Electronic Supplementary Information

For

A Sensitive Fluorescent Sensor for the Detection of Endogenous Hydroxyl Radical in Living Cells, Bacteria and Direct Imaging of its Ecotoxicity in Living Zebra Fish

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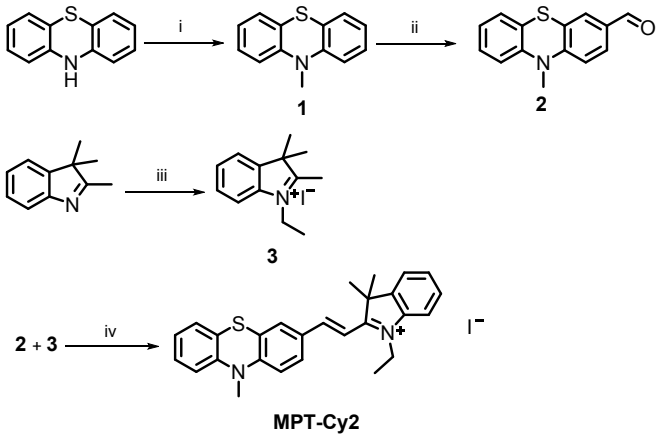
Dye synthesis

The synthetic route for **MPT-Cy2** (3-[2-(1',3',3'-thimethyl-indolium-2'-yl)vinyl]-10- methyl-phenothiazine) is outlined in Scheme S1.



Scheme 1. Synthetic route of dye **MPT-Cy2**. (i) NaH, DMF, CH_3I, room temperature, 2 h, 78%. (ii) $POCl_3$, DMF, 95 °C, 12 h, 65 %; iii) Toluene, CH_3CH_2I, reflux, 10 h, 92%; iv) Piperidine, Ethanol, reflux, 12 h 72%.

Intermediate products **1**, **2** and **3** were synthesized according to literature procedures. [1, 2]

Synthesis of 3-[2-(1',3',3'-thimethyl-indolium-2'-yl) vinyl]-10- methyl-phenothiazine (MPT-Cy2)

1-Ethyl-2, 3, 3- trimethyindolenium quaternized salt **3** (1.58 g, 5.0 mmol), and aldehyde **2** (2.5 g, 4.5 mmol) were added to a 100 mL flask with 50 mL ethanol, followed by catalytic piperidine (1.0 mL). The resulting mixture was stirred for 12 h under reflux. The residue was recrystallized from ethanol to give the desired product in 70% yield. **^{1}H-NMR** (400 MHz, d6-DMSO) δ 8.36 (d, J = 16.0 Hz, 1H), 8.11 (m, 2H), 7.88 (t, J = 7.2 Hz, 2H), 7.59 (m, 3H), 7.26 (m, 2H), 7.09 (m, 3H), 4.69 (d, J = 8.0 Hz, 2H), 3.45 (s, 3H), 1.78 (s, 6H), 1.45 (t, 3H). **^{13}C-NMR** (101 MHz, DMSO) δ181.16, 153.34, 147.52, 144.17, 141.77, 139.93, 134.41, 130.15, 129.89, 129.10, 128.31, 127.43,

124.69, 124.02, 123.43, 118.21, 117.53, 116.35, 114.53, 108.64, 106.11, 73.81, 52.15, 31.17, 26.84, 14.18, 11.42. Mp: 210-212 ^{0}C. **HRMS-ESI**: *m/z* calcd. M^+ for $C_{27}H_{27}N_2S^+$, 411.1889; found, 411.1861.

3-[2-(1',3',3'-thimethyl-indolium-2'-yl)vinyl]-5-oxo-10-methyl-phenothiazine (OMPT-Cy2)

^{1}H NMR (400 MHz, d6-DMSO) δ 9.02 (s, 1H), 8.60 (d, *J* = 1.2 Hz, 2H), 8.09 (s, 1H), 7.84 (t, 2H), 7.79 (m, 4H), 7.64 (m, 2H), 7.44 (d, 1H), 4.72 (m, 2H), 3.92 (s, 3H), 1.84 (s, 6H), 1.49 (t, 3H). **^{13}C-NMR** (101 MHz, DMSO) δ 206.94, 181.16, 153.34, 150.12, 144.17, 144.06, 140.93, 133.52, 129.65, 128.99, 128.65, 128.32, 127.33, 124.29, 123.26, 123.11, 121.43, 116.18, 115.26, 110.13,55.48, 52.35, 37.26, 31.15, 26.25, 19.02, 14.12. **HRMS-ESI**: *m/z* calcd. M^+ for $C_{27}H_{27}N_2OS^+$, 427.1839; found, 427.1856.

