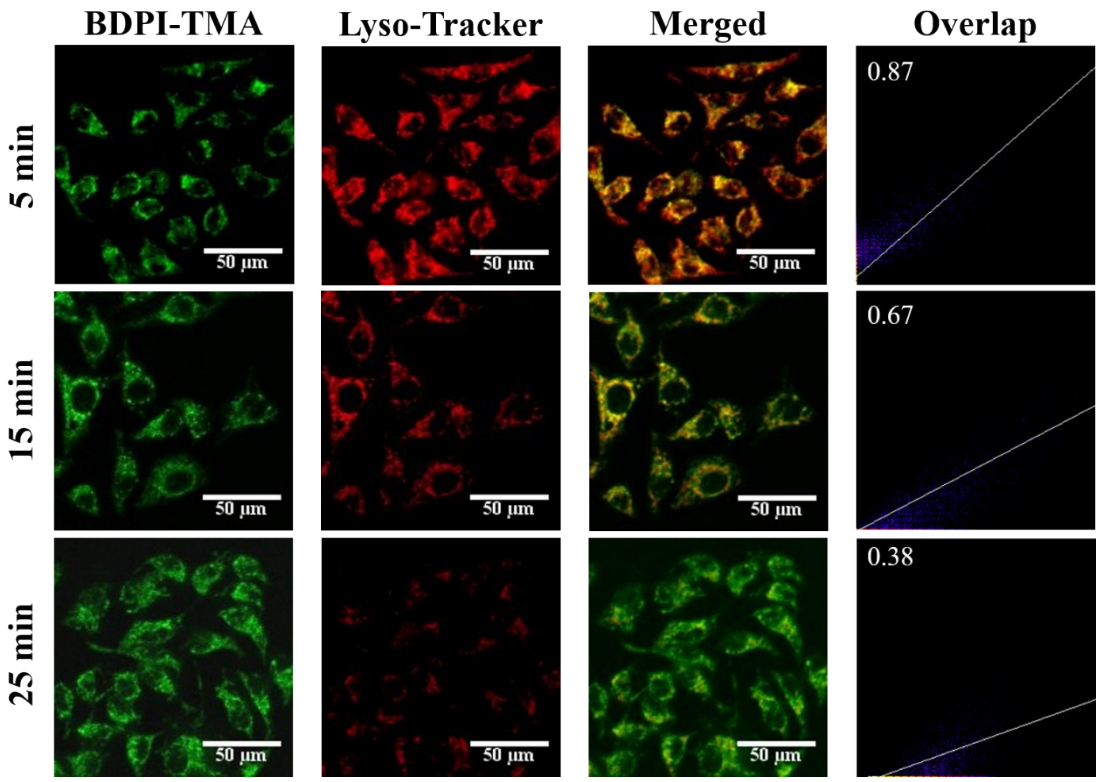

Fig. S27 CLSM images of HeLa cells incubated with Lyso-Tracker Red and BDPI-TMA NPs for different durations and merged images. Scale bar, 50 μm. Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm); Red channel: Lyso-Tracker (λ_{ex} = 633 nm, λ_{em}: 660~730 nm).

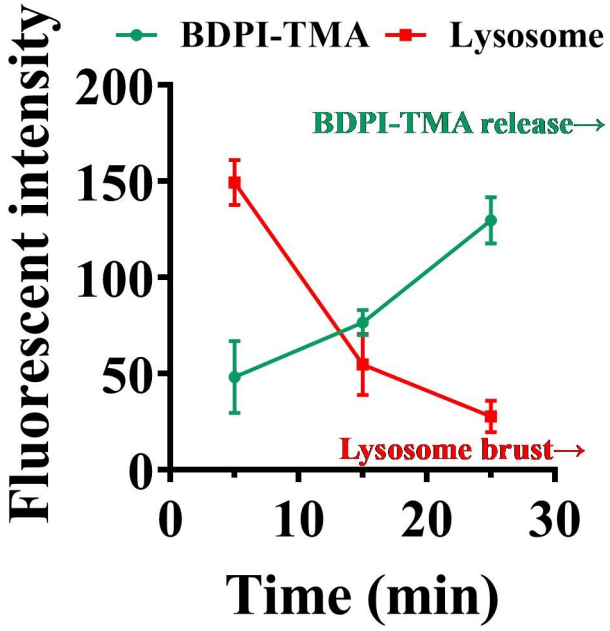

Fig. S28 Fluorescence intensity of HeLa cells incubated with Lyso-Tracker Red and BDPI-TMA NPs for different durations. Error bars represent the mean ± S.D., n = 3.

