Supporting Information for

Multicationic AIEgens for Unimolecular Photodynamic Theranostics and Two-Photon Fluorescence Bioimaging

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Table of contents

Experimental Section

1. Materials and Instruments

All reagents in the organic synthesis were purchased from Energy Chemical (China) and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. Unless otherwise noted all reagents with analytical purity were used as received. The starting material SM1 was synthesized according to our previous report.[1] 4', 6'-diamidino-2-phenylindole (DAPI), 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl tetrazolium bromide (MTT) were purchased from Boster Biological Teachnology Co., Ltd. MitoTracker Deep Red FM, Lysotracker Red DND-99 and Golgi-Tracker Red (BODIPYTR® C5-Ceramide) was obtained from YEASEN Biotechnology Co., Ltd (Shanghai). Fetal bovine serum (FBS) was provided by M&C gene technology (Beijing) Co., Ltd. Calf Thymus DNA (ctDNA) were obtained from Sigma Aldrich (Shanghai, China). Bovine serum albumin (BSA) was purchased from Biosharp.

All nuclear magnetic resonance (NMR) spectra were recorded on an Agilent 400-MR 400 MHz spectrometer operated in the Fourier transform mode. $CDCl_3$ and DMSO-d_6 were used as the solvent. High-resolution mass spectroscpy (HRMS) was conducted on a SolariX 7.0T mass spectrometer (BrukerDaltonics, USA). UV-Vis absorption spectra were acquired on a TU-1810DSPC UV/Vis spectrophotometer (Puxi General Instrumental Company, China). Samples were charged into a quartz cuvette with 1 cm optical path at room temperature. Fluorescence spectra were recorded on a HITACHI F4600 fluorescence spectrophotometer. Isothermal titration calorimetry (ITC) traces were measured by MicroCal ITC200 (GE Healthcare Life Science). One-photon confocal imaging was performed using Olympus FV3000 confocal laser scanning

microscope. Two-photon fluorescent images were collected by using FluoView FVMPE-RS system. Transmission electron microscopy (TEM) images were recorded on a Hitachi HT7700 Transmission Electron Microscope system with an accelerating voltage of 120 kV (BPCI and TPCI) and a Tecnai G2 F30 transmission electron microscope (FEI,USA) with an acceleration voltage of 300 kV (TPCB).

2. Synthesis Methods

2-(4-(bis(4-(pyridin-4-yl)phenyl)amino)benzylidene)malononitrile (BPC): Compound SM**1** (660 mg, 1.54 mmol), malononitrile (1.02 g, 15.4 mmol), ammonium acetate (1.78 g, 23.2 mmol) were placed in a dry two-neck round bottom flask. Under nitrogen, acetic acid (40 mL) were added. The solution was heated to 120°C for 4 hours. After that, the mixture was poured into water to get precipitates. After dried, the crude product was purified by silica gel column eluting with DCM/ethanol (v/v = 50/1) to get an orange solid (450 mg, yield: 62%). 1H NMR (400 MHz, cdcl3) δ 8.67 (dd, J = 4.5, 1.6 Hz, 4H), 7.80 (d, J = 9.0 Hz, 2H), 7.72 – 7.61 (m, 4H), 7.58 (s, 1H), 7.50 (dd, J = 4.5, 1.6 Hz, 4H), 7.36 – 7.28 (m, 4H), 7.17 – 7.07 (m, 2H). 13C NMR (101 MHz, cdcl3) δ 157.82, 152.48, 150.38, 147.00, 145.99, 135.43, 132.91, 128.56, 126.65, 124.11, 121.26, 120.29, 114.76, 113.70.

4,4'-(((4-(2,2-dicyanovinyl)phenyl)azanediyl)bis(4,1-phenylene))bis(1-methylpyridin-1-ium) iodide (BPCI): In a 50 mL round-bottom flask, compound **BPC** (80.0 mg, 0.17 mmol) and methyl iodide (2.4 g, 1.0 mL, 17.0 mmol) were dissolved in 20 mL acetonitril. The mixture was stirred and heated to 40 ^{o}C for 1 h and then refluxed for 12 h. After cooling to room temperature, the mixture was poured into diethyl ether to get

precipitates. The precipitates were then washed with diethyl ether three times and dried under reduced pressure to afford the product as an orange powder (100 mg, yield: 78%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (d, J = 6.9 Hz, 4H), 8.46 (d, J = 6.8 Hz, 4H), 8.40 (s, 1H), 8.13 (d, J = 8.7 Hz, 4H), 7.94 (d, J = 8.9 Hz, 21H), 7.38 (d, J = 8.8 Hz, 4H), 7.24 (d, J = 8.9 Hz, 2H), 4.29 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 160.07, 153.40, 151.37, 148.77, 145.96, 133.30, 130.42, 130.17, 126.39, 123.89, 123.09, 115.28, 114.33, 78.03, 47.43. HRMS (ESI$^+$): m/z calcd for $C_{34}H_{27}N_5^{2+}$, 252.6128 ; found: 252.6128.

4-Methoxy-N-(4-methoxyphenyl)-N-phenylaniline (1): 4-iodoanisole (7.20 g, 30.76 mmol), aniline (1.14 mL, 12.49 mmol), ophenanthroline (0.45 g, 2.30 mmol), cuprous iodide (0.48 g, 2.52 mmol) and potassium hydroxide (5.60 g, 100.00 mmol) were dissolved in a round-bottom flask in 100 mL toluene. The mixture was refluxed under N_2 atmosphere and the progress of the reaction was monitored by thin layer chromatography (TLC). When the reaction was completed, the mixture was filtered and the solvent was removed under vacuum. The residue was then purified by column chromatography on silica gel column (hexane/dichloromethane, v/v = 3/1) to afford the product 2 as a light yellow powder (2.9 g). Yield: 76 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (t, J = 7.9 Hz, 1H), 7.04 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.1 Hz, 1H), 6.90 – 6.72 (m, 2H), 3.79 (s, 3H).

4-(Bis(4-methoxyphenyl)methyl)benzaldehyde (2): To the stirred solution of 1 (2.6 g, 8.5 mmol) in DMF (40 mL), POCl3 (1.1 mL,. 12.0 mmol) was added at 0 °C under Ar. The reaction was performed at 90 °C and monitored by TLC. When the reaction is

completed, 50 mL ice water was added slowly to quench the reaction and the raw product was extracted using dichloromethane and water. The organic layers were combined and dried using anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure, and the residue was purified by chromatography on a silica gel column with hexane/dichloromethane (v/v = 1/1) to give compound 3 as a dense yellow oil (2.5 g). Yield: 88 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.75 (s, 1H), 7.63 (d, *J* = 8.7 Hz, 2H), 7.20 – 7.03 (m, 4H), 6.93 – 6.86 (m, 4H), 6.85 (d, *J* = 8.8 Hz, 2H), 3.82 (s, 6H).