Preparation of stock solutions for generation of ROS[3-6]

(a) H_2O_2

H_2O_2 was diluted appropriately in water. The concentration of H_2O_2 was determined based on the molar extinction coefficient at 240 nm (43.6 M^{-1} cm^{-1}). Then, a H_2O_2 stock solution in water was prepared.

(b) •OH

To a solution of H_2O_2 in 100 μM sodium phosphate buffer at pH 7.4 as a cosolvent, the $FeSO_4$ solution (10 μM) was added at room temperature. Then, •OH was generated from Fe^{2+} and H_2O_2 (Fenton reaction).

(c) OCl^-

NaOCl solution was diluted appropriately in 0.1 M NaOH aq. The concentration of OCl^- was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}). Then, a OCl^- stock solution in 0.1 M NaOH aq. was prepared.

(d) Generation of $•O_2^-$

Superoxide ($•O_2^-$) was added as solid KO_2.

(e) $^1O_2$

A solution of $NaMoO_4$ was added to a solution of H_2O_2 in 0.1 M sodium phosphate buffer at pH 7.4 as a cosolvent at room temperature.

Live cell incubation

HeLa cells were cultured in DEME (Invitrogen) supplemented with 10% FCS (Invitrogen). One day before imaging cells were seeded into 24-well flatbottomed plates. The next day, the cells were incubated with 8.0 μM dye for 40 min at 37 °C under 5% CO_2 and washed with phosphate-buffered saline (PBS) three times.

Hela cells pre-treated with PMA (2 ng mL^{-1}) for 40 min and then incubated with **MPT-Cy2** (8 μM) plus MitoTracker Deep Red FM (1 μM) for 30 min at 37 ^{0}C. The cells were washed with PBS buffer and the fluorescence images were acquired

Strains and growth conditions

Four bacteria were grown aerobically in standard luria-bertani medium (LB) or anaerobically in

lactate medium (LM) containing 2.0 g/l lactate, 2.0 g/l yeast extract, 12.8 g/l $Na_2HPO_4 \cdot 7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, and 1.0 g/l NH_4Cl at 33°C (as described previously).[7] The decabromodiphenyl ether (5 M) was performed to bacteria at 37 °C for 2 h for each assay in triplicate.

TiO_2NP Exposure

Commercially available TiO_2 particles were obtained from Aladdin, China. Stock solutions (0.5 mg mL^{-1}) of the NPs were prepared by sonication in water for 30 min. Zebra fish were continuously exposed to TiO_2NP suspensions and illumination (14 h per day) for 5 days spanning initial embryogenesis through larval development. The photocatalytic experiments were performed using a solar simulator apparatus (Model No. 92190) with a 1600-W xenon lamp. All the data are expressed as mean ± standard deviation (SD) of three independent experiments.

Zebra fish embryos and zebra fish husbandry and exposure

Zebra fish embryos were collected immediately after fertilization and placed into 48-well plates and treated with concentrations of 0.5 mg mL^{-1} TiO_2NP (5 nm and 40nm) suspensions and illumination. Zebra fish were maintained at 25 °C under a constant temperature of 20 ± 1 °C with a 16:8 h light: dark photoperiod.[8-7] The zebrafish embryos were exposed to 0.5 mg/mL concentrations of the 5 and 40 nm diameter nanoparticles in beakers with 20 fish per beaker over a period of 5 days and their survival rate was monitored. The exposure, collection, and analysis experiment was replicated 3 times.

Zebrafish and zebrafish embryos were incubated with the **MPT-Cy2** probe (5 μM) after culture in aerated tap water containing TiO_2NPs of 5 or 40 nm diameter for 30 min. Zebra fish embryos and zebra fish were washed with PBS buffer and the fluorescence images were acquired

Fluorescence imaging

Fluorescence imaging in cells, tumor slices and zebrafish were obtained with spectral confocal multiphoton microscopes (Zeiss LSM 700 confocal laser scanning microscope).

Photostability

MPT-Cy2, and **OMPT-Cy2** were dissolved in DMSO-water (5:5 v) at a concentration of 10.0 μM, respectively. The solutions were irradiated under a 500 W iodine tungsten lamp for 2 h at a distance of 250 mm away. An aqueous solution of sodium nitrite (50.0 g/L) was placed between the samples and the lamp as a heat filter. The photostabilities were expressed in the terms of remaining absorption (%) calculated from the changes of absorbance at the absorption maximum before and after irradiation by iodine tungsten lamp.

