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Porphyrin photosensitized metal–organic framework for cancer cell apoptosis and caspase responsive theranostics

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General

Materials and Reagents. Zinc acetate dihydrate ($\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$), 1,3,5-benzene-tricarboxylic acid (BTC), and dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). $\alpha,\beta,\gamma,\delta$ -Tetrakis(1-methylpyridinium-4-yl)porphyrin *p*-toluenesulfonate (TMPyP) was obtained from Tokyo Chemical Industry Co., Ltd. Trypsin, lysozyme, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), pepsin, 3-(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), 1,3-diphenylisobenzofuran (DPBF), methylene blue (MB), (3-glycidyloxypropyl)trimethoxysilane (GPTS), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and vitamin C were purchased from Sigma-Aldrich (USA). H_2N -poly(ethylene glycol) (PEG)-folate (M.W. 3400) was obtained from Nanocs (USA). Human caspase-3 recombinant protein and TUNEL apoptotic kit were purchased from millipore (Billerica, MA, USA). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (USA). Caspase-3 inhibitor (Z-DEVD-FMK) was purchased from Santa Cruz (USA). LysoTracker Deep Red, Hoechst 33342 and MitoTracker Deep Red FM were obtained from Invitrogen (Carlsbad, CA, USA). Phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4 and 1.41 mM KH_2PO_4 . All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water ($\geq 18 \text{ M}\Omega$, Milli-Q, Millipore).

Caspase-3 substrate peptide SGDEVDK (Cy3-labelled) was synthesized and purified by Sangon Biological Engineering Technology & Co., Ltd. (Shanghai, China).

Apparatus. Powder X-ray diffraction (PXRD) data was recorded using a X'TRA diffractometer (ARL, Switzerland). Dynamic light scattering (DLS) was observed on a 90 Plus/BI-MAS equipment (Brook Haven, USA). The transmission electron microscopic (TEM) images were obtained on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). The scanning electron microscopic (SEM) images were obtained from an S-3400N scanning electron microscope (Hitachi, Japan). The nitrogen isotherm was obtained on Micromeritics ASAP2020 (USA) at 77 K. Absorption spectra were recorded on an UV-3600 UV-Vis-NIR spectrophotometer (Shimadzu, Japan). Infrared (IR)

spectra were recorded on a Nicolet NEXUS870 Fourier transform infrared (FT-IR) spectrometer (Madison, WI). Zeta potential analysis was performed on a Zetasizer instrument (Nano-Z, Malvern, UK). Fluorescence spectra were measured on an F-7000 spectrometer (HITACHI, Japan). Confocal fluorescence imaging of cells was performed on a TCS SP5 confocal laser scanning microscope (Leica, Germany). Fluorescence/phosphorescence lifetime was determined on an FLS920 spectrometer (Edinburgh, UK) equipped with laser and microsecond pulse as excitation light source, respectively. Flow cytometric analysis was performed on a Coulter FC-500 flow cytometer (Beckman-Coulter). Phototoxicity assay of the nanoprobe was performed using a 660 nm laser (Changchun Laser Optoelectronics Technology Co., Ltd., China).

Synthesis of PS@MOF and PS@MOF Probe. PS@MOF was synthesized with a one-pot incorporation of porphyrin into the cage of a variant MOF of HKUST-1.^{S1} Briefly, Zn(OAc)₂·2H₂O (6.6 mg), BTC (10.5 mg), and TMPyP (2.0 mg) were added to 2.4 mL mixture of DMF (2.0 mL) and H₂O (0.4 mL) in a 25-mL Teflon liner, which was heated to 85 °C for 12 h under vacuum. The mixture was cooled and filtered, and the resulting deep green solid was washed with methanol and dried in a vacuum to obtain PS@MOF.