4-(Bis(4-hydroxyphenyl)amino)benzaldehyde (3): A solution of compound 2 (2.2 g, 6.6 mmol) in 30 mL dichloromethane was added slowly with BBr3 (4.96 g, 19.8 mmol) at 0 °C and stirred under N_2 atmosphere for 30 min. The reaction mixture was stirred for another 24 h at room temperature and quenched by 30 mL methanol. The solvents were then removed under reduced pressure and the raw product was extracted using ethyl acetate and water. The extract was dried by Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel column with hexane/ethyl acetate (v/v = 1/2) to give compound 4 (1.8 g) as a yellow powder. Yield: 90 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H), 9.59 (s, 2H), 7.77 – 7.41 (m, 2H), 7.22 – 7.03 (m, 4H), 6.85 – 6.74 (m, 4H), 6.61 (d, *J = 8.8 Hz*, 2H).

4-(Bis(4-((6-bromohexyl)oxy)phenyl)amino)benzaldehyde (4): A solution of 3 (457 mg, 1.5 mmol) and 1,2-dibromoethane (1.72 mL, 3.74 g, 20.0 mmol) in acetonitrile (25 mL) was added with K_2CO_3 (1.66 g, 12.0 mmol). The reaction mixture was stirred and refluxed under N_2 atmosphere. The reaction was monitored by TLC until the

consumption of the starting materials. Then the reaction mixture was concentrated and extracted using ethyl acetate and water. The extract was dried by Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel column with hexane/ethyl acetate (v/v = 2/1) to give compound 5 (530 mg) as a yellow powder. Yield: 68 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.77 (s, 1H), 7.75 – 7.52 (m, 2H), 7.22 – 7.03 (m, 4H), 7.01 – 6.66 (m, 6H), 4.29 (t, *J* = 6.2 Hz, 4H), 3.65 (t, *J* = 6.2 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.32, 155.71, 153.77, 139.48, 131.40, 128.05, 128.02, 117.16, 115.91, 68.10, 29.05. HRMS (ESI^+): m/z calcd for $C_{23}H_{21}Br_2NO_3$, 519.9901; found, 519.9939.

Synthesis of (2Z,2'Z)-2,2'-(1,4-phenylene)bis(3-(4-(bis(4-(2-bromoethoxy)phenyl) amino)phenyl)acrylonitrile) (5): A mixture of compound 4 (519.23 mg, 1.00 mmol), 1,4-phenylenediacetonitrile (51.48 mg, 0.33 mmol) and potassium tert-butoxide (561.00 mg, 5.00 mmol) were dissolved in 30 mL methanol. This mixture was stirred for 24 h at reflux temperature under an atmosphere of nitrogen. After cooling down to room temperature, the solvent was removed under reduced pressure. The raw product was extracted three times using DCM and water. The organic layers were combined and dried by anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure, and the residue was purified by chromatography on a silica gel column with hexane/dichloromethane (v/v = 1/6) to give compound 5 as an orange solid (58 mg). Yield: 15 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 9.0 Hz, 4H), 7.66 (s, 4H), 7.42 (s, 2H), 7.16 – 7.06 (m, 8H), 6.94 – 6.78 (m, 12H), 4.28 (t, *J* = 6.2 Hz, 8H), 3.64 (t, *J* = 6.2 Hz, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.33, 150.67, 141.83, 140.00,

135.06, 130.88, 129.48, 127.59, 125.96, 124.96, 118.44, 115.89, 105.76, 68.18, 29.07. HRMS (ESI$^+$): m/z calcd for $C_{56}H_{46}Br_4N_4O_4$, 1158.0212; found, 1158.0205.

Synthesis of 2,2',2'',2'''-((((((1Z,1'Z)-1,4-phenylenebis(2-cyanoethene-2,1-diyl)) bis (4,1-phenylene))bis(azanetriyl))tetrakis(benzene-4,1-diyl))tetrakis(oxy))tetrakis (N,N,N-trimethylethan-1-aminium) (TPCB): In a 50 mL round-bottom flask, compound 5 (40.0 mg, 0.035 mmol) and trimethylamine (1 mL in 2.0 mol/L THF solution, 2.0 mmol) were dissolved in 20 mL acetonitrile. The mixture was stirred at room temperature for 24 h. After the reaction was completed, the mixture was poured into diethyl ether to get precipitates. The precipitates were then washed with diethyl ether three times and dried under reduced pressure to afford the product as a red powder (45 mg, yield: 94 %). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 7.93 (s, 2H), 7.88 – 7.61 (m, 8H), 7.18 (d, J = 8.9 Hz, 8H), 7.04 (d, J = 8.9 Hz, 8H), 6.73 (d, J = 8.9 Hz, 4H), 4.44 (s, 8H), 3.77 (s, 8H), 3.16 (s, 36H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 155.38, 151.00, 142.85, 139.73, 134.71, 131.40, 128.37, 126.26, 124.84, 119.03, 117.35, 116.58, 104.73, 64.57, 62.38, 53.58. HRMS (ESI$^+$): m/z calcd for $C_{68}H_{82}N_8O_4^{4+}$, 268.6609 ; found, 268.6601.

3. Time-dependent density functional theory (TD-DFT) Simulation

Quantum mechanics calculation on TPBT was performed with TD-DFT B3LYP method on 6-311g(d,p) basis set, Gaussian 09 program.

4. Phototoxicity and Darktoxicity Measurement.

The cytotoxicity of the PSs in the absence and presence of white light irradiation was assessed by MTT assays. HeLa cells were seeded in 96-well plates and cultured in standard medium containing 10% FBS and 1% antibiotics (penicillin, 10,000 U mL^{-1},

streptomycin 10 mg/mL) for 24 h (37 ºC, 5% CO_2). The cells were then incubated with PS solutions at various concentrations in the dark for 24 h. The mixtures were discarded and added with fresh standard DMEM before it is exposed or unexposed (in dark) to white light (300 − 800 nm, 4 mW cm^{-2}) for 30 min. The cells were further cultured for 6 h after irradiation, and then 10 μL of freshly prepared MTT (5 mg mL^{-1}) solution was added into each well. The MTT solution was carefully removed after 3 h of incubation, and DMSO (150 μL) was added into each well to dissolve all the formazan formed. The absorbance of MTT at 570 nm was measured by the microplate reader (Varioskan LUX, Thermo Scientific, USA). Cell viability was expressed by the ratio of the absorbance of the cells incubated with PS to that of the cells incubated with culture normal medium. Each experiment was repeated at least three times.

5. Cellular Imaging by CLSM.

Living HeLa cells: HeLa cells were cultured in chamber (LAB-TEK, Chambered Cover Glass System) at 37 °C. After 80% confluence, the medium was replaced with PS in DMEM (10 μM) and incubated for 4 h. Then a solution of MitoTracker Deep Red FM (100 nM) or Lysotracker Red DND-99 (100 nM) was added and incubated for 30 min. After that, the cells were washed with PBS for three times and imaged by CLSM. Golgi-Tracker Red staining was done according to the manual instruction after the cells incubated with TPCI (10 μM) for 4h. Confocal imaging was performed using Olympus FV3000 microscope with ×100 objective lens.

Fixed HeLa cells: HeLa cells were cultured in chamber (LAB-TEK, Chambered Cover Glass System) at 37 °C. After 80% confluence, the medium was replaced with PS in

DMEM (10 μM) and incubated for 4 h. The cells were then treated with DAPI for 5 min after fixed by 4% paraformaldehyde and washed twice with the PBS buffer. Subsequently, the fixed cells were observed by CLSM at different time.