Table S1 Spectral properties of **MPT-Cy2** and **OMPT-Cy2** in different solvents

Dyes	solvents	λ_{abs} (nm)	λ_{em} (nm)	$\varepsilon\times10^4$ (nm)	Φ (%)
MPT-Cy2	DMSO	392/ 552	487	2.56	0.0006
	H_2O	385/ 545	476	2.55	0.0005
	acetone	383/ 545	475	2.46	0.0007
	methanol	391/ 551	477	3.21	0.0005
	DCM	406/ 580	491	3.10	0.0004
OMPT-Cy2	DMSO	346/ 492	483/ 628	5.67	0.051
	H_2O	347/ 455	477/ 593	5.12	0.013
	acetone	347/ 479	476/ 604	5.98	0.037
	methanol	342/ 475	475/ 620	5.10	0.039
	DCM	351/ 479	488/ 623	5.24	0.035

Table S2 Physicochemical Characterization of TiO_2 NPs

Parameters	TiO_2 NPs-rutile	TiO_2 NPs- anatase
particle size (nm)	40 nm	5 nm
purity	99.8%	99.8%
surface area (m^2/g)	30	147
zeta potential (mV at pH 7.5)	-32.4	-41.6

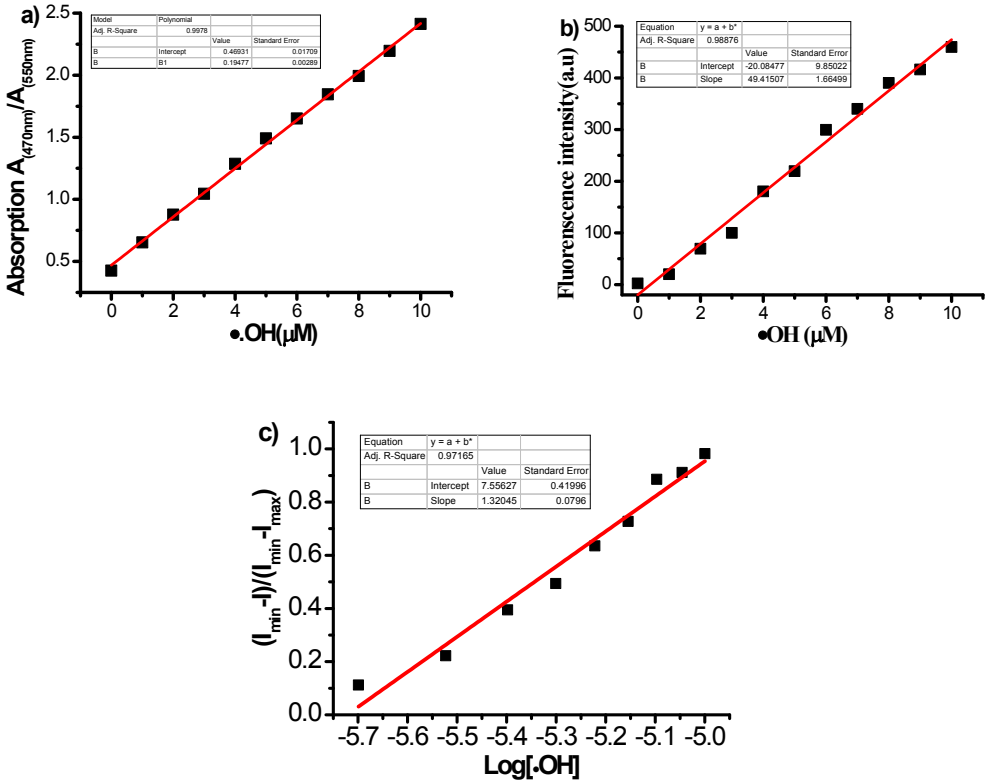


Fig. S1. a) Plot of the absorption intensity ratios at 470 nm and 550nm of **MPT-Cy2** (10 μM) upon addition of •OH (0–10 μM); b) the fluorescence spectra of **MPT-Cy2** (10 μM) upon the addition of •OH (0-10 μM); c) the response of the fluorescence signal to changing •OH concentrations. A linear regression curve was then fitted to these fluorescence intensity data, and the point at which this line crossed the ordinate axis was considered as the

detection limit (1.16×10^{-6} M). Y = 7.55627 + 1.32045 * X, R = 0.97165. The detection limit was calculated based on the method reported in the previous literature. [10] Conditions: each spectrum was recorded after 3 min in water.