To prepare PS@MOF probe, the PS@MOF was firstly functionalized with GPTS. Briefly, 1 mg PS@MOF was dispersed in 4 mL ethanol by sonication, and the dispersion was mixed with 4 mL of 2% (v/v) ethanolic solution of GPTS and heated to 60 °C for 12 h. After washed with ethanol several times, 1.0 mg of the epoxy-terminated PS@MOF-GPTS was dispersed in 1.0 mL water and added in the mixture of Cy3-labelled caspase-3 substrate peptide solution (0.5 mg mL⁻¹, 100 μL) and H₂N-PEG-FA solution (1.0 mg mL⁻¹, 50 μL), which was then incubated at room temperature in dark overnight to form the FA and peptide functionalized PS@MOF nanocomposite, named as PS@MOF probe. The unbound excess peptide and H₂N-PEG-FA were removed by filtration through a 10 kDa MWCO centrifugal filter (Millipore) at 10000 rpm for 10 min until the filtrate became free of rose-colored peptide. The obtained probe was resuspended in PBS and stored at 4 °C. Similar procedure

was used to prepare the composite of PS@MOF and H₂N-PEG-FA as the peptide-free probe of PS@MOF-FA.

Loading Amounts of TMPyP and Peptide on the Probe. The amount of TMPyP loaded in MOF cage was determined by UV-Vis spectroscopy. The absorbance of all the supernatant from the preparation of PS@MOF was converted to the concentration of corresponding TMPyP with a linear calibration curve. The calibration curve was obtained with known concentrations of TMPyP (**Fig. S4**). Finally, the loading percentage of TMPyP in MOF cage was obtained by dividing the subtracted concentration by the original concentration. The amount of the peptide loaded on the probe surface was obtained by fluorescence measurements of Cy3 labeled to the peptide with a similar procedure (**Fig. S5**).

Formation and Evaluation of Singlet Oxygen. Upon the suitable laser irradiation and the absorption of photons, the energy-enriched porphyrin derivatives render back the excess energy and return to its ground state. Because of this process, the excess energy can be released to surrounding substrates. Importantly, a fraction of the excited singlet state molecules is transformed via intersystem crossing into the relatively long-lived excited triplet state, which can transfer its energy to ground-state molecular oxygen to form highly reactive singlet oxygen.^{S2}

DPBF was used as a singlet oxygen (¹O₂) indicator to evaluate the ¹O₂ generation. The ¹O₂ quantum yield (Φ_{Δ}) was detected through monitoring the oxidation of DPBF with a UV-Vis spectrophotometer. Methylene blue (MB) was used as the typical standard for the determination of the singlet oxygen quantum yields.^{S3} Moreover, MB has strong absorption at around 664 nm, which is consistent with the radiation wavelength of the 660-nm laser used in all the experiments. Briefly, an oxygen-saturated solution of TMPyP, MOF, PS@MOF, PS@MOF probe and MB individually containing 60 μ M DPBF was prepared in the dark and irradiated with a 660 nm laser at a power of 100 mW cm⁻² in an interval of 30 s. The quantum yields were calculated with eq. S1 using MB (Φ_{Δ} = 0.52) as the standard.^{S4}

$$\Phi_{\Delta(x)} = \Phi_{\Delta(\text{std})} \times \frac{S_x}{S_{\text{std}}} \times \frac{F_{\text{std}}}{F_x} \quad \text{eq. S1}$$

where subscripts x and std designate the sample and MB, respectively, S stands for the slope of plot of the absorbance of DPBF (at 418 nm) vs. irradiation time, and F stands for the absorption correction factor, which is given by $F = 1 - 10^{-\text{OD}}$ (OD represents the optical density of sample and MB at 660 nm).

Fluorescence and Phosphorescence Quantum Yields. For determination of fluorescence quantum yield (Φ_F) of TMPyP and PS@MOF, Rhodamine 101 in ethanol ($\Phi_{F(s)} = 1.00$) was used as a standard. The $\Phi_{F(x)}$ value was calculated according to eq. S2.^{S5}

$$\Phi_{F(x)} = \Phi_{F(s)} \times \frac{A_s}{A_x} \times \frac{F_x}{F_s} \times \left(\frac{n_x}{n_s}\right)^2 \quad \text{eq. S2}$$

where A is the absorbance, F is the area under the fluorescence emission curve, n is the refractive index of the solvent used in the measurement, and the subscripts s and x represent the standard and sample, respectively.