6. Measurement of Two-Photon Absorption Cross-Section

Two-photon cross-sections (δ) of the samples were obtained by the two-photon excited fluorescence (2PEF) method.[2] The samples were excited with laser pulses of 140 fs produced by the mode-locked Ti:Sapphire laser with a repetition rate of 80 MHz, whose data was collected by Ocean Optics-USB4000. The concentration of sample solution was 0.5 mM in DMSO (Dimethyl sulfoxide). Rhodamine B in methanol was used as a reference. δ was further calculated from the following equation:

$$\frac{\delta_2}{\delta_1} = \frac{F_2 \eta_1 c_1 n_1}{F_1 \eta_2 c_2 n_2}$$

Where δ_1 and δ_2 are the 2PA cross-sections, F_1 and F_2 are the integrated fluorescence intensities measured at the same power of the excitation beam, η_1 and η_2 are the fluorescence quantum yields, c_1 and c_2 are the concentrations, n_1 and n_2 are the refractive indexes of solvents (1 corresponds to Rhodamine B, 2 is the sample).

7. 2PFM Imaging of C. elegans

All C. elegans were cultured on standard Nematode Growth Medium (NGM) plates seeded with OP50 and maintained at 20 °C incubators. After 36 h starvation, C. elegans were fed by TPCI (500 μM, 200 μL) in M9 buffer dried on the medium. After different light treatment, the worms were immobilized by 2.5 mM levamisole (Sigma-Aldrich) in M9 buffer for further 2PFM Imaging.

8. Statistical Analysis

Quantitative data were expressed as mean ± standard deviation (s.d.). Statistical comparisons were made by paired Student's t-test (between two groups). P value < 0.01 was considered statistically significant difference. The statistical calculations were carried out by using GraphPad Prism.

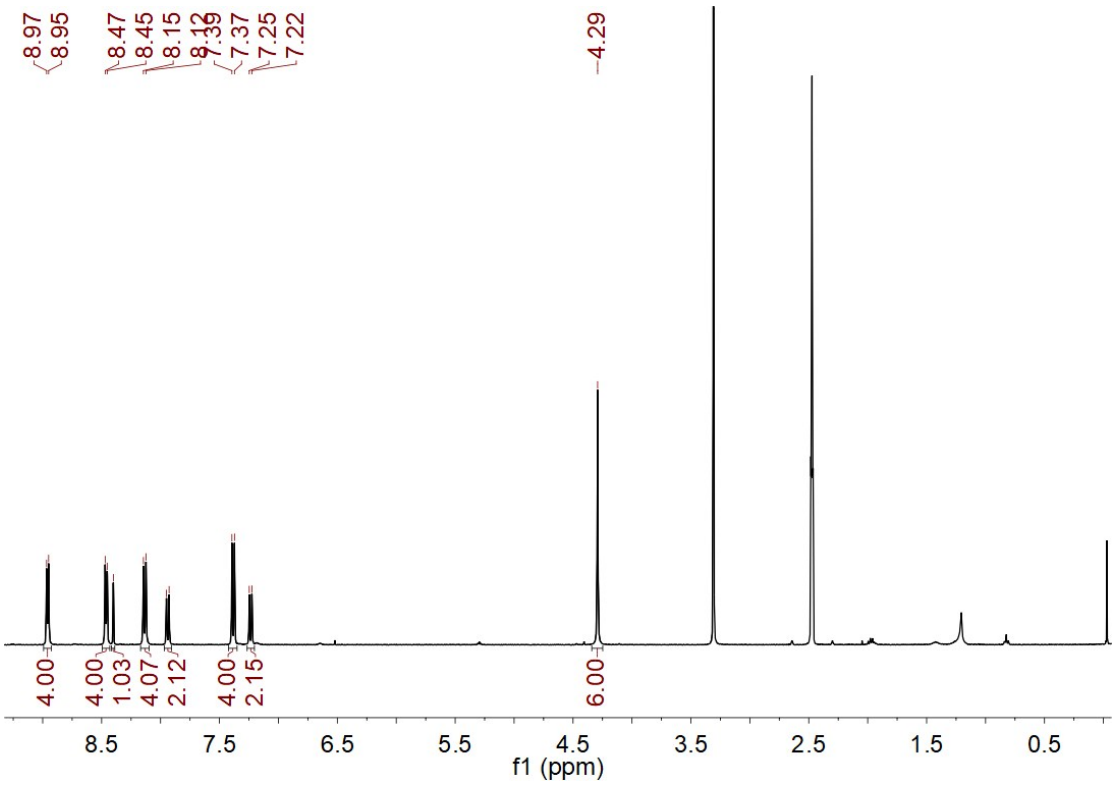

Fig. S1 ^{1}H NMR spectrum of BPCI in $CDCl_3$.

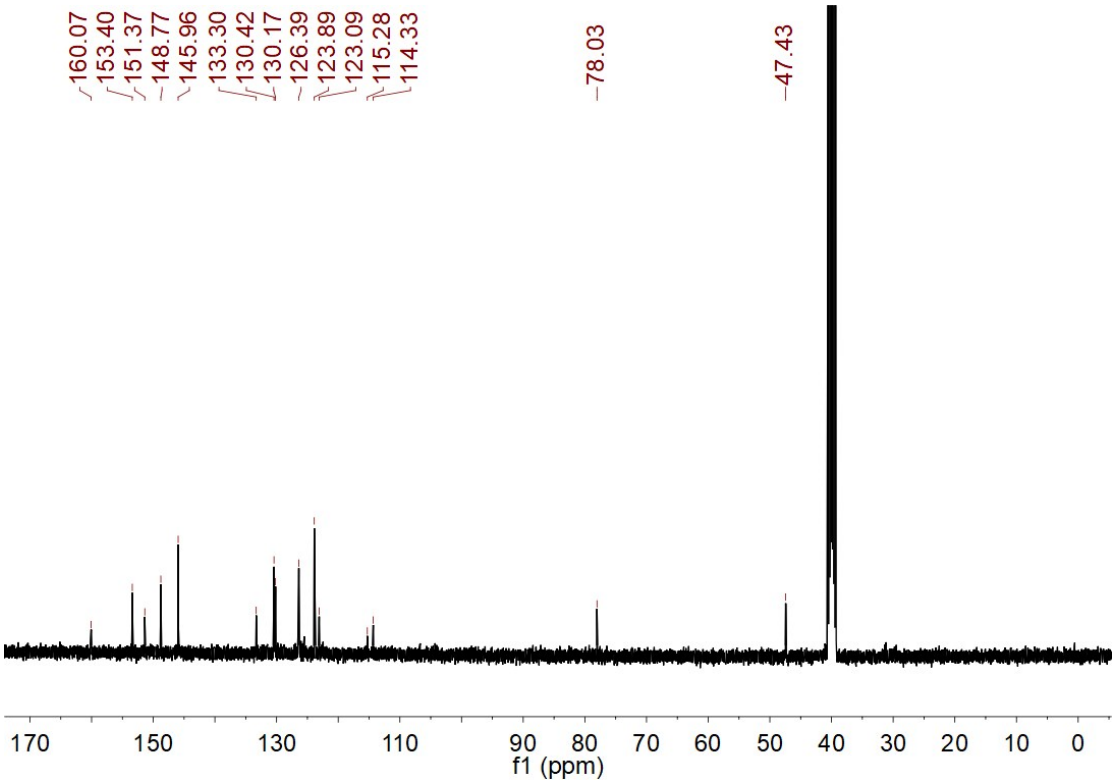

Fig. S2 ^{13}C NMR spectrum of BPCI in $CDCl_3$.