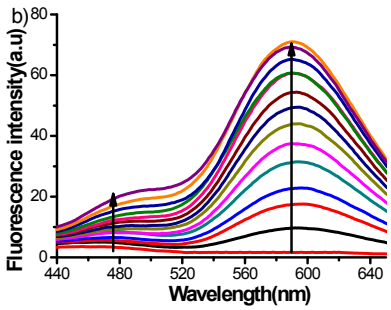


Fig. S2. Changes in the fluorescence emission spectrum of **MPT-Cy2** (10μM) with increases of the •OH concentration (0–30μM) at 595nm in water, λ_{ex} = 350 nm.

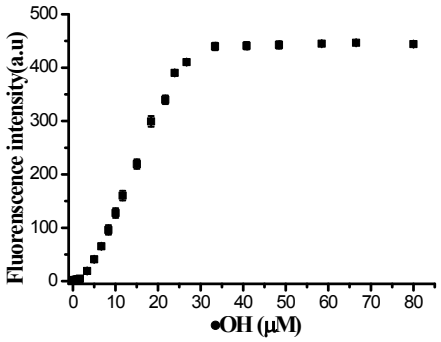


Fig. S3. Changes in the fluorescence spectrum of **MPT-Cy2** (30 μM) upon addition of •OH (0-80 μM). Each spectrum was recorded after 3 min in water.

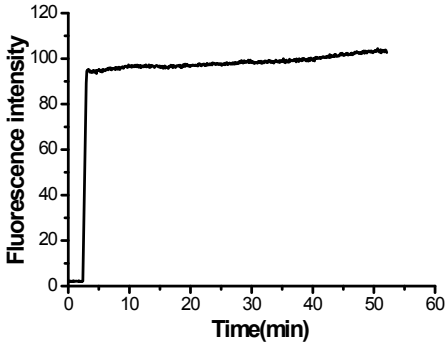


Fig. S4.Time dependent fluorescence intensity changes of **MPT-Cy2** (10 μM) at 590 nm in the presence of 50 equiv •OH in water (60min).

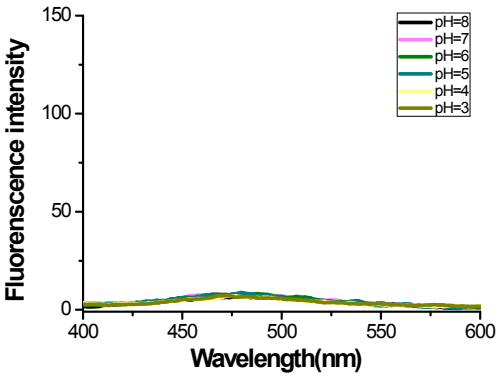


Fig. S5.Influence of pH on fluorescence for MPT-Cy2 (20 μM) from pH=3 to pH= 8.

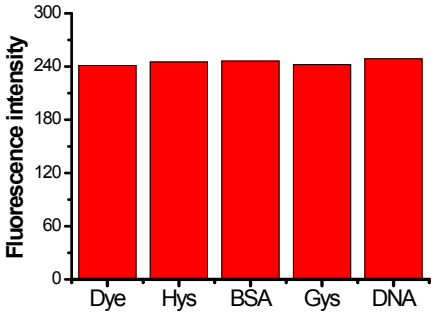


Fig. S6. The fluorescence spectra titration of **OMPT-Cy2** (2.0 μM) in PBS buffer (20.0 mM pH = 7.4) by Hys(25.0 mM); Cys (200 μM); BSA (200 μM); DNA (200 μM) during 120 min.

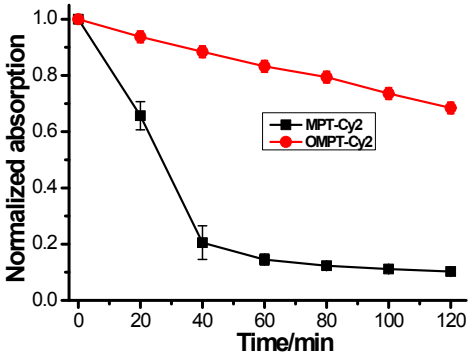


Fig. S7. Photo-fading of dyes (**MPT-Cy2** and **OMPT-Cy2)** in solvent mixture with the ratio of DMSO-water 5: 5 v/v with radiation by a 500 Wiodine-tungsten lamp. **MPT-Cy2**: λ_{abs} = 520 nm, **OMPT-Cy2**: λ_{abs} = 460 nm.

