Electronic Supplementary Material (ESI) for Materials Chemistry Frontiers.

Supplementary Information

Hydrogen peroxide-responsive AIE probe for imaging-guided organelle targeting and photodynamic cancer cell ablation

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Experimental Section

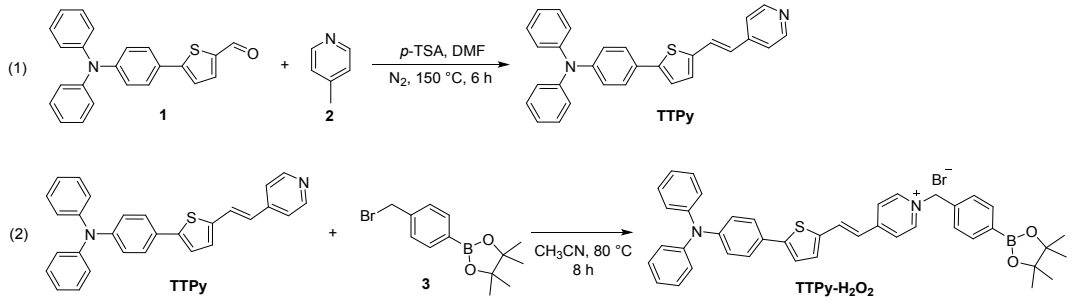
Main Materials

All the chemicals and biological reagents for synthesis and analysis were purchased from Energy, Bide and Sigma-Aldrich Chemical Reagent Ltd., and used without further purification unless specified requirement. Human hepatoma cells (HepG2), human cervical carcinoma cells (HeLa) and human normal liver cell (LO2) were purchased from American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G (P) (100 U mL^{-1}), streptomycin (S) (100 U mL^{-1}), Mito-tracker Deep Red (DR) and HCS Lipid TOX™ Deep Red (DR) Neutral Lipid Stain were from Invitrogen™ (USA). Calcein AM/ (PI) kit was from KeyGEN BioTECH (China). 3-(4,5-dimethylthiazol-2 -yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma (USA). Phorbol myristate acetate (PMA) was from Aladdin (China). DCFH-DA was from Beyotime (China).

Instruments

Nuclear magnetic resonance (NMR) spectra were measured on Bruker AVANCE III 400MHZ and 500MHZ NMR spectrometers. High resolution mass spectrometer (HRMS) was tested on a Thermo Exactive Focus Q. UV-vis absorption spectra were measured on a PerkinElmer Lambda 950 spectrophotometer. Fluorescence spectra were recorded on an Edinburgh FS5 fluorescence spectrophotometer. Quantum yield was determined by a Quanta-integrating sphere. Confocal laser scanning microscopy images were collected on a confocal laser scanning microscope (CLSM, ZEISS-LSM900) and analyzed by using ZEN 3.2 software. CCK-8 assays were conducted on a BioTek microplate reader.

Synthetic procedure for the preparation of TTPy and TTPy-H2O2



Synthesis of TTPy. The starting material 1 is either commercially available or prepared through literature reported methods. A solution of 1 (355 mg, 1 mmol) and *p*-toluenesulfonic acid (206 mg, 1.2 mmol) was refluxed under nitrogen in dry DMF (10 mL) at 150 °C, then 1.76 mL 4-methylpyridine (100 µL, 1 mmol) was added via syringe and reacted for 6 h. Then the mixture was cooled to room temperature and extracted with DCM and water. The crude product was purified by a silica gel column chromatography using DCM/methanol (99:1 v/v) as eluent. Bright orange powder of TTPy was obtained in 76% yield (326 mg). ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.53 (d, *J* = 5.2 Hz, 2H), 7.50 (d, *J* = 8.8 Hz, 2H), 7.42 (d, *J* = 16.0 Hz, 1H), 7.34 – 7.26 (m, 6H), 7.18 (d, *J* = 3.8 Hz, 1H), 7.15 – 7.04 (m, 9H), 6.81

(d, *J* = 16.0 Hz, 1H). ^{13}C NMR (151 MHz, CD_2Cl_2) δ 150.68, 148.35, 147.87, 145.34, 144.78, 140.60, 130.11, 129.91, 128.12, 127.01, 126.58, 125.28, 125.18, 123.93, 123.69, 123.35, 120.93. ESI HRMS: calcd. for [C29H22N2S+H]+: 431.1576, found 431.1520.

Synthesis of TTPy-H_2O_2. 4-Bromomethylphenylboronic acid pinacol ester (150 mg, 0.5 mmol) and TTPy (215 mg, 0.5 mmol) was refluxed under nitrogen in dry acetonitrile (10 mL) at 80 oC for 8 h. After cooling to room temperature, the mixture was added into 50 mL of ice-cooled diethyl ether with vigorous stirring. The precipitates were separated by centrifugation and washed three times with diethyl ether and dried in a vacuum at 40 ^{o}C to a constant weight. Eventually, TTPy-H_2O_2 was obtained as deepred powder in 88% yield (320 mg). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 9.08 (d, *J* = 6.6 Hz, 2H), 8.00 (d, *J* = 15.7 Hz, 1H), 7.92 (d, *J* = 6.6 Hz, 2H), 7.78 (d, *J* = 8.0 Hz, 2H), 7.56 (d, *J* = 8.1 Hz, 2H), 7.46 – 7.40 (m, 3H), 7.28 (dd, *J* = 8.8, 7.1 Hz, 4H), 7.17 (d, *J* = 3.9 Hz, 1H), 7.08 (d, *J* = 7.5 Hz, 6H), 6.99 (d, *J* = 8.8 Hz, 2H), 6.75 (d, *J* = 15.8 Hz, 1H), 6.05 (s, 2H), 1.30 (s, 12H). ^{13}C NMR (101 MHz, CD_2Cl_2) δ 154.06, 149.92, 149.09, 147.59, 144.37, 139.11, 136.84, 136.16, 135.88, 135.28, 129.97, 129.00, 127.34, 127.06, 125.61, 124.29, 124.07, 123.88, 122.94, 120.38, 84.61, 63.37, 25.19. ESI HRMS: calcd. for $[C_{42}H_{40}BN_2O_2S]^+$: 647.2898, found 647.2922.

Preparation of Various ROS and RNS

The stock H_2O_2 and NaOCl solution was purchased form Sigma-Aldrich reagent company. The concentration of H_2O_2 was measured by Fluorimetric Hydrogen Peroxide Assay Kit.

Superoxide ($O_2^{\cdot-}$) was generated from xanthine/xanthine oxidase system. Xanthine oxidase solid was added firstly. After xanthine oxidase was dissolved, xanthine in 1.6 M NaOH was added and the mixtures were stirred at 25°C for 1 h.