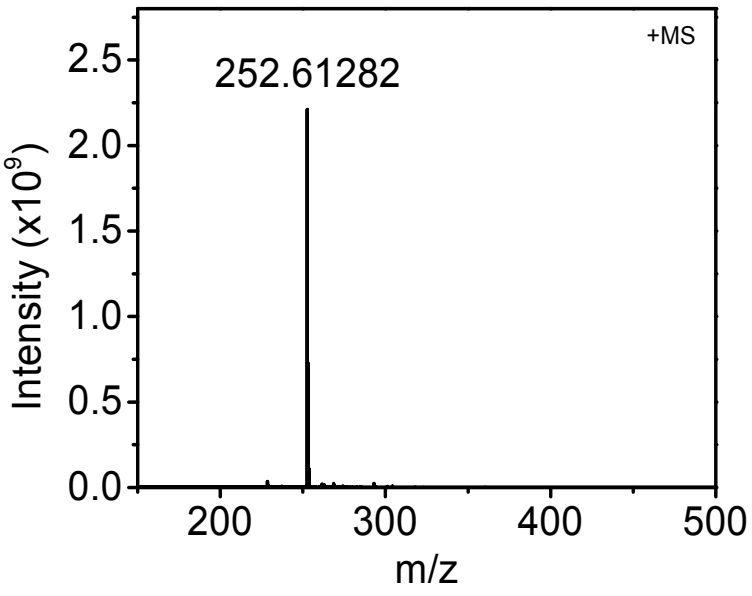

Fig. S3 HRMS mass spectrum of BPCI.

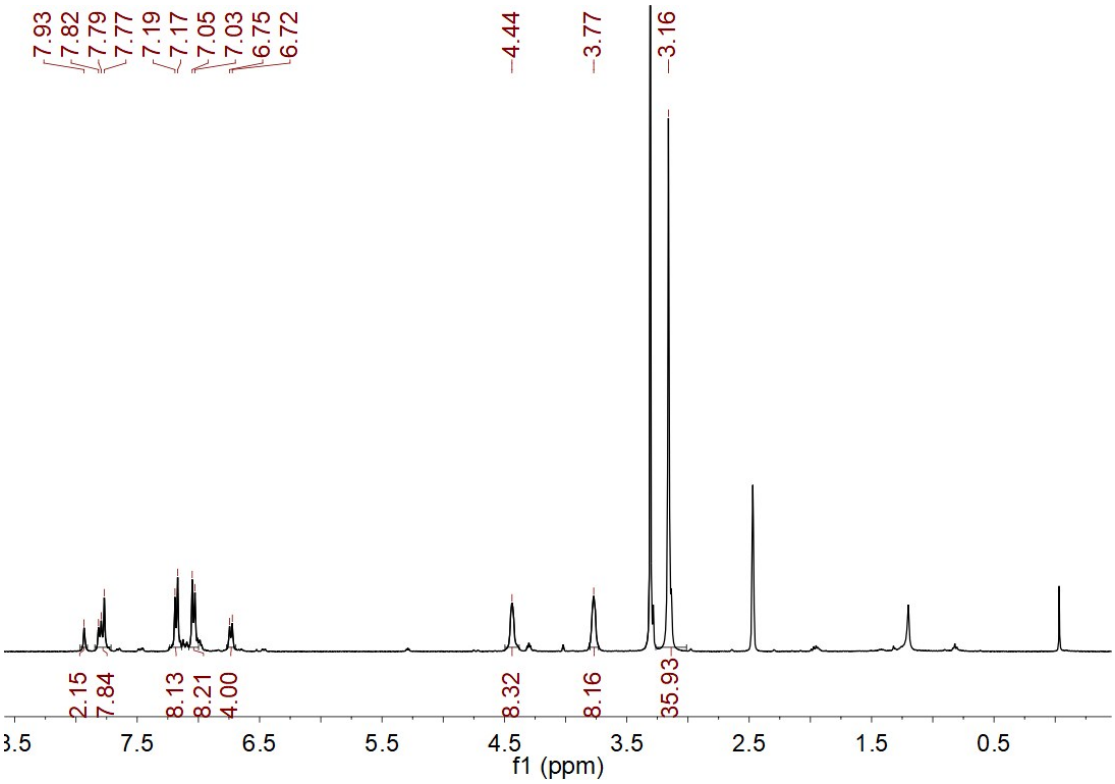

Fig. S4 ^{1}H NMR spectrum of TPCB in DMSO-*d6*.

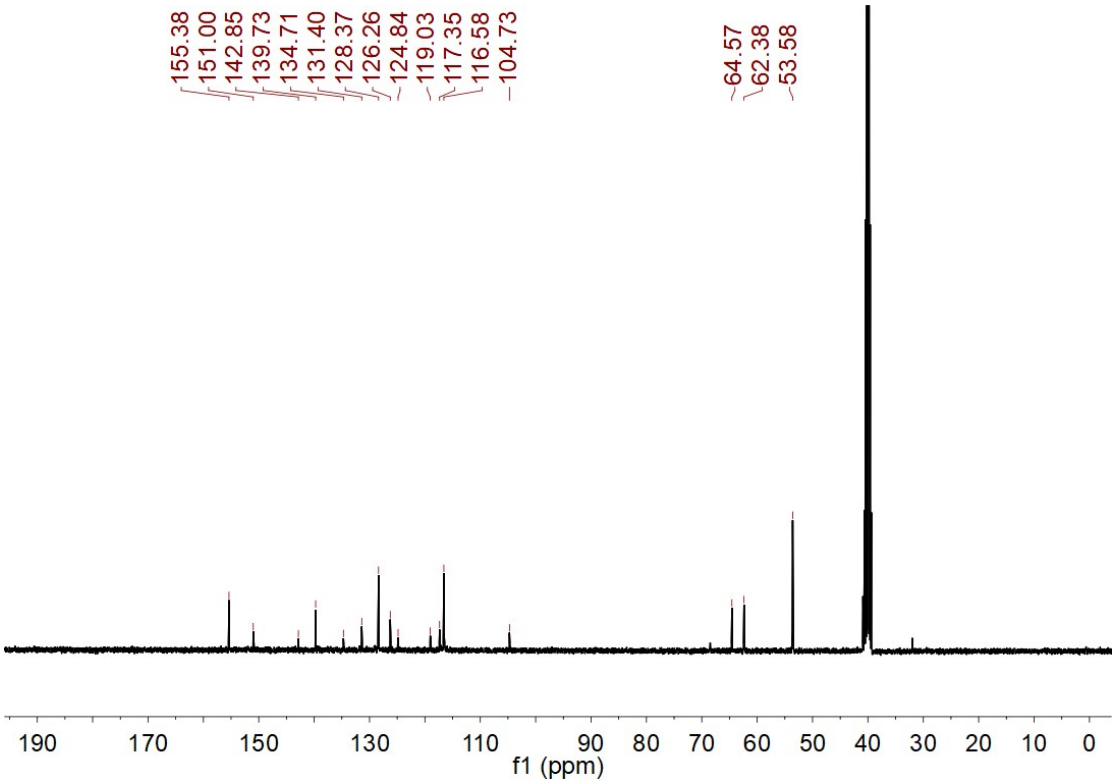

Fig. S5 ^{13}C NMR spectrum of TPCB in DMSO-*d6*.