For determination of phosphorescence quantum yield (Φ_P) of PS@MOF, the Φ_P value was calculated according to eq. S3.

$$\frac{\Phi_{P(\text{PS@MOF})}}{\Phi_{P(\text{PS})}} = \frac{A_{\text{PS}}}{A_{\text{PS@MOF}}} \times \frac{P_{\text{PS@MOF}}}{P_{\text{PS}}} \quad \text{eq. S3}$$

where $\Phi_{P(\text{PS})}$ is the Φ_P of TMPyP, which can be acquired from relative study,^{S6} A is the absorbance, and F is the area under the phosphorescence emission curve.

In Vitro Detection of Caspase-3 Activity. PS@MOF probe (2.0 μM PS equiv.) was incubated with different amounts of recombinant caspase-3 protein in caspase assay buffer (100 μL) containing 40 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS and 10 mM DTT at 37 °C for the designed time, and the change of fluorescence intensity was measured. The fluorescence spectra were collected from 550 to 700 nm under an excitation of 511 nm.

Cell Culture. Human cervical carcinoma HeLa cell lines and immortalized human epidermal HaCaT cell lines were obtained from KeyGEN Biotech (Nanjing, China). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 $\mu\text{g mL}^{-1}$ streptomycin and 100 U mL^{-1} penicillin, at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. Cell numbers were determined with a Petroff-Hausser cell counter (USA).

Cell Extract for *in Vitro* Caspase Assay. After HeLa cells were incubated with PS@MOF-FA (2.0 μM PS equiv.) for 8 h and then irradiated with the laser for 15 min, 2.0×10^6 cells were dispensed in a 1.5-mL Eppendorf tube, washed twice with ice-cold PBS, and resuspended in 200 μL of ice-cold caspase lysis buffer containing pH 7.4 HEPES-NaOH, 0.1% sucrose, 1% CHAPS, 2 mM EDTA and 10 mM DTT. The mixture was incubated for 30 min on ice and centrifuged at 10000 rpm for 1 min at 4 °C to obtain the supernatant as cell extract for caspase analysis. As control, HeLa cells were pretreated with caspase-3 inhibitor (10 μM) before treatment with PS@MOF-FA and light irradiation.

Cell Colocalization Assay. For colocalization assay of the PS@MOF probe-transfected HeLa cells, HeLa cells were seeded into 35-mm confocal dishes (1×10^4 per dish) and incubated for 24 h at 37 °C. After incubation with PS@MOF probe (2.0 μM PS equiv.) for 8 h and washed with PBS, the cells were further incubated with 5.0 $\mu\text{g mL}^{-1}$ Hoechst 33342 (a nucleus dye), 75 nM LysoTracker Deep Red (a lysosome dye) or 500 nM MitoTracker Deep Red FM (a mitochondrial dye) for 15 min. Hoechst 33342 was excited with a 405-nm laser diode and the emission was collected from 420 to 480 nm. Both LysoTracker Deep Red and MitoTracker Deep Red FM were excited with a HeNe 633-nm laser and the emissions were collected from 660 to 720 nm.

Confocal Fluorescence Imaging and Flow Cytometric Assay. HeLa or HaCaT cells were incubated with PS@MOF probe (1.0 μM PS equiv.) at 37 °C for 8 h, and rinsed three times with PBS. Then the cells were irradiated with a 660 nm laser for 15 min to perform confocal fluorescence imaging at different postirradiation times. The fluorescence of cells was collected from 555 to 610 nm on the microscope with the excitation wavelength of 514 nm for Cy3. All images were digitized

and analyzed with Leica Application Suite Advanced Fluorescence (LAS-AF) software package. Flow cytometry was used to evaluate the specificity of the probe to cancer cells. After treatment with the probe and laser irradiation as described above, HeLa or HaCaT cells were trypsinized, harvested, rinsed with PBS and resuspended, and subjected to flow cytometric assay over FL2 channel.