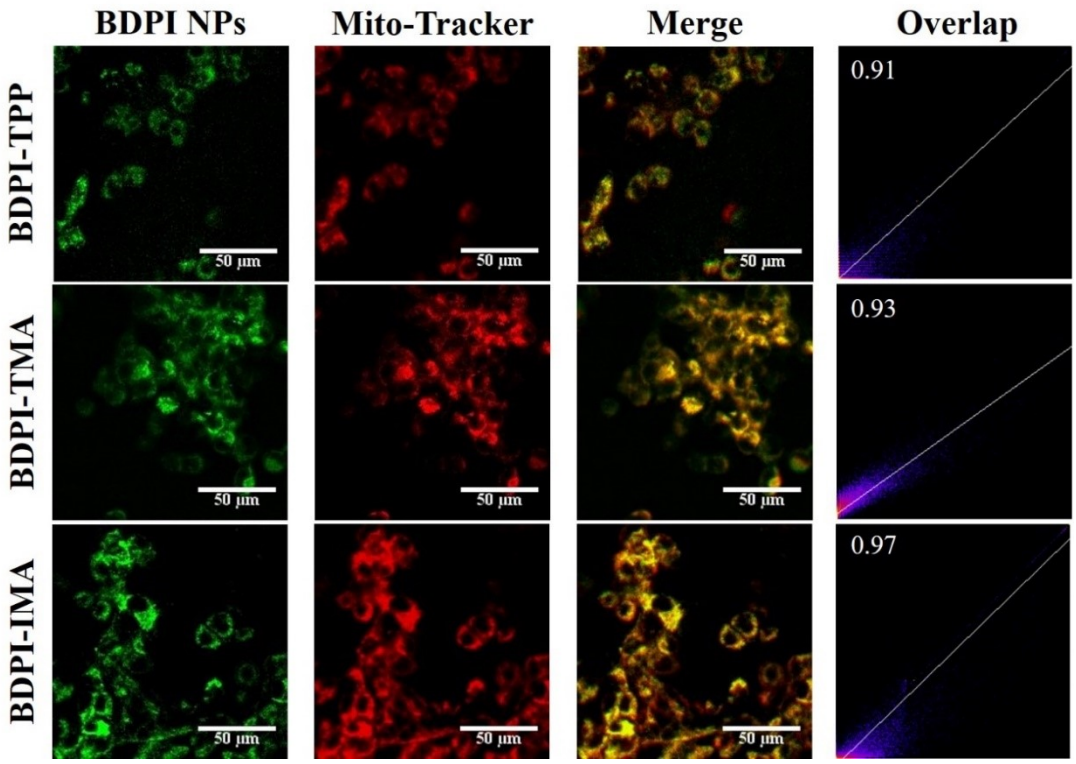

Fig. S29 CLSM images of 4T1 cells incubated with Mito-Tracker Deep Red 633 and BDPI NPs and merged images. Scale bar, 50 μm. Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm); Red channel: Mito-Tracker (λ_{ex} = 633 nm, λ_{em}: 660~730 nm).