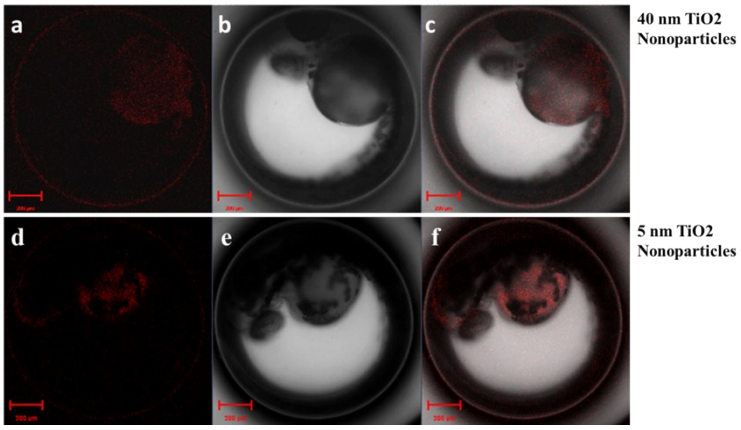


Fig. S8. Confocal fluorescence microscopic images of zebra fish embryos were incubated with the **MPT-Cy2** probe (5μM) after culture in aerated tap water containing two different sized nanoparticles (5nm and 40nm) for 40 min. (a-b) Fluorescence image of the embryo with 5 μM **MPT-Cy2** after culture in aerated tap water containing 40 nm sized nanoparticles; (c-d) Fluorescence image of the embryo with 5 μM **MPT-Cy2** after culture in aerated tap water containing 5nm sized nanoparticles.

In vitro testing: the fluorescent substance, **OMPT-Cy2**, was generated when •OH was added slowly to the **MPT-Cy2** solution. To confirm the formation of this substance, the partial MS and HPLC spectra of the reaction of **PTE-Cy2** with •OH are shown in Fig S8 and S9.

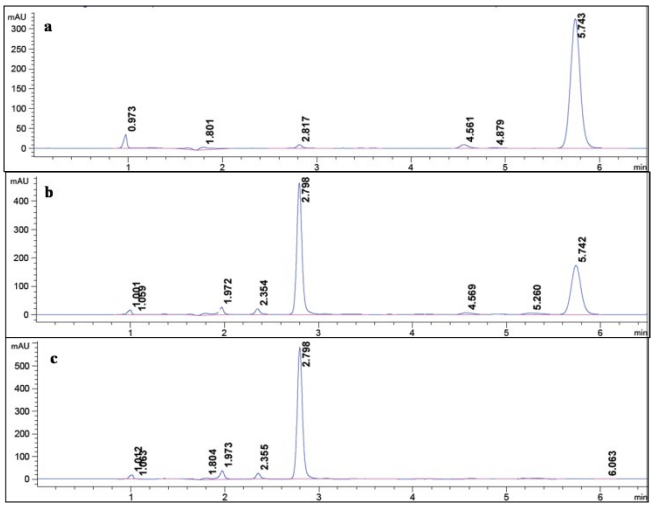


Fig. S9. HPLC chromatograms of probe **MPT-Cy2** after reaction with •OH, a) just **MPT-Cy2** (10 μM) exist in the sulution, b) **MPT-Cy2** (10 μM) upon addition of •OH (6 μM) for 10 min, c) **MPT-Cy2** (10 μM) upon addition of •OH (15 μM) for 10 min.

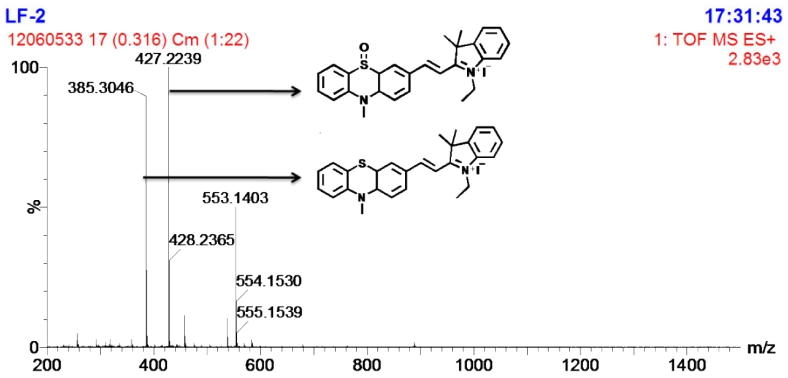


Fig. S10. MS monitoring oxidition of the •OH with **MPT-Cy2** process.