Imaging of Cellular ROS. After HeLa cells were incubated with PS@MOF probe for 8 h, they were further incubated with 10 μM DCFH-DA as the ROS probe for 30 min and irradiated with a 660 nm laser at a power of 100 mW cm^{-2} for 15 min to perform the confocal fluorescence imaging, which was excited at the wavelength of 448 nm and collected from 505 to 550 nm. The probe-induced generation of ROS was also examined in the presence of vitamin C (3 mM) as the ROS scavenger.

Dark Toxicity and Phototoxicity Assay. MTT assay was carried out to investigate the dark toxicity and phototoxicity of TMPyP, PS@MOF probe and Ce6. HeLa cells (5×10^5 per well) were seeded into two 96-well plates in 200 μL fresh medium and incubated at 37 $^\circ\text{C}$ for 24 h. After rinsing with PBS, HeLa cells were incubated with 200 μL culture media containing serial concentrations of the photodynamic therapy (PDT) agents for 8 h. Meanwhile, the cells were incubated with 200 μL culture medium without PDT agents as the control. One plate was kept in dark to investigate the dark toxicity, and another plate was irradiated using a 660 nm laser at a power of 100 mW cm^{-2} for 15 min. Afterward, the cells were grown for another 6 h. Then, 50 μL of 5 mg mL^{-1} MTT solution was added to each well. After 4-h incubation, the medium containing unreacted MTT was removed carefully, and 150 μL DMSO was added to each well to dissolve the crystals formed by the living cells. After the cell plate was vibrated for 15 min, the absorbance at a wavelength of 560 nm was measured with a microplate reader. The relative cell viability (%) was calculated by $(A_{\text{test}}/A_{\text{control}}) \times 100$.

Cell Apoptosis Monitoring. HeLa cells (1.0×10^4) were seeded into 35-mm confocal dishes and incubated for 24 h at 37 $^\circ\text{C}$. The medium was then replaced with fresh culture medium containing PS@MOF probe (1.0 μM PS equiv.) and incubated at 37 $^\circ\text{C}$ for 8 h. After the irradiation with a 660-

nm laser at a power of 100 mW cm^{-2} for 15 min and incubated for another 2 h, the cells were treated with TUNEL kit according to the manufacturer's instruction. The apoptotic fluorescence and probe fluorescence of HeLa cells was visualized with a confocal laser scanning microscope.

For flow cytometric evaluation, 5.0×10^5 HeLa cells were seeded in a 6-well plate for 12 h containing 2 mL fresh DMEM in each well. These cells were then incubated with PS@MOF probe ($0.5 \text{ }\mu\text{M}$ PS equiv.) or Ce6 ($0.5 \text{ }\mu\text{M}$) for 8 h and then treated with laser irradiation for 15 min. The resulting cells were collected, stained with the mixture of $5.0 \text{ }\mu\text{L}$ Annexin V-FITC and $5.0 \text{ }\mu\text{L}$ propidium iodide (PI) for 15 min, and analyzed with flow cytometry over FL1 (Annexin V-FITC) and FL3 (PI) channels.