Hydroxyl radicals (·OH) and tert-butoxy radicals (·OtBu) were generated by reaction of Fe^{2+} with H_2O_2 or TBHP respectively.[2]

Nitric oxide (NO) was generated from *N,N*-Di-sec-butyl-*N,N*-dinitrosol-1,4 -phenylenediamine (BNN6).

Reduced glutathione (GSH), ascorbic acid (Vc), cysteine (Cys), glucose (Glu), arginine (Arg), $Fe_2(SO_4)_3$, $CaCl_2$ and $MgSO_4$ were obtained from commercial sources and used without additional purification.

The measure concentration: 1: Blank, 2: 200 μM ClO^-, 3: 200 μM $O_2^{\cdot-}$, 4: 200 μM TBHP, 5: 200 μM ·OH, 6: 100 μM H_2O_2, 7: 200 μM NO, 8: 10 mM Cys, 9: 10 mM GSH, 10: 10 mM Vc, 11: 10 mM Glu, 12: 10 mM Arg, 13: 10 mM Fe^{3+}, 14: 10 mM Ca^{2+}, 15: 10 mM Mg^{2+}.

Determination of the detection limit (*LOD*) of TTPy-H_2O_2 towards addition of H_2O_2

Based on the linear fitting in Fig. 1j, *C* is estimated as follows:

$$LOD = 3\sigma/B$$

Where σ is the standard deviation obtained from three individual fluorescence measurements (I_{590}) of TTPy-H_2O_2 (10 μM) without H_2O_2, *B* is the slope obtained after linear fitting the titration curves within certain ranges.

Cell cultures, H_2O_2-activated TTPy-H_2O_2 treatment, intracellular ROS, in vitro PDT efficiency estimation and cell viability

Cell Culture. HepG2 and LO2 cells were cultured in RPMI 1640 medium supplemented with

10% FBS and 1% PS. HeLa cells were cultured in DMEM medium with 10% FBS and 1% PS. The cell culture medium was changed every 3 days and all the cells were cultured at 37 °C and 5% CO_2 in a humidified atmosphere.

Cell Imaging. The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. The HeLa cells were incubated with different concentrations of TTPy-H_2O_2 (0, 2, 5, 10 μM) or TTPy (0, 2, 5, 10 μM) for 30 min, imaging was recorded by a confocal laser scanning microscope (CLSM) (LSM900, Carl Zeiss, Germany). TTPy-H_2O_2: Ex = 405 nm, Em = 410-550 nm; TTPy: Ex = 488 nm, Em = 650-700 nm.

The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. TTPy-H_2O_2 (5 μM) was incubated with the HeLa cells for 30 min. After the removal of TTPy-H2O2, the cells were washed three times with PBS, and Mito-tracker DR (2 μM) was incubated with the cells for 15 min. TTPy (5 μM) was used to stained HeLa cells for 30 min. After the removal of TTPy, the cells were washed three times with PBS and incubated with HCS Lipid TOX™ DR Neutral Lipid Stain (2 μM) for 30 min. The imaging of the cells was recorded by a CLSM. Mito-tracker DR: Ex = 640 nm, Em = 650-700 nm ; HCS Lipid TOX™ DR Neutral Lipid Stain: Ex = 640 nm, Em = 650-700 nm.

Intracellular H_2O_2 Detection. The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. The HeLa cells were incubated with TTPy-H_2O_2 (5 μM) for 30 min. The medium containing TTPy-H_2O_2 was removed and the cells were washed three times with PBS, followed the addition of different concentrations of H_2O_2 (0, 25, 50 and 100 μM). The cells were incubated for 1 h. Imaging was recorded by a CLSM.

The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. TTPy-H_2O2 (5 μM) were used to stain the HeLa cells for 30 min. Imaging was recorded by CLSM after TTPy-H_2O_2-treated cells were incubated with H_2O_2 (50 μM) at different time points.

To characterize the intracellular distribution of TTPy-H_2O_2, HeLa cells were treated with PMA to stimulate the production of endogenous H_2O_2. The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. The HeLa cells were incubated with TTPy-H_2O_2 (5 μM) for 30 min, followed incubation with PMA (2 μg mL^{-1}) at different time points (0, 90 and 120 min) after TTPy-H_2O_2 medium was removed and the cells were washed three times with PBS.

Intracellular ROS Detection. HeLa cells were seeded in confocal dishes for 24 h. HeLa cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the darkness after the treatment by TTPy-H_2O_2 (5 μM) treated for 30 min. The fluorescence intensity was measured using a CLSM, with one-photon (488 nm) excitation. DCFH-DA: E_x = 488 nm, E_m = 505-525 nm.

Cytotoxicity Assay by Calcein-AM/PI Kit. Calcein-AM/PI kit was used to analyze the cell viability and morphology of HeLa cells. The HeLa cells (5×10^4 cells mL^{-1}) were seeded in confocal dishes and incubated for 24 h. TTPy-H_2O_2 (0, 5 and 10 μM) was added in the culture medium and incubated with the cells for 30 min. 50 μM H_2O_2 was used to treat the HeLa

cells for 1 h. The treated cells were washed three times with PBS and treated with or without light irradiation for 10 min, then the cells were cultured for 2-10 h. Calcein-AM (2 μM)/PI (8 μM) was used to stain the HeLa cells for 30 min. Calcein-AM/PI mixture medium was removed and the cells were washed once with PBS, 1 mL fresh DMEM medium was added before observing by CLSM. Calcein-AM: E_x = 488 nm, E_m = 500-550 nm; PI: E_x = 561 nm, E_m = 630-700 nm.

MTT assay. Cell viability was carried out by MTT assay. HepG2, HeLa and LO2 cells were cultured in 96-well plates (1×10^4 cells 200 μL^{-1}) for 24 h. Different concentrations of TTPy-H_2O_2 (0, 2, 5 and 10 μM) and TTPy (0, 2, 5 and 10μM) were incubated with the cells for 30 min. Then, the cells were cultured for another 24 h after treatment with or without light irradiation (16 mW cm^{-2}) for 10 min and 20 min, respectively. 100 μL of fresh DMEM medium containing 10 μL MTT (5 mg mL^{-1}) was added after the culture medium in each 96-well plate removed completely. The cells were further incubated for 4 h. 100 μL of DMSO was added into each well after the DMEM/MTT medium was removed. OD was measured on a Synergy H1 microplate reader (BioTek, USA), at an optical absorbance of 570 nm. Five replicate measurements were obtained for each sample.