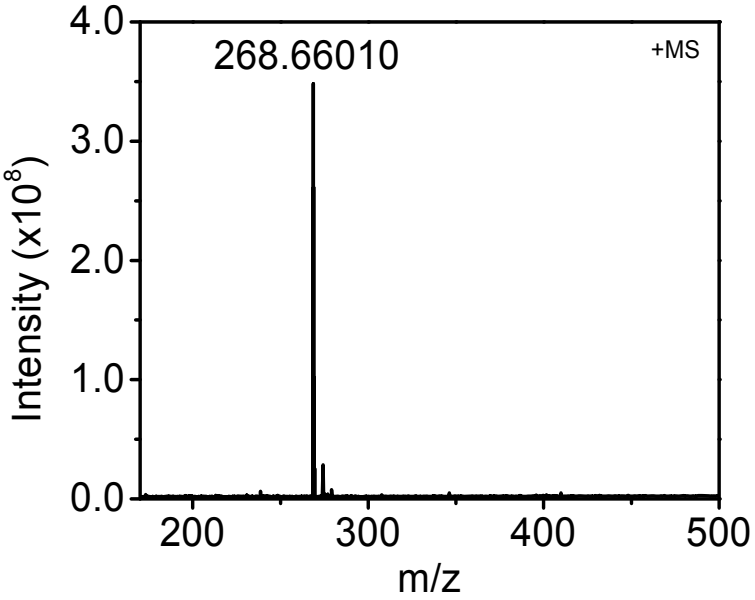

Fig. S6 HRMS mass spectrum of TPCB.

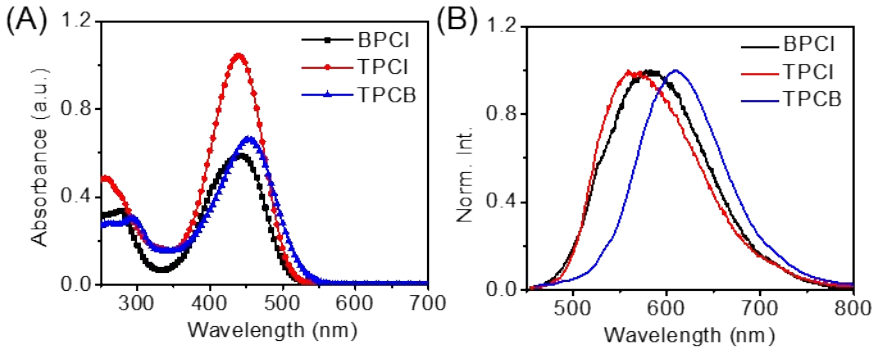

Fig. S7 (A) UV-vis absorption spectra of the three PSs in water. (B) Normalized FL spectra of the three PSs in water. [PS] = 10 μM. E_x: 450 nm.

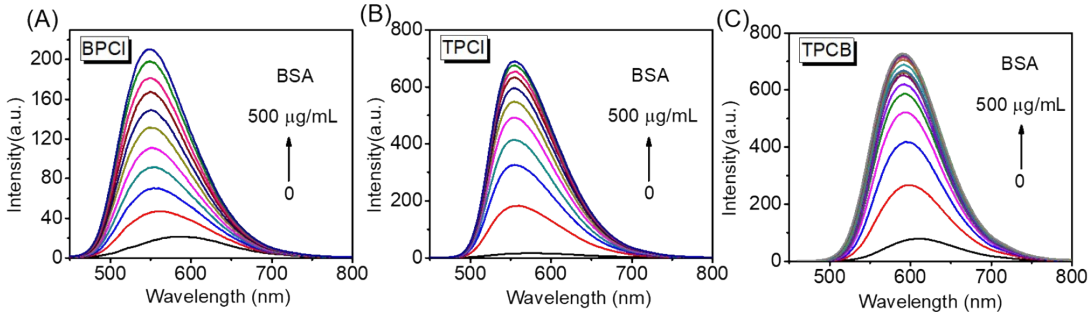

Fig. S8 The FL spectroscopic titration of BPCI (A), TPCI (B) and TPCB (C) by stepwise addition of BSA in water. [PS] = 10 μM, λ_{ex} = 450 nm.

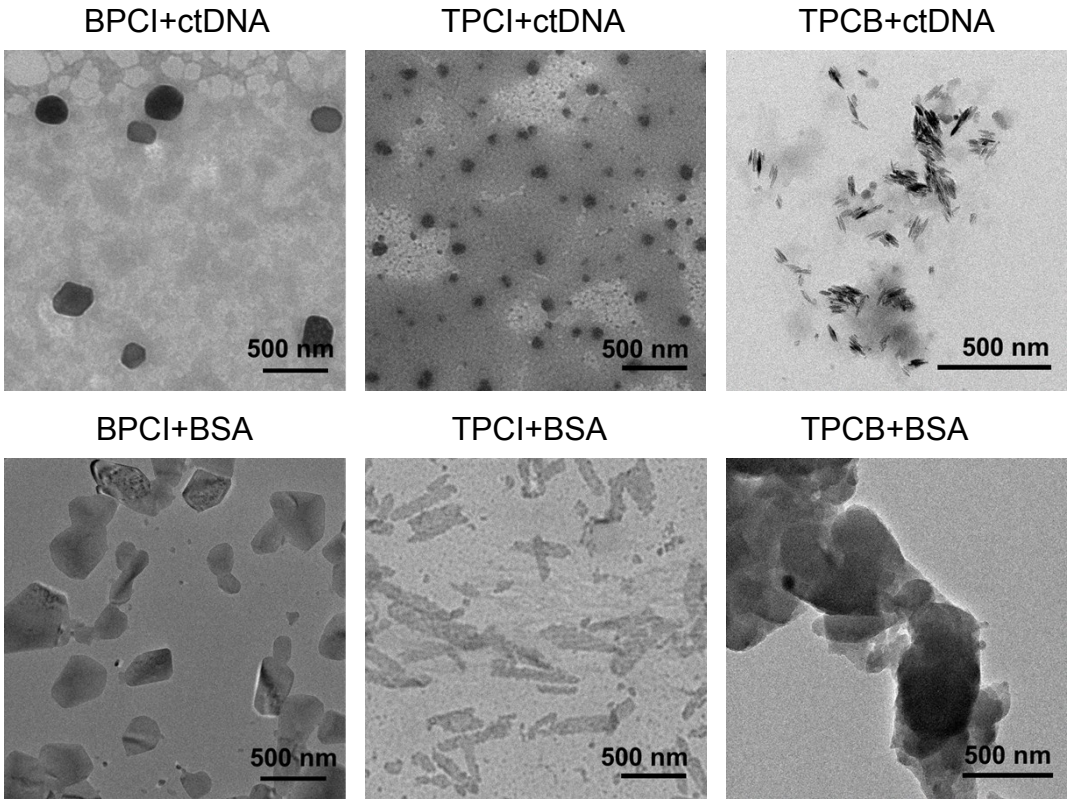

Fig. S9 TEM images of BPCI, TPCI and TPCB with the addition of ctDNA and BSA, respectively. [PS] = 10 μM, [ctDNA] = 22 μg/mL, [BSA] = 500 μg/mL.