Supplementary Figures

Characterization of PS@MOF

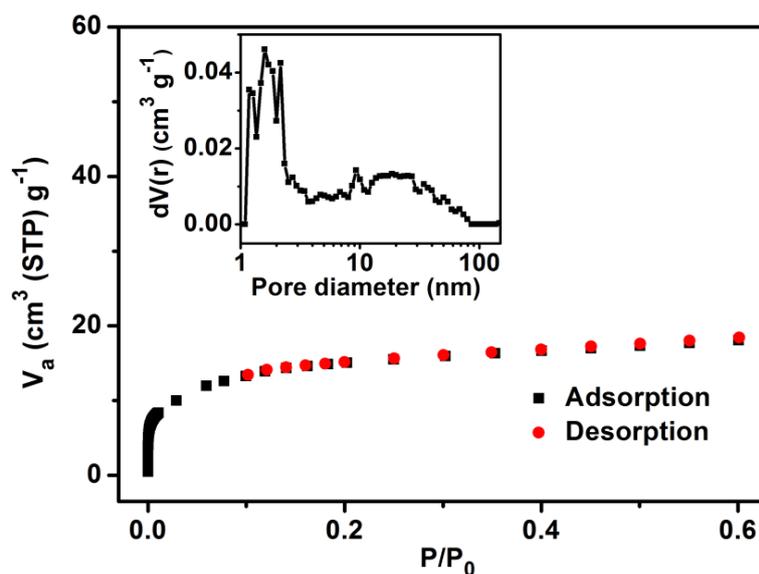


Fig. S1 N_2 adsorption (black)/desorption (red) isotherm of PS@MOF. Inset: Pore size distribution.

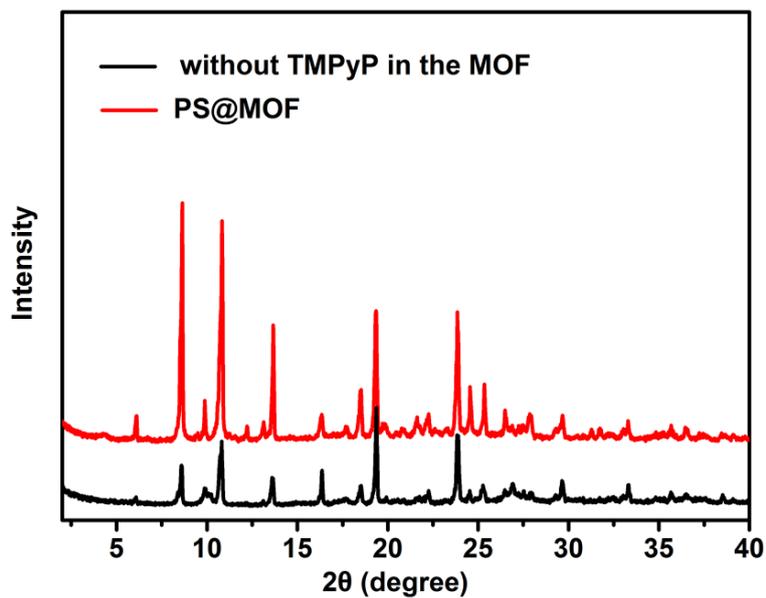


Fig. S2 PXRD patterns of PS-free MOF and PS@MOF.

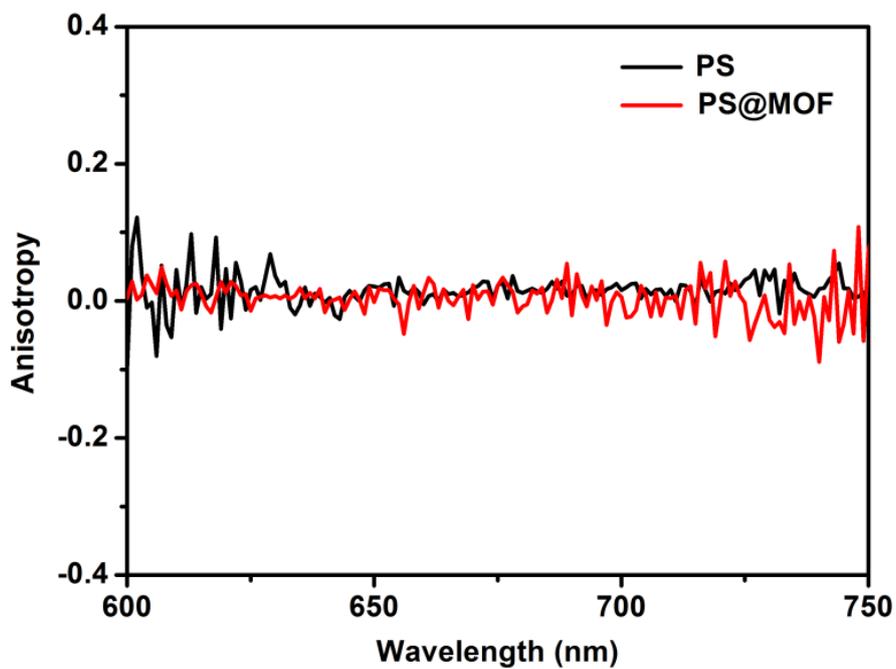


Fig. S3 Fluorescence anisotropy measurements of TMPyP (PS) and PS@MOF at an excitation wavelength of 440 nm.