Supplementary Figure & Table

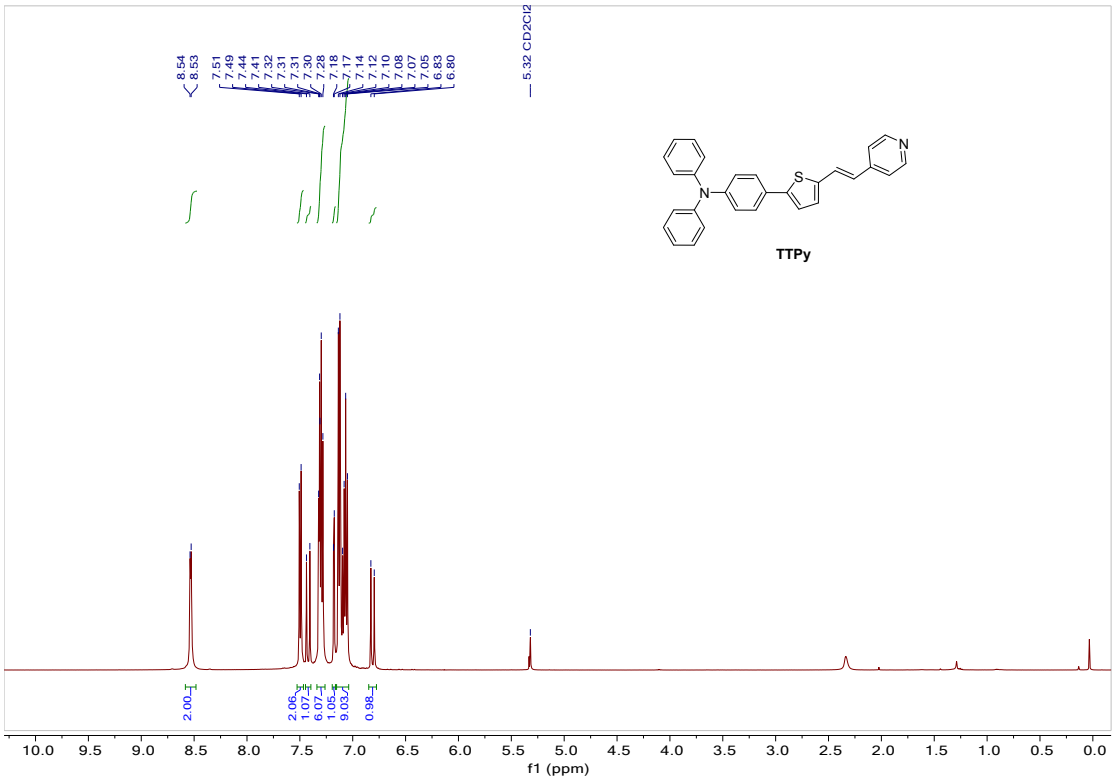


Figure S1. ^{1}H NMR spectrum of TTPy.

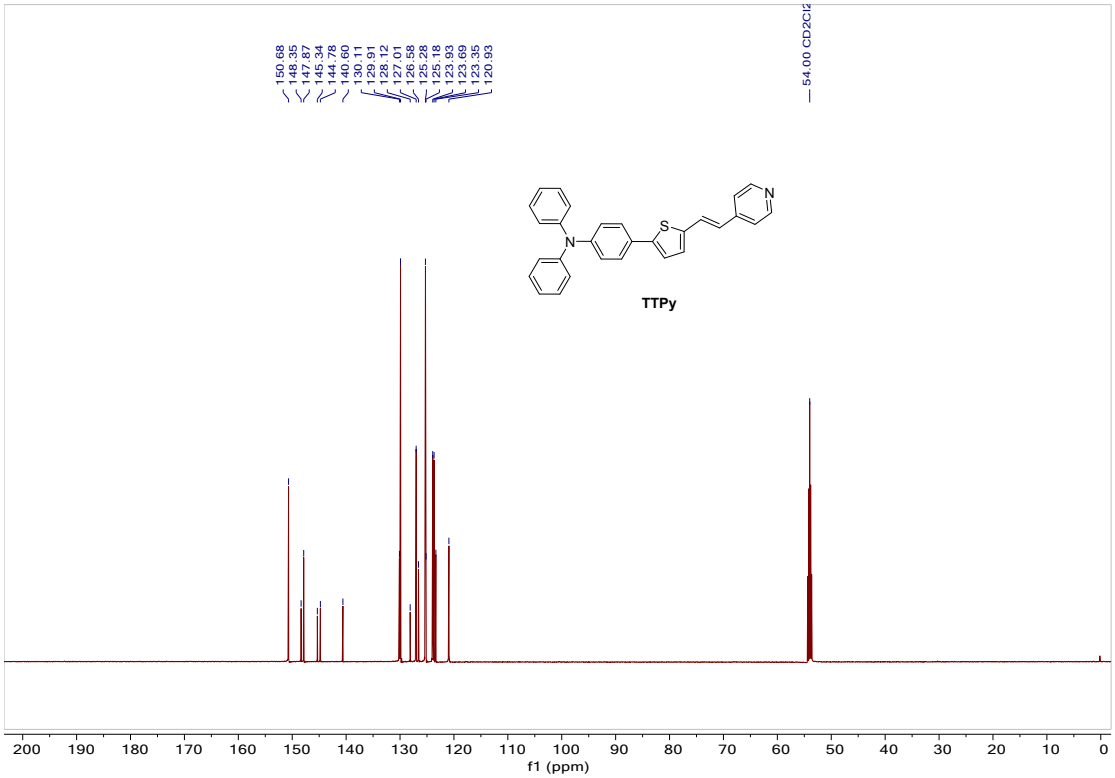


Figure S2. ^{13}C NMR spectrum of TTPy.

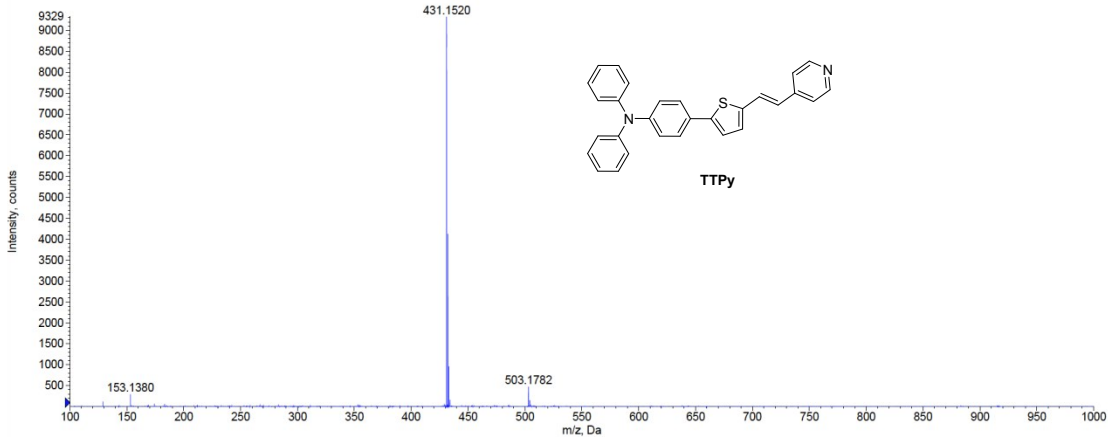


Figure S3. HRMS spectrum of TTPy.