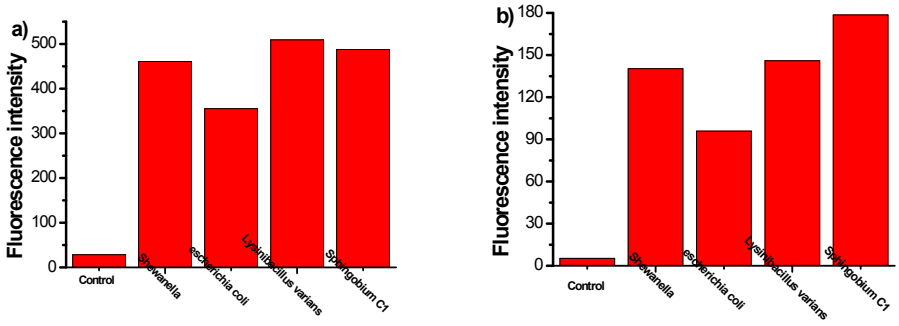


Fig. S11. Changes in the fluorescence intensity of DCFH-DA (a) and **MPT-Cy2** (b) (10μM) with four species of bacteria in recovered supernatants.

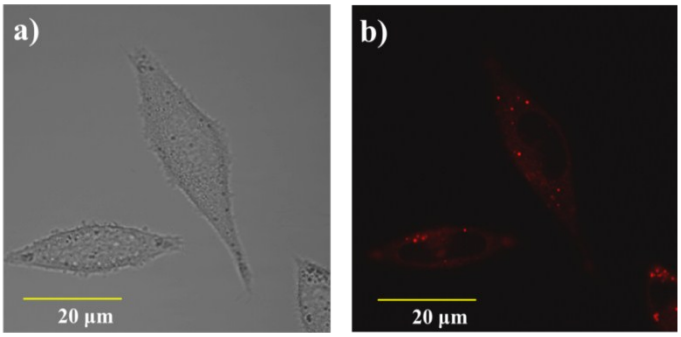


Fig. S12. The sensor **MPT-Cy2** (20 mM) was loaded into HeLa cells for 2.5 hours without PMA pre-treated.

We monitored the changes of the fluorescence in the cells for 4 hours without PMA pre-treated. There was almost non-fluorescent when the sensor **MPT-Cy2** (10 μM) was loaded into HeLa cells for 40 min. As shown in Fig.S12, there was only a little fluorescence signals came out gradually 2.5 hours later.

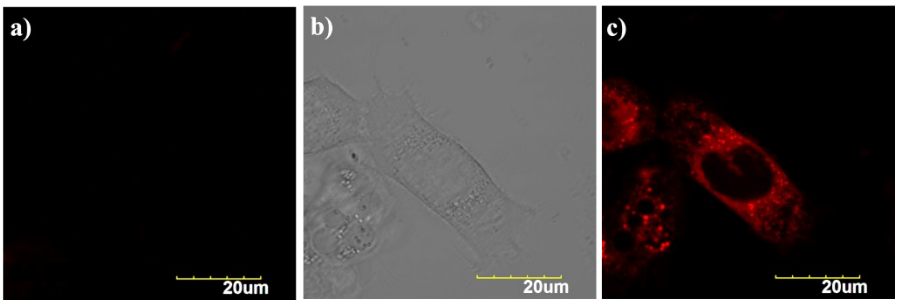


Fig. S13. HeLa cells pre-treated with TEMPOL (5 mM) for 1 h and then incubated with PMA (2 ng mL^{-1}) for another 1 h and finally with **MPT-Cy2** (8 μM) for 40 min.