Loading of TMPyP and Peptide on PS@MOF Probe

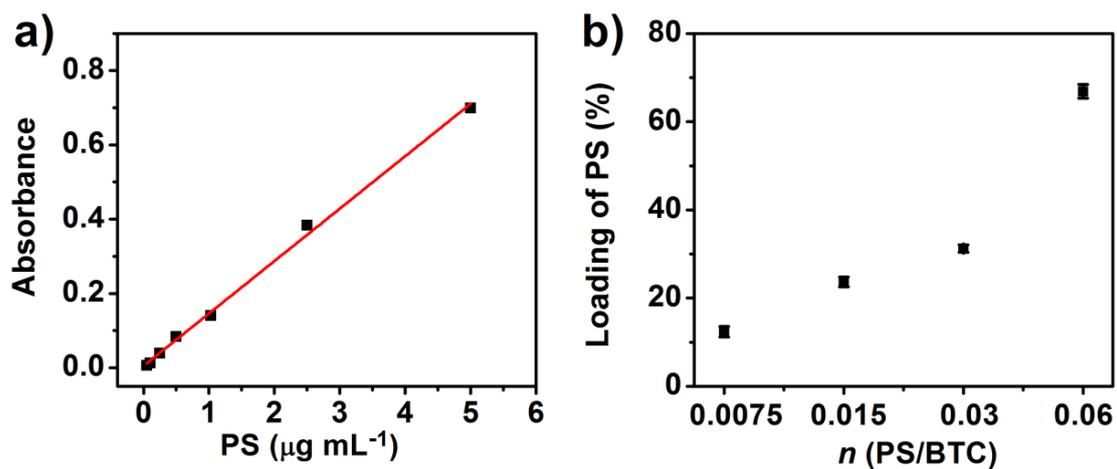


Fig. S4 (a) Plot of absorbance vs. the concentration of TMPyP. (b) Loading of TMPyP in MOF cage varied as the molar ratio of TMPyP to BTC added in the reaction mixture.

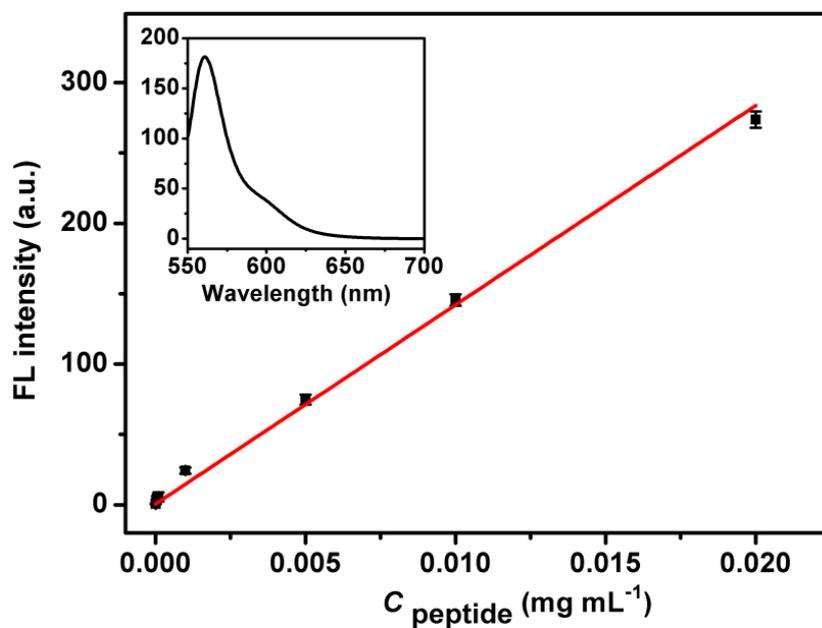


Fig. S5 Plot of fluorescence intensity vs. peptide concentration. Inset: Fluorescence spectrum of the supernatant containing excess peptide collected after probe preparation.