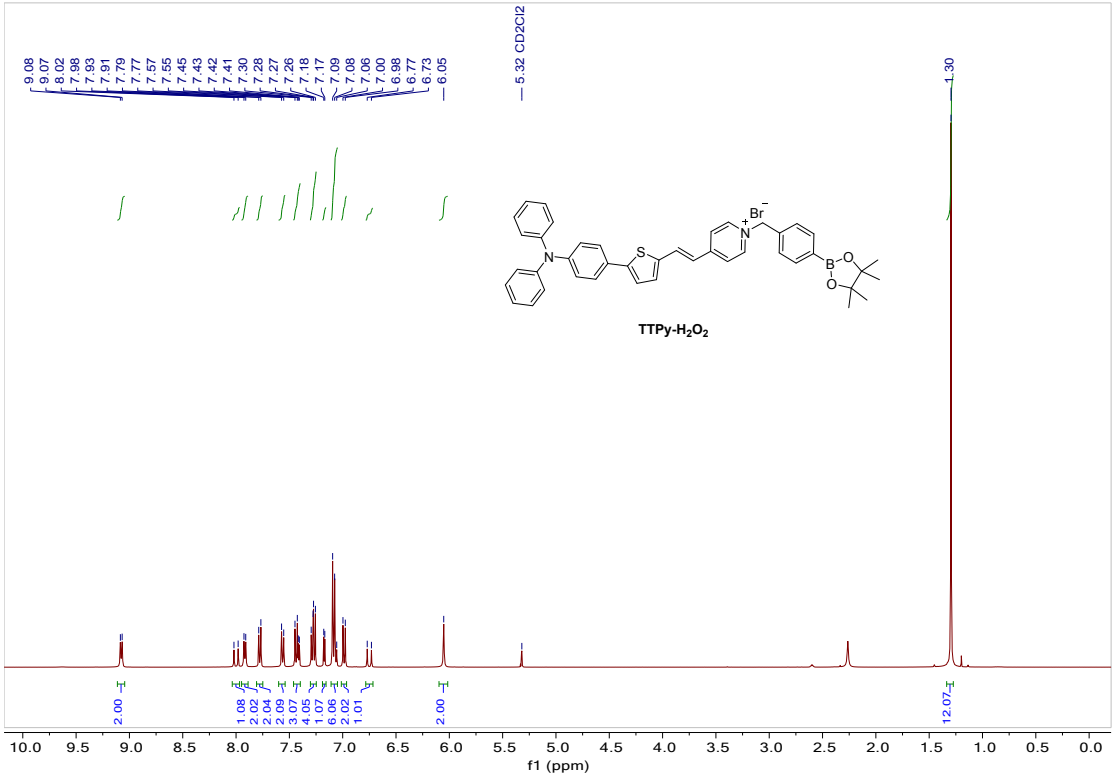


Figure S4. ^{1}H NMR spectrum of TTPy-H_2O_2.

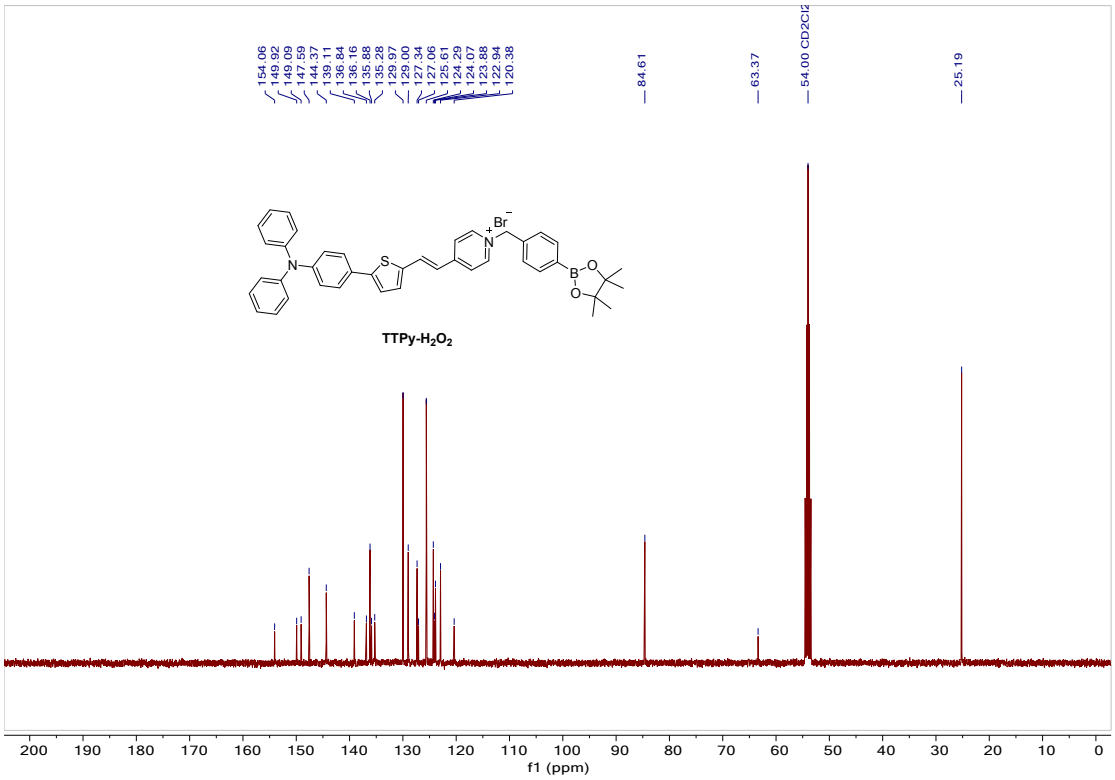


Figure S5. ^{13}C NMR spectrum of TTPy-H_2O_2.

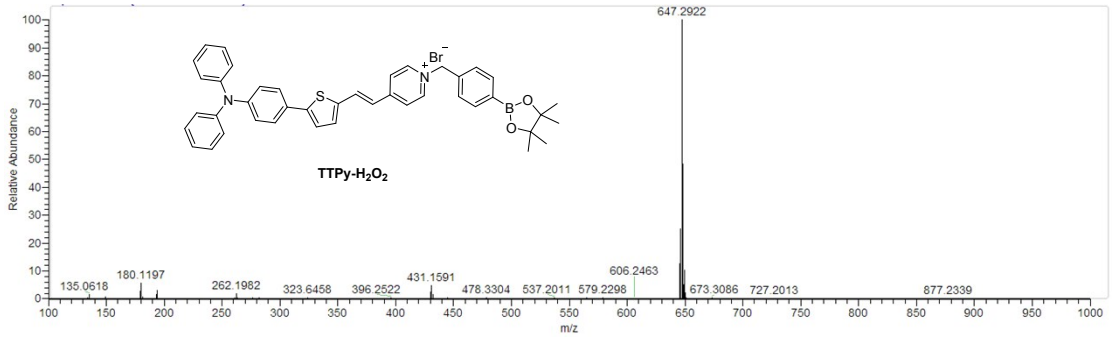


Figure S6. HRMS spectrum of TTPy-H_2O_2.