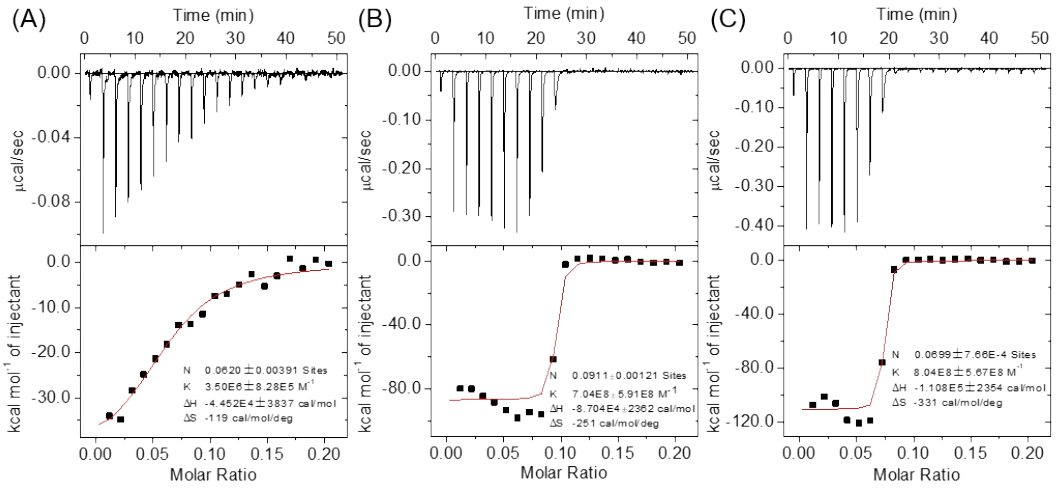

Fig. S10 Calorimetric curves for titration of BPCI (A), TPCI (B) and TPCB (C), respectively, with serial injections of oligonucleotide (20 μM) and corresponding

binding isotherm as a function of oligonucleotide / PS molar ratio at 25 °C. [PS] = 20 μM.

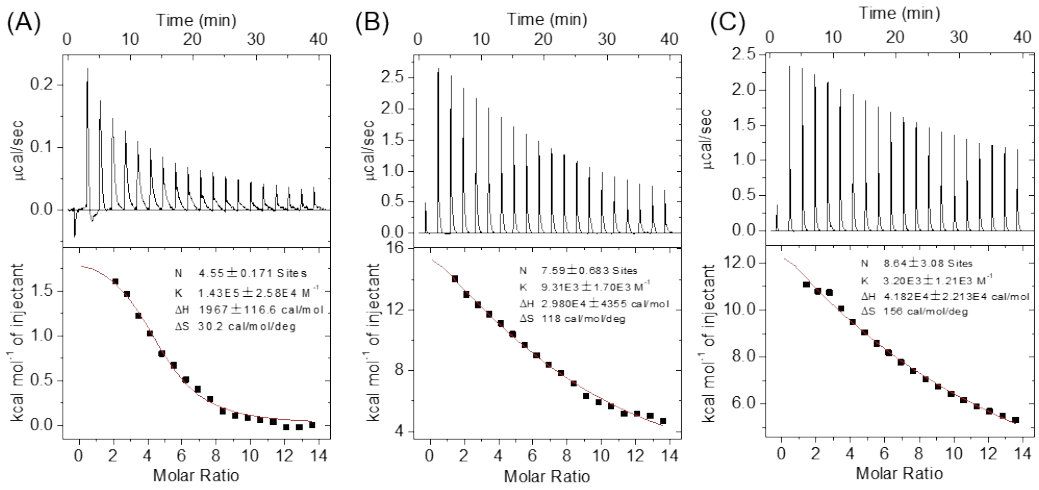

Fig. S11 Calorimetric curves for titration of BSA with serial injections of BPCI (A), TPCI (B) and TPCB (C), respectively, and corresponding binding isotherm as a function of PS / BSA molar ratio at 25 °C. [BSA] = 15 μM, [PS] = 1 mM.

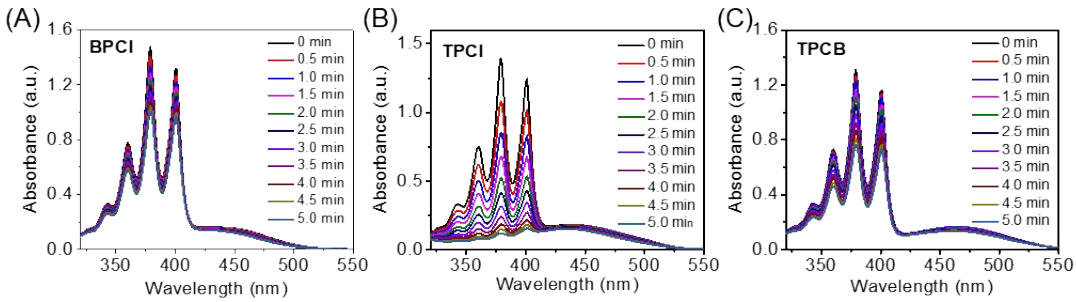

Fig. S12 UV−vis absorption spectra of ABDA in BPCI (A), TPCI (B) and TPCB (C) solution irradiated for different durations with white light irradiation (300−800 nm, 4 mW cm^{-2}). [PS] = 4 μM, [ABDA] = 100 μM.

Table S1 The singlet and triplet excited states of BPCI, TPCI and TPCB revealed by TD-DFT calculations.