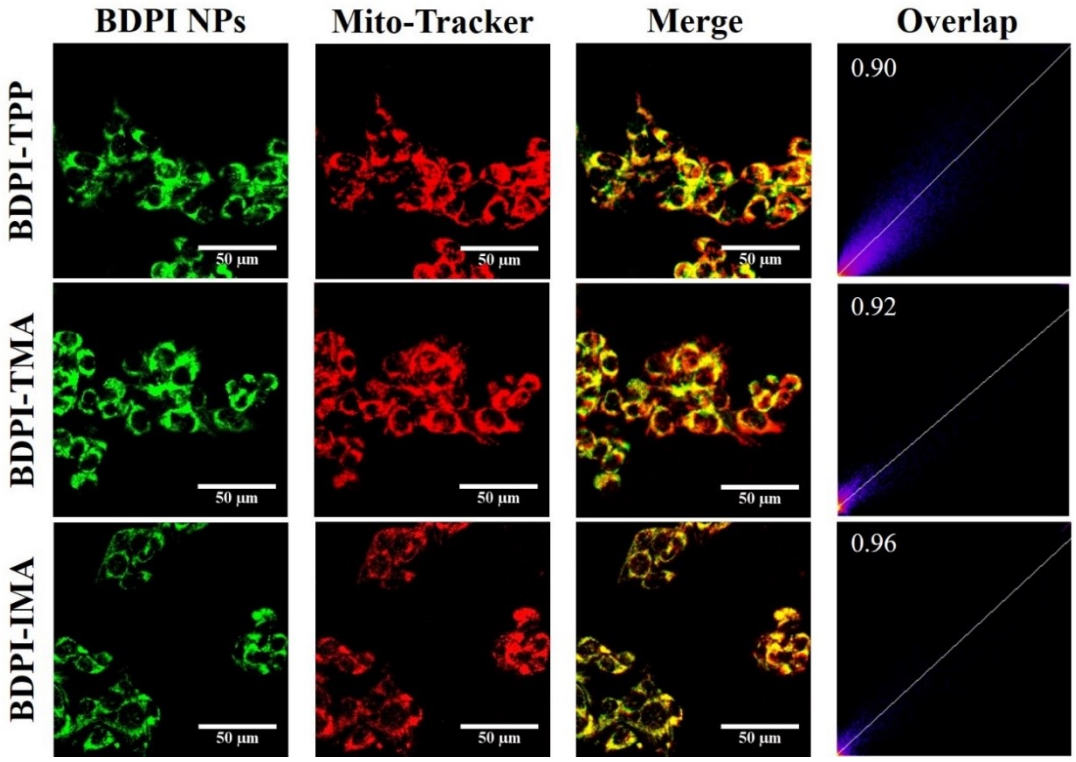

Fig. S30 CLSM images of B16-F10 cells incubated with Mito-Tracker Deep Red 633 and BDPI NPs and merged images. Scale bar, 50 μm. Green channel: BDPI (λ_{ex} = 488 nm, λ_{em}: 510~550 nm); Red channel: Mito-Tracker (λ_{ex} = 633 nm, λ_{em}: 660~730 nm).

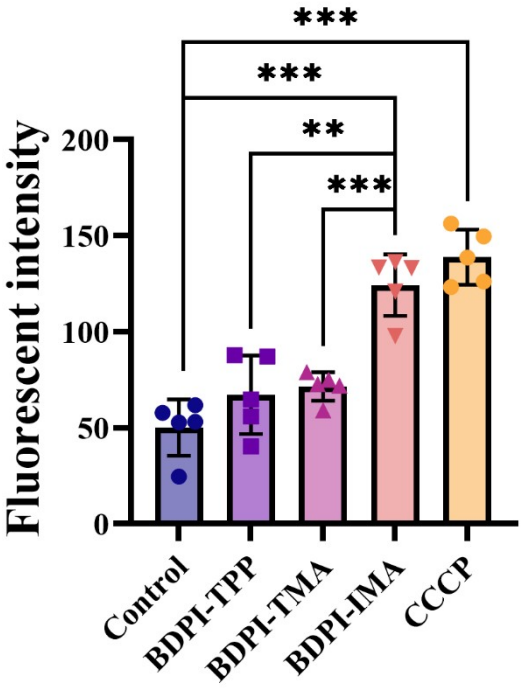

Fig. S31 Fluorescence of MMP in HeLa cells using the JC-1 assay. Error bars represent the mean ± S.D., n = 5. **p = 0.0012, ***p < 0.001.

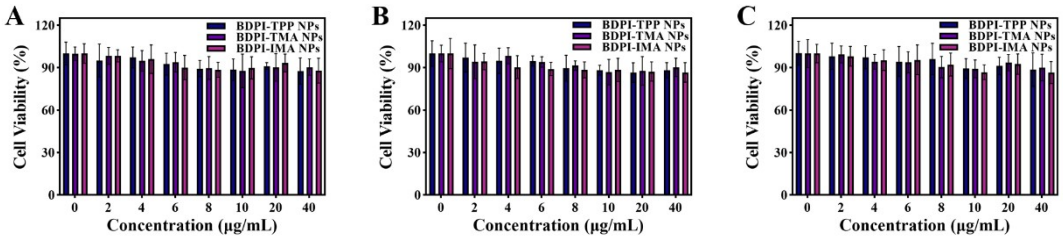

Fig. S32 Cell viability of (A) HeLa cells, (B) 4T1 cells and (C) B16-F10 cells treated with BDPI NPs. The cells were incubated with BDPI-TPP NPs, BDPI-TMA NPs and BDPI-IMA NPs respectively for 12 h.