Table S1. The optical properties of TTPy and TTPy-H_2O_2. [a,b]

	Abs	$E_{m,\ DMSO/PBS}$	$QY_{soln.}$	$E_{m,\ solid}$	QY_{solid}
TTPy-H_2O_2	490 nm	670 nm	0.8 %	700 nm	2.4 %
TTPy	410 nm	590 nm	83.5 %	535 nm	2.1 %

[a] Absorption and emission maximum in DMSO/PBS buffer (3:7 v/v, pH 7.4); [b] All the fluorescence quantum yields are determined by a calibrated integrating sphere system.

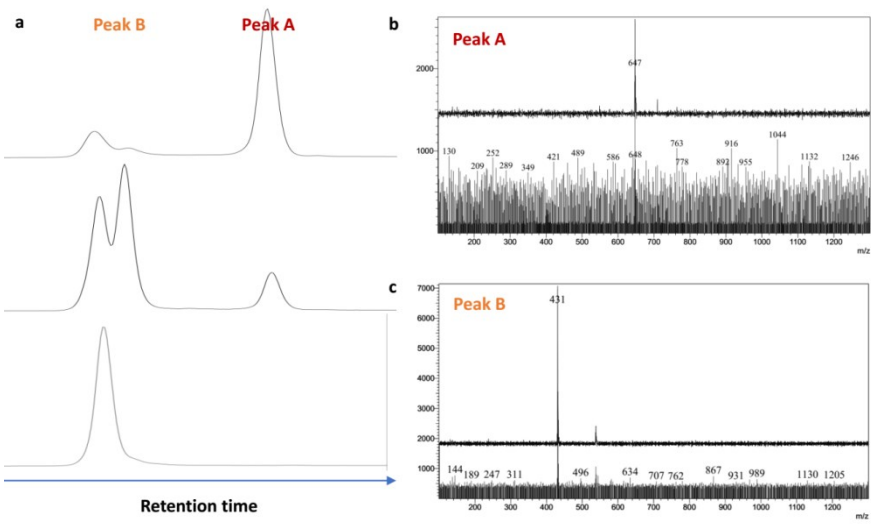


Figure S7. Reaction monitoring after adding H_2O_2 for TTPy-H_2O_2 by LC-MS.

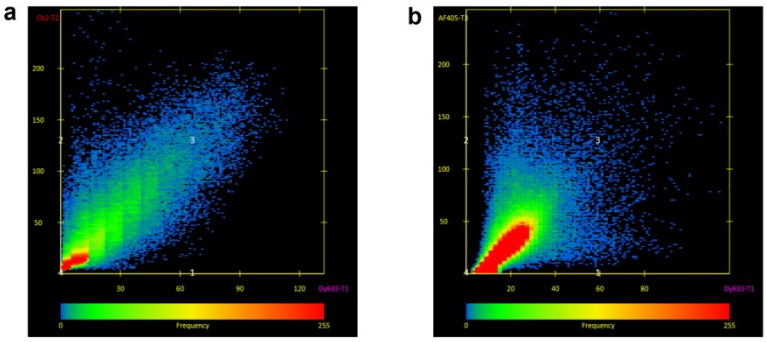


Figure S8. The scatter plots of correction coefficient in the organelle colocalization experiments. (a) The scatter plots of correction coefficient between TTPy-H_2O_2 and commercial MitoTracker Deep Red (Figure 2a). The Person's colocalization coefficient is 0.88; (b) The scatter plots of correction coefficient between TTPy-H_2O_2 and commercial lipid droplet Tracker Deep Red which treated with H_2O_2 (50 μM) for 1 hour (Figure 2b). The Person's colocalization coefficient is 0.85.

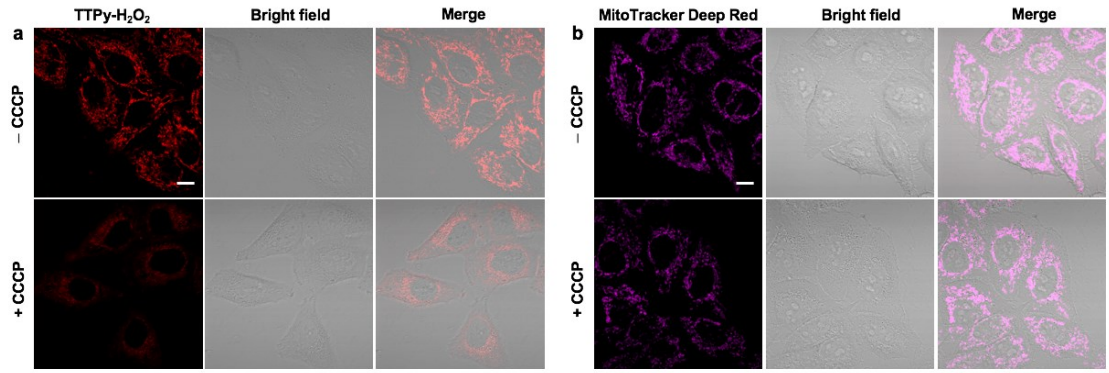


Figure S9. The CLMS fluorescence images of CCCP-pretreated and untreated HeLa cells stained with TTPy-H_2O_2 (1 μM) and commercial MitoTracker Deep Red (500 nM). The emission filter of MitoTracker Deep Red: λ_{ex} = 640 nm, λ_{em} = 650-700 nm; TTPy-H_2O_2: λ_{ex} = 488 nm, λ_{em} = 650-700 nm. Scale bar: 10 μm.