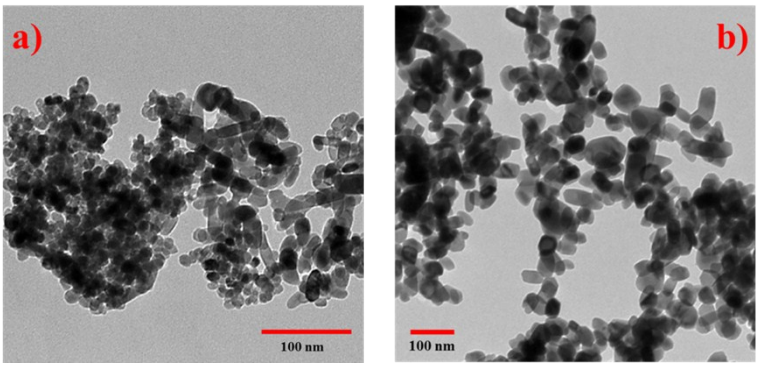
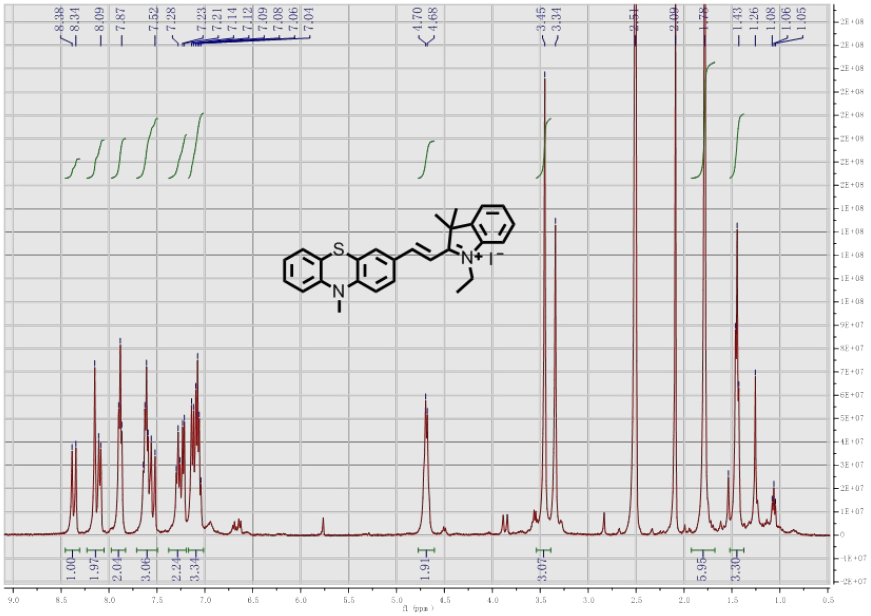


Fig. S14. TEM images of 5 nm TiO_2NPs (a) and 40 nmTiO_2NPs (b).



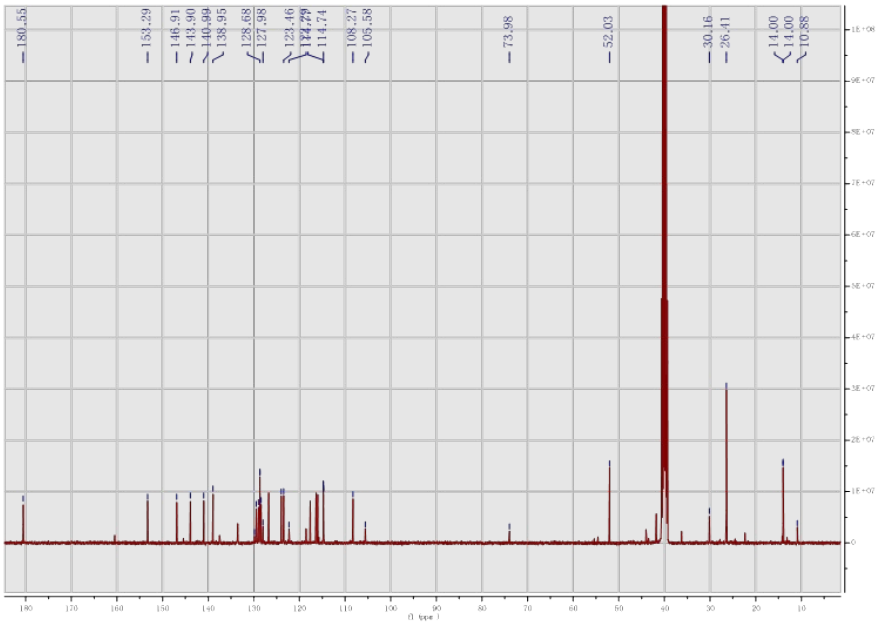
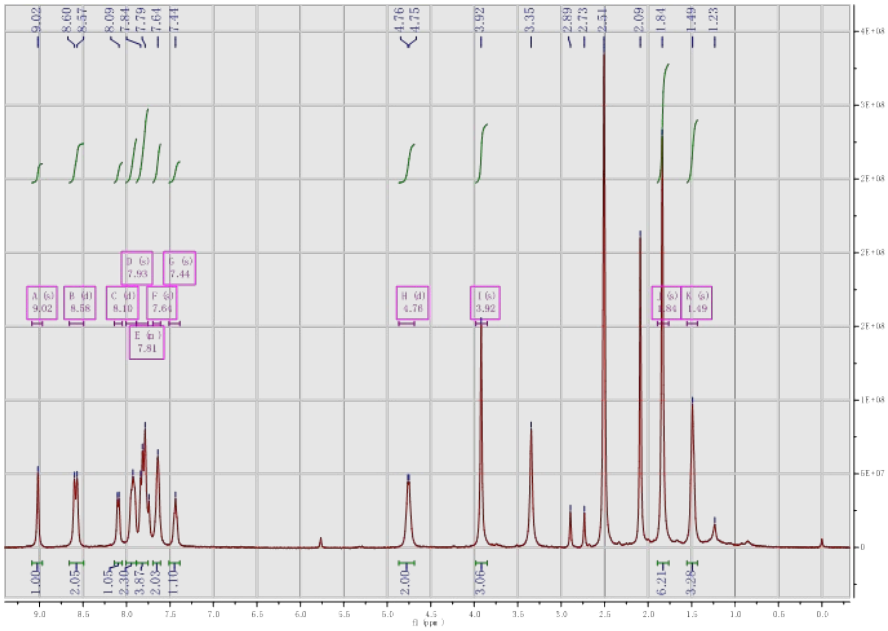