Stability of PS@MOF Probe

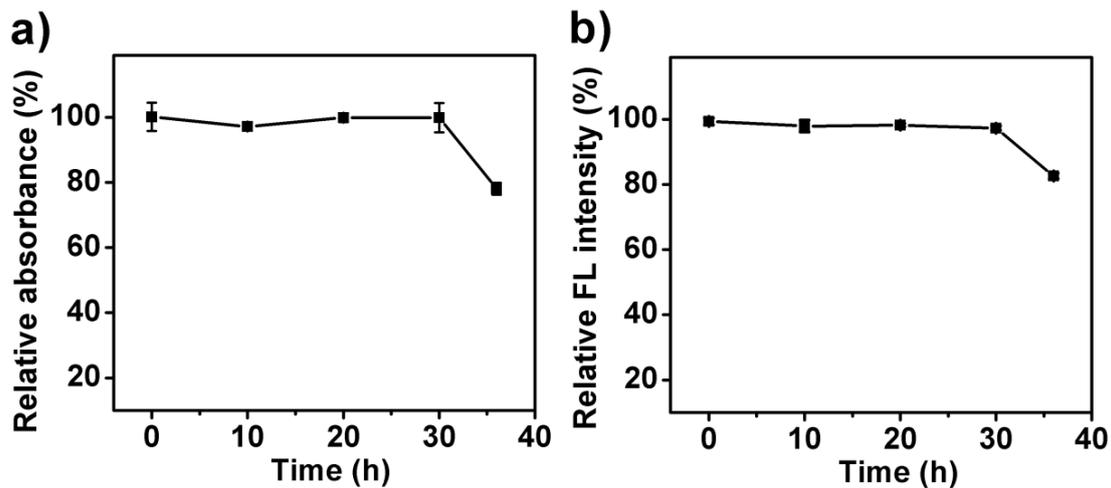


Fig. S6 Stability assays of PS@MOF probe in PBS for (a) TMPyP in MOF cage and (b) peptide on the probe.

Fluorescence and Phosphorescence of PS@MOF

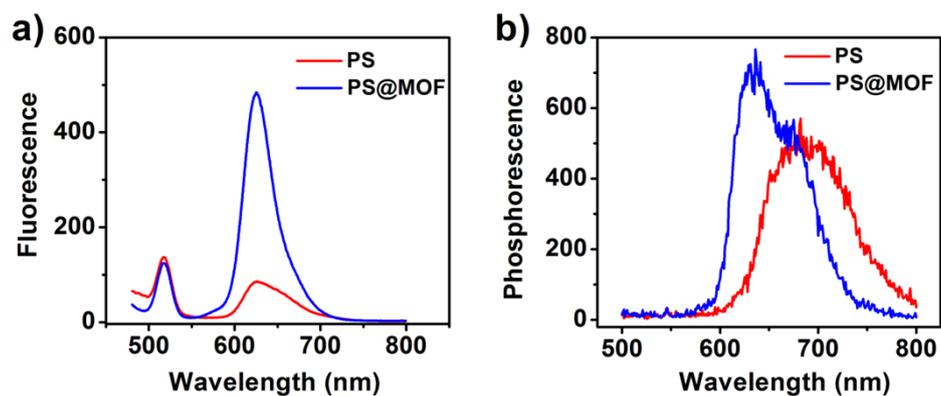


Fig. S7 Fluorescence and phosphorescence spectra of TMPyP and PS@MOF with an excitation wavelength of 440 nm.