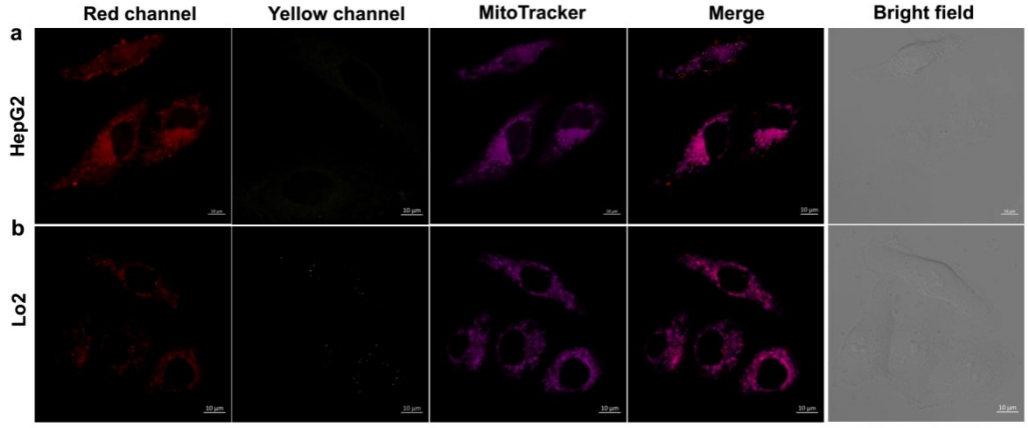


Figure S10. The organelle colocalization of TTPy-H_2O_2 (5 μM) with commercial MitoTracker in HepG2 cells (a) and LO2 cells (b). Red channel: λ_{ex} = 488 nm, λ_{em} = 650-730 nm; Yellow channel: λ_{ex} = 405 nm, λ_{em} = 410-550 nm; Commercial tracker channel: λ_{ex} = 633 nm, λ_{em} = 650-700 nm. Scale bar: 10 μm.

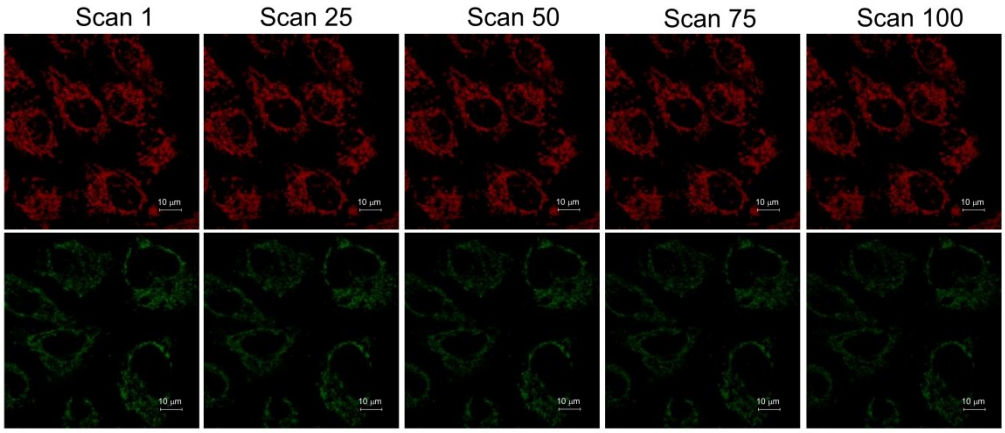


Figure S11. Photostability of TTPy-H_2O_2 and commercial MitoTracker Green. λ_{ex} = 488 nm, 0.6% laser power. Scale bar: 10 μm.

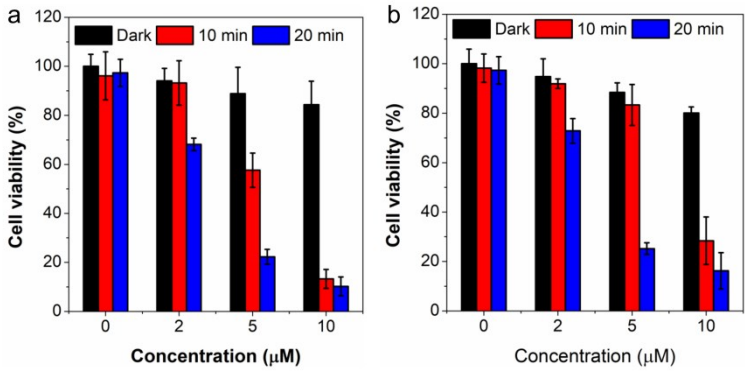


Figure S12. Viability of cells stained with different concentrations of TTPy-H_2O_2 in the absence or presence of white light irradiation. (a) HepG2 cells; (b) LO2 cells.

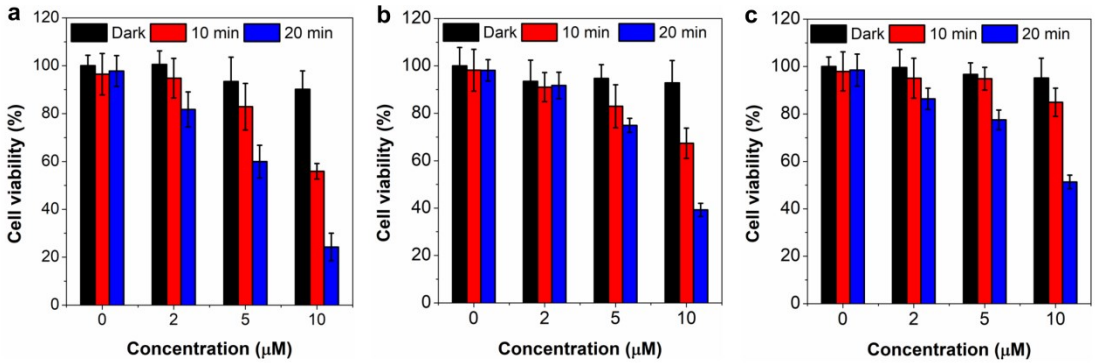


Figure S13. Viability of cells stained with different concentrations of TTPy in the absence or presence of white light irradiation. (a) HeLa cells; (b) HepG2 cells; (c) LO2 cells.

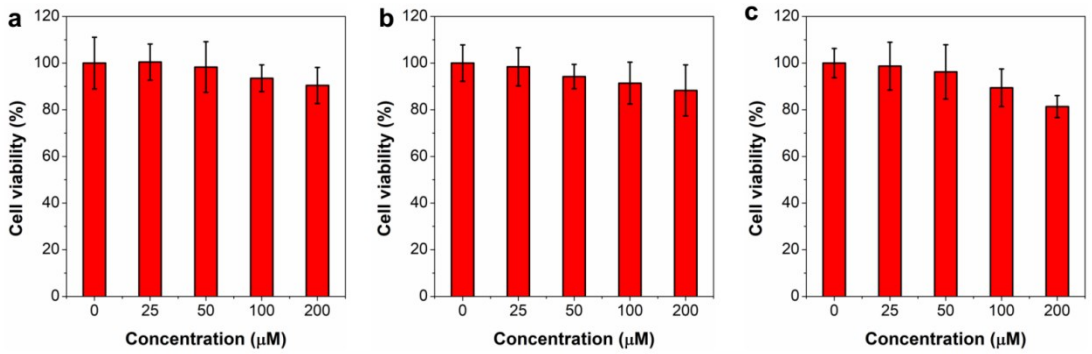


Figure S14. Viability of cells stained with different concentrations of H_2O_2 in the different cell lines. (a) HeLa cells; (b) HepG2 cells; (c) Lo2 cells.