	BPCI		TPCI		TPCB	
	Singlet (eV)	Triplet (eV)	Singlet (eV)	Triplet (eV)	Singlet (eV)	Triplet (eV)
1	2.2636	1.838	2.0022	1.7725	2.7321	1.9833
2	2.5441	2.0499	2.0158	1.7732	3.0434	2.3012
3	3.023	2.4743	2.1818	1.9367	3.3309	2.9438
4	3.1821	2.8939	2.2243	1.9886	3.3868	3.0291
5	3.2546	3.1058	2.2802	2.1579	3.449	3.0462
6	3.2834	3.1322	2.3455	2.1799	3.4743	3.0783
ΔE_{ST}	0.2137 ($S_1 \rightarrow T_2$)		0.0136 ($S_1 \rightarrow T_4$)		0.4309 ($S_1 \rightarrow T_2$)	

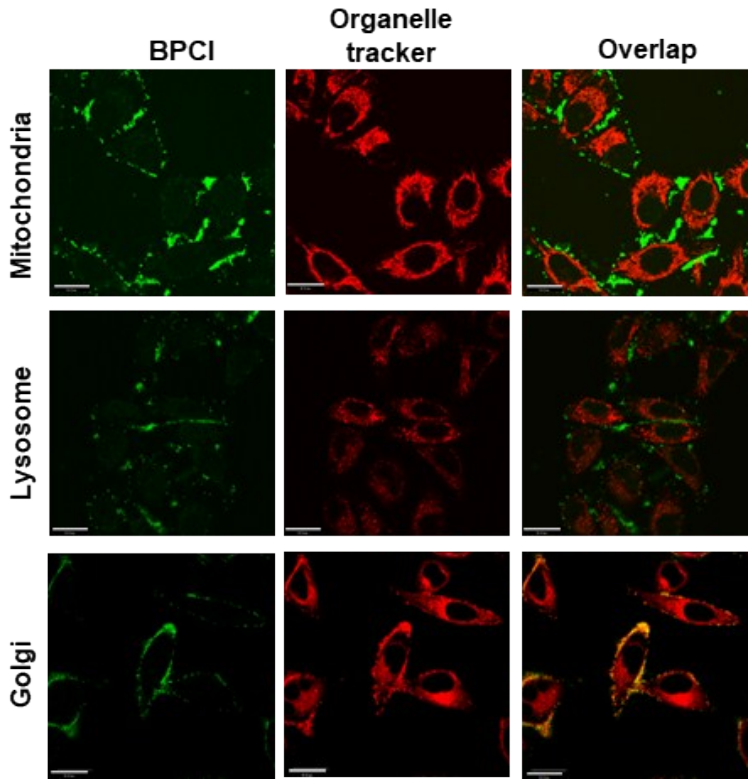

Fig. S13 Subcellular co-localization of BPCI (10 μM) with different organelle trackers, using MitoTracker Deep Red FM, LysoTracker® Red DND and Golgi-Tracker Red respectively. Excitation at 488 nm and signal collection at 500 − 570 nm for TPCI; excitation at 561 nm and signal collection at 600 − 670 nm for LysoTracker® Red DND; excitation at 594 nm and signal collection at 610 − 700 nm for Golgi-Tracker Red; excitation at 640 nm and signal collection at 650 − 750 nm for MitoTracker Deep Red FM, Scale bar: 20 μm.

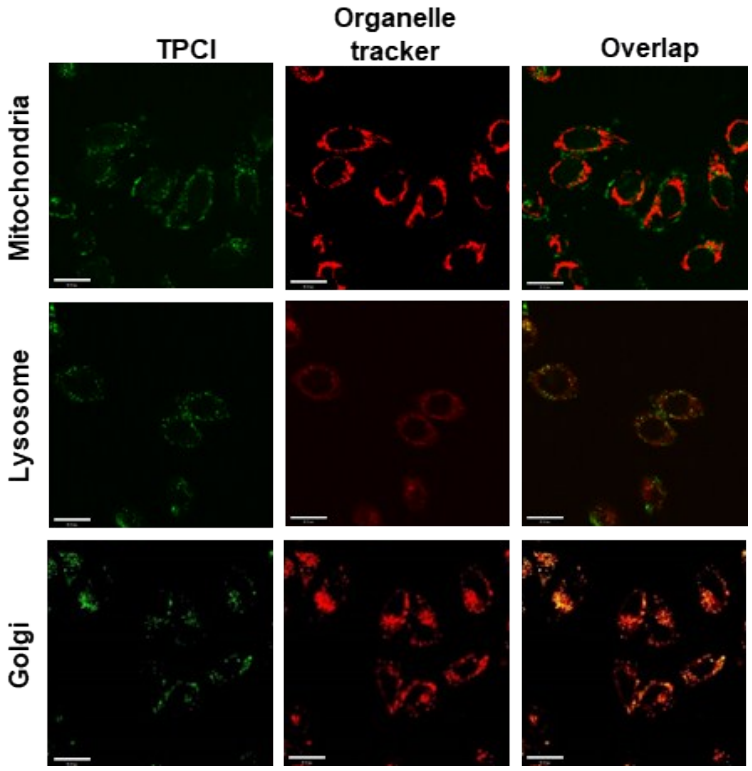

Fig. S14 Subcellular co-localization of TPCI (10 μM) with different organelle trackers, using MitoTracker Deep Red FM, LysoTracker® Red DND and Golgi-Tracker Red respectively. Excitation at 488 nm and signal collection at 500 − 570 nm for TPCI; excitation at 561 nm and signal collection at 600 − 670 nm for LysoTracker® Red DND; excitation at 594 nm and signal collection at 610 − 700 nm for Golgi-Tracker Red; excitation at 640 nm and signal collection at 650 − 750 nm for MitoTracker Deep Red FM, Scale bar: 20 μm.[1]

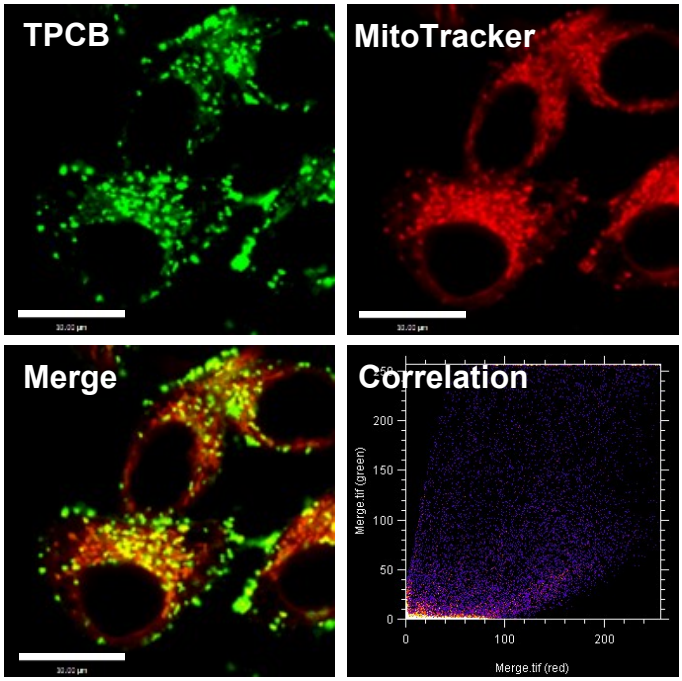

Fig. S15 CLSM images of TPCB-pretreated living HeLa cells co-stained with MitoTracker Deep Red FM. [TPCB] = 2 μM. Green channel for TPCB (λex = 488 nm, λem = 550-610 nm), Red channel for MitoTracker Deep Red FM (λex = 640 nm, λem = 650-750 nm). Scale bar: 10 μm.