Fig. S15. ^{1}H and ^{13}C NMR spectra of **MPT-Cy2** in DMSO.



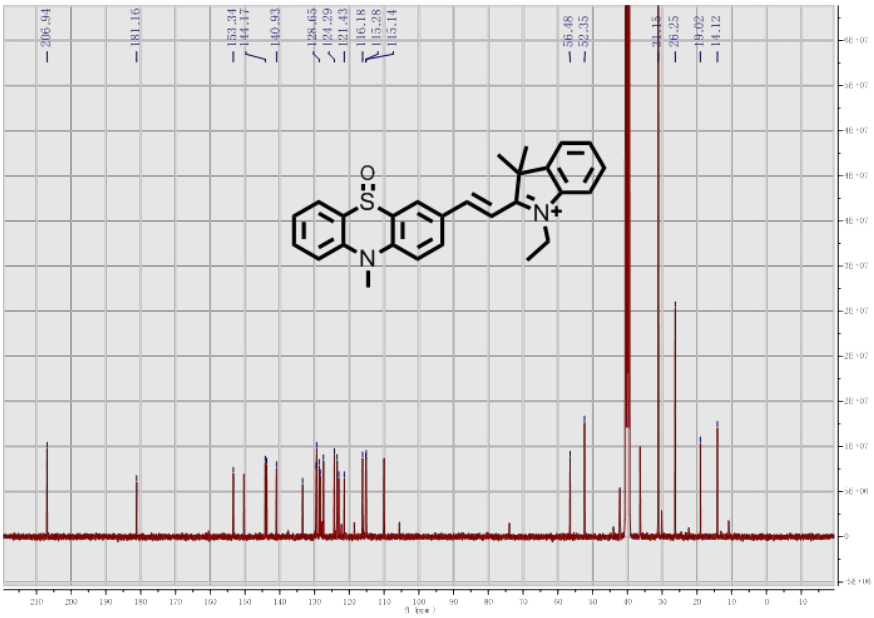


Fig.S16. ^{1}H and ^{13}C NMR spectra of O**MPT-Cy2** in DMSO.

References

1) H. Spreitzer, J. Daub, *Chem. Eur*, **1996**, 9, 1150-1158.
2) B.J. Coe, J.A. Harris, I. Asselberghs, K. Clays, G. Olbrechts, A. Persoons, J.T. Hupp, R.C. Johnson, S.J. Coles, M.B. Hursthouse, K. Nakatani, *Adv. Funct. Mater*. **2002**, 12, 110-116.
3) D. Oushiki, H. Kojima, T. Terai, M. Arita, K. Hanaoka, Y. Urano, T. Nagano, *J Am Chem Soc* **2010**, *132*, 2795-2801.
4) L. Yuan, W. Lin, J. Song, *Chem. Commun. (Camb)* **2010**, *46*, 7930-7932.
5) S. M. Chen, J. X. Lu, C. D. Sun, H. M. Ma, *Analyst.* **2010**, *135*, 577-582.
6) K. Kundu, S. F. Knight, N. Willett, S. Lee, W. R. Taylor, N. Murthy, *Angew. Chem. Int. Edit.* **2009**, *48*, 299-303.
7) X. J. Chen, M. Y. Xu, J. B. Wei, G. P. Sun, *Appl Microbiol Biotechnol*. 2010, 86, 743–751.
8) Fish, Early-life Stage Toxicity Test, OECD guideline for testing of chemicals, Adopted by the Council on 17th July 1992.
9) Guideline 202 on "Daphnia sp., Acute Immobilisation Test and Reproduction Test", OECD/OCDE, 13 April 1992.
10) M. Shortreed, R. Kopelman, M. Kuhn, and B. Hoyland. *Anal. Chem.* 1996, 68, 1414-1418.