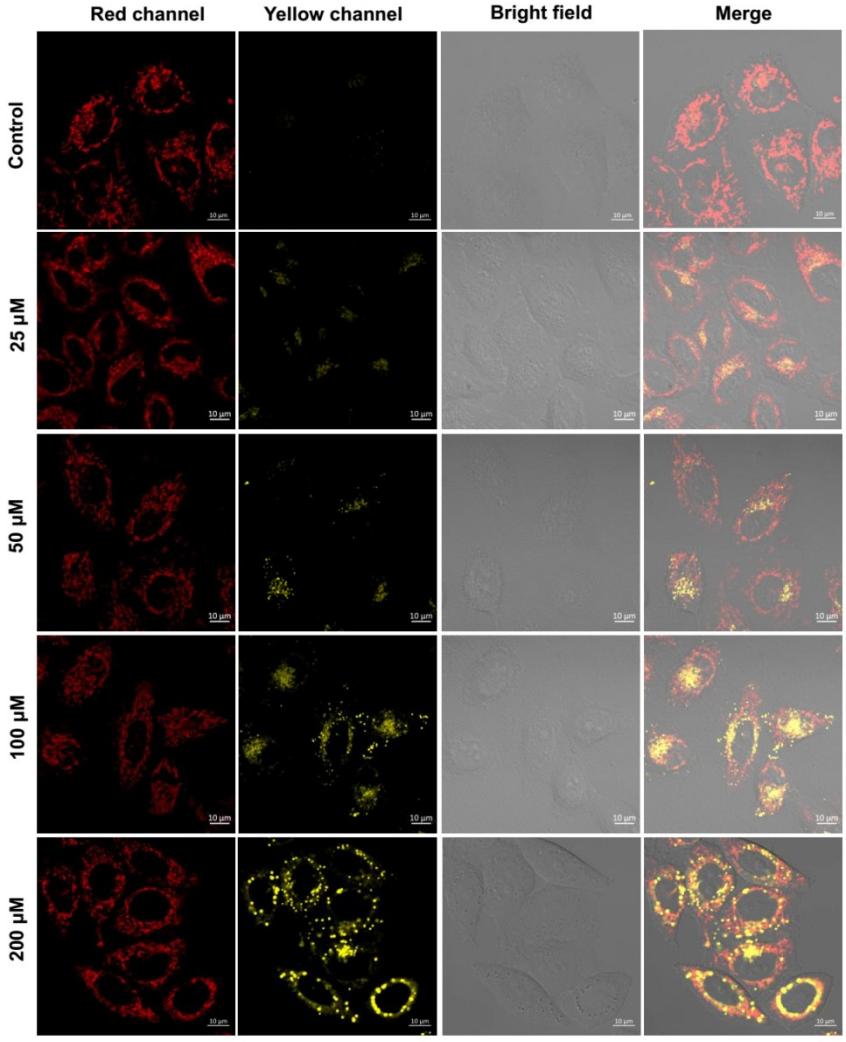


Figure S15. Confocal fluorescence imaging of TTPy-H_2O_2 (5 μM) incubated with H_2O_2 at different concentrations for 1 h in HeLa cells (control, 25 μM, 50 μM, 100 μM and 200 μM). Red channel: λ_{ex} = 488 nm, λ_{em} = 650-700 nm; Yellow channel: λ_{ex} = 405 nm, λ_{em} = 410-550 nm; Scale bar: 10 μm.

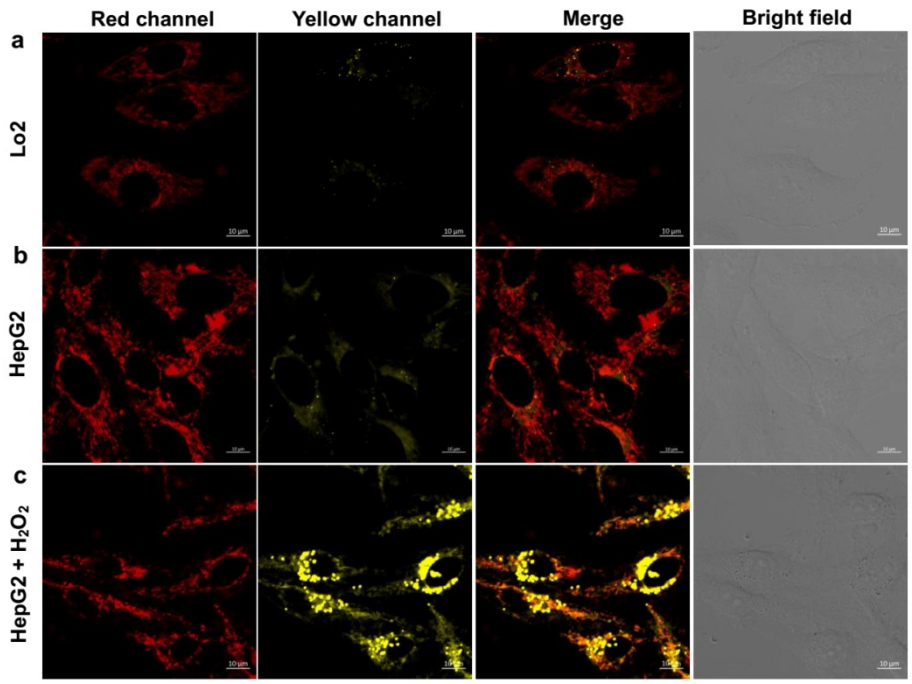


Figure S16. Confocal fluorescence imaging of TTPy-H_2O_2 (5 μM) in different cell lines. (a) Staining of TTPy-H_2O_2 for 30 min in LO2 cells; (b) Staining of TTPy-H_2O_2 for 30 min in HepG2 cells which were treated with H_2O_2 (50 μM) for 1h (c). Red channel: λ_{ex} = 488 nm, λ_{em} = 650-700 nm; Yellow channel: λ_{ex} = 405 nm, λ_{em} = 410-550 nm; Scale bar: 10 μm.