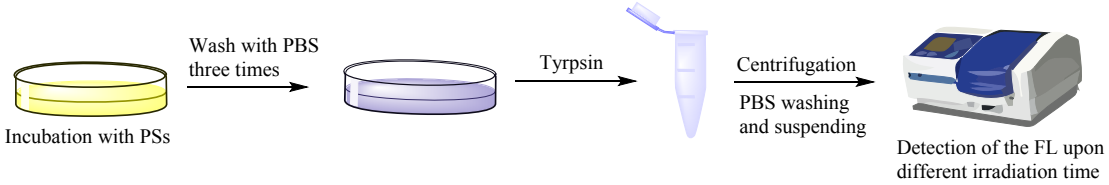

Fig. S16 Procedures for photodynamic theranostics detection upon white light irradiation. The cells were incubated with PS solutions for 4h, then washed by PBS three times. After that, the cells were digested with trypsin and collected with three times washing. The suspending cells in cuvettes was then receiving the PDT, and their FL response was recorded by a FL spectrophotometer. [PS] = 10 μM.

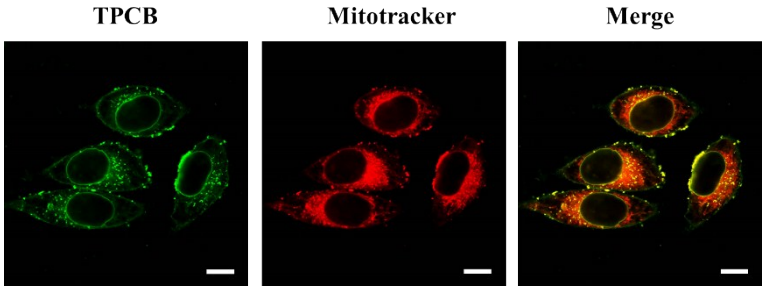

Fig. S17 CLSM images of TPCB-pretreated fixed HeLa cells co-stained with MitoTracker Deep Red FM. [TPCB] = 2 μM. Green channel for TPCB (λ_{ex} = 488 nm, λ_{em} = 550-610 nm), Red channel for MitoTracker Deep Red FM (λ_{ex} = 640 nm, λ_{em} = 650-750 nm). Scale bar: 10 μm.

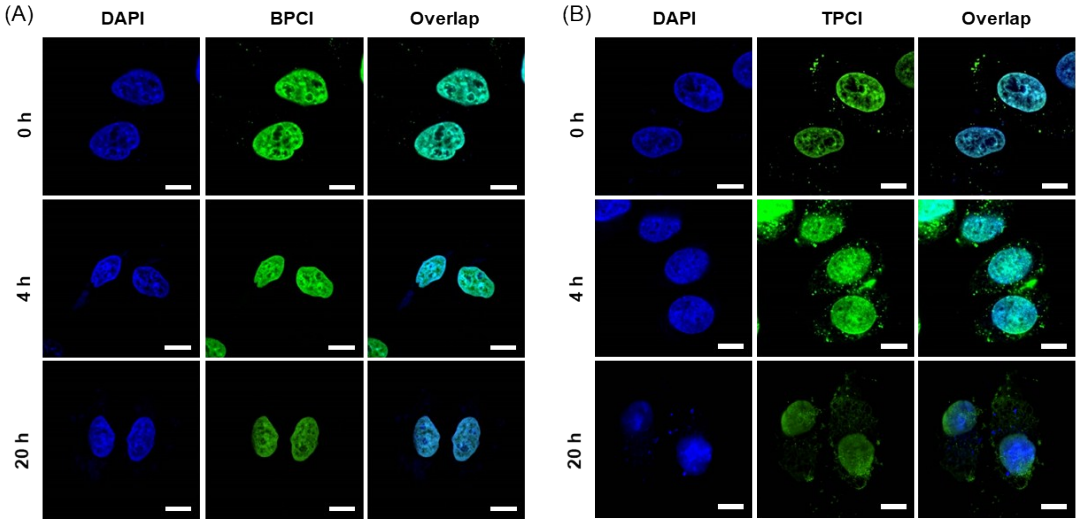

Fig. S18 CLSM images of BPCI (A) or TPCI (B) pretreated fixed HeLa cells at different time points. Cells were co-stained with DAPI for 5 min. Blue channel for DAPI (λ_{ex} = 405 nm, λ_{em} = 415-470 nm), Green channel for BPCI and TPCI (λ_{ex} = 488 nm, λ_{em} = 500-570 nm).

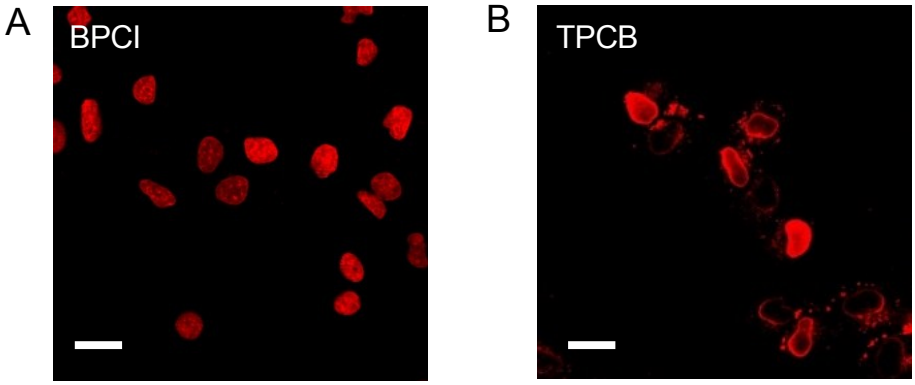

Fig. S19 Two-photon fluorescent microscopic images of BPCI (A) and TPCB (B) pretreated Hela cells after been fixed for 6h (λ_{ex} = 800 nm for BPCI and λ_{ex} = 840 nm for TPCB, respectively). Scale bar: 20 μm.

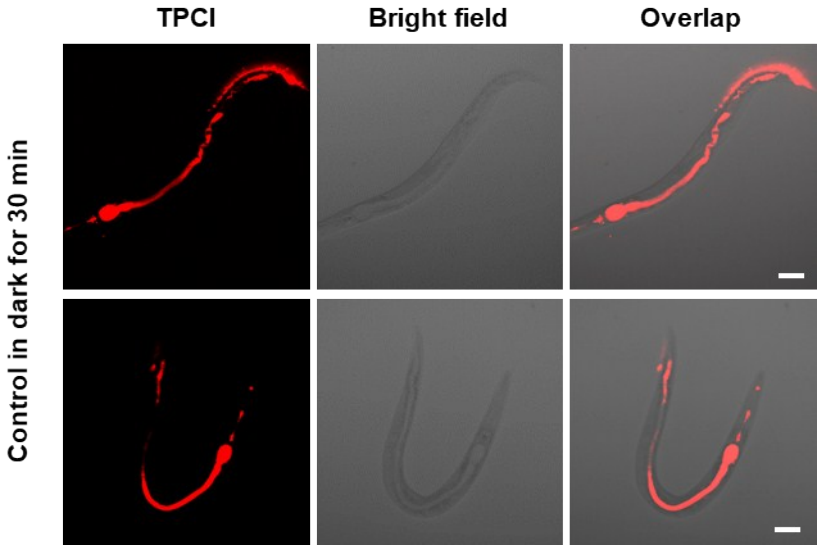

Fig. S20 Two-photon (λ_{ex} = 840 nm) fluorescent microscopic images of caenorhabditis elegans in the control group before (up) and after (down) placing in dark for 30 min. Scale bar: 50 μm.

Reference

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