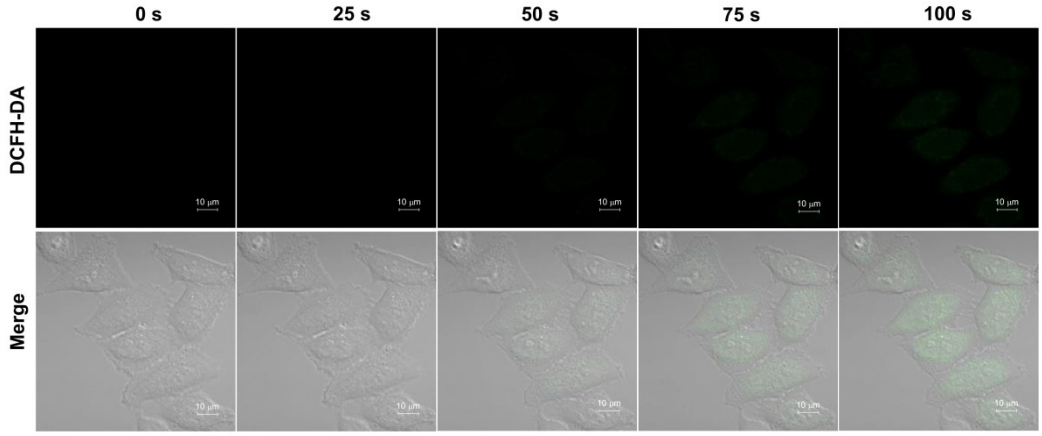


Figure S17. Confocal fluorescence imaging of DCFH-DA (10 μM) upon 488nm laser irradiation. Scale bar: 10 μm.

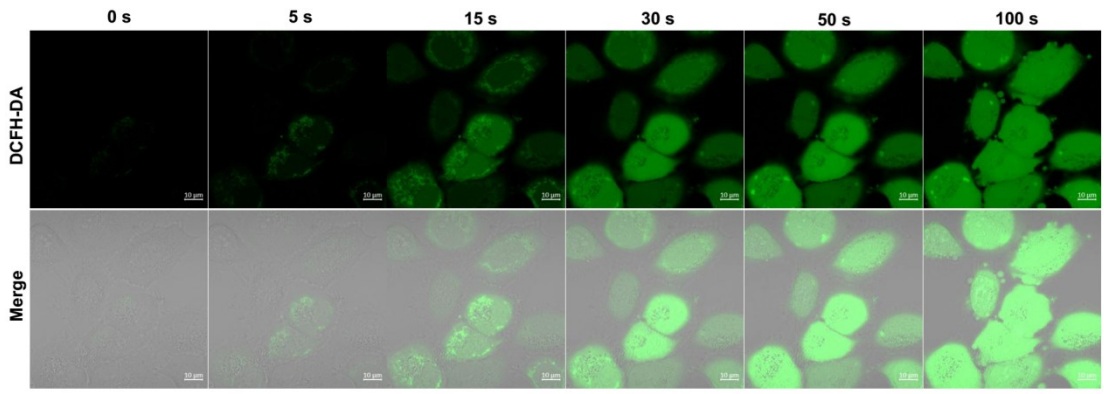


Figure S18. Confocal fluorescence imaging of intracellular ROS level. HeLa cells incubation with TTPy-H_2O_2 for 30 min, followed by the staining with DCFH-DA (10 μM) upon 488nm laser irradiation. Scale bar: 10 μm.

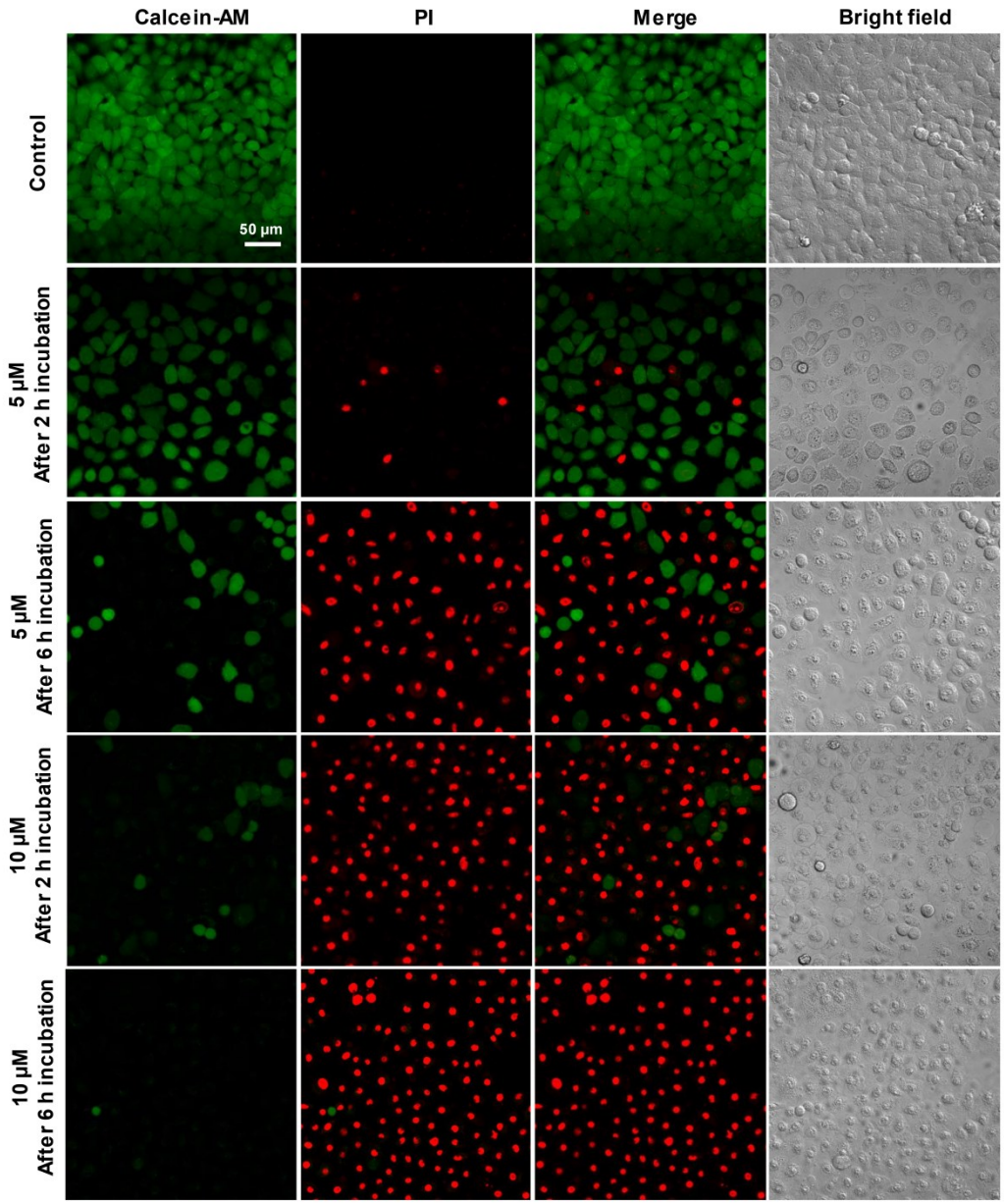


Figure S19. Live/dead cell staining with Calcein-AM/PI in HeLa cells. The cells are treated with different concentrations of TTPy-H_2O_2, then irradiated by white light for 10 min. After incubation for different times (2h and 6 h), the CLMS imaging was carried out. Scale bar: 50 μm.