In Vitro Assays of Caspase-3

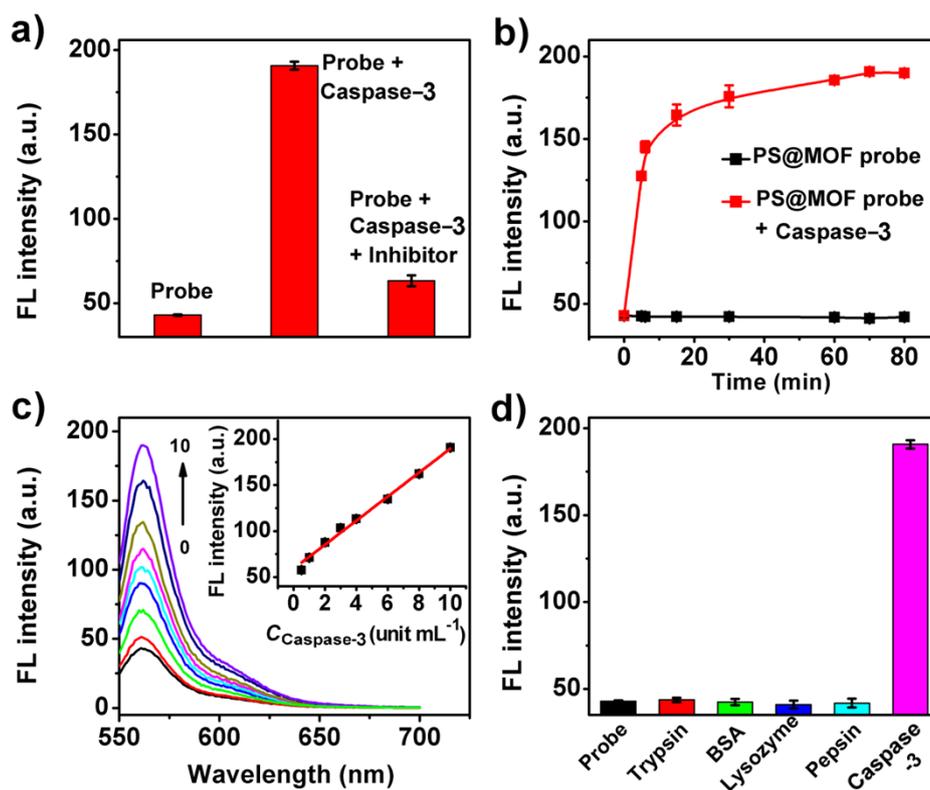


Fig. S8 (a) Fluorescence responses of PS@MOF probe to caspase-3 (10 unit mL⁻¹) in absence and presence of caspase-3 inhibitor (10 μM) at 562 nm under 511-nm excitation. (b) Time-dependent fluorescence responses of PS@MOF probe in absence and presence of caspase-3 (10 unit mL⁻¹). (c) Fluorescence spectra of PS@MOF probe after incubation with 0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 8.0 and 10 unit mL⁻¹ (from bottom to top) caspase-3 for 60 min. Inset: plot of fluorescence intensity vs. caspase-3 concentration. (d) Fluorescence responses of PS@MOF probe to caspase-3 (10 unit mL⁻¹) and other proteins (2.0 μM). The concentration of PS@MOF probe is equivalent to 2.0 μM TMPyP.

Quenching Effect of PS@MOF on Dyes

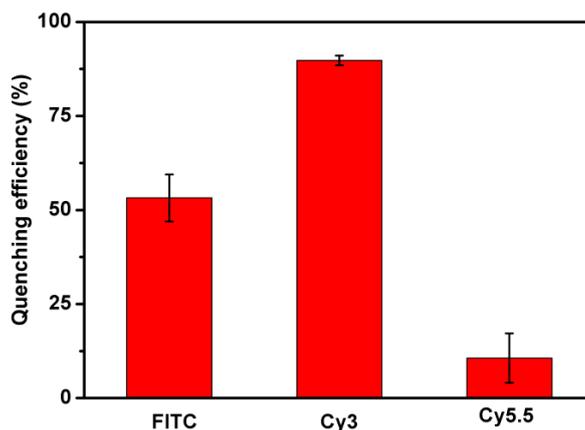


Fig. S9 Quenching efficiency of PS@MOF on different dyes with corresponding emission wavelengths.

Colocalization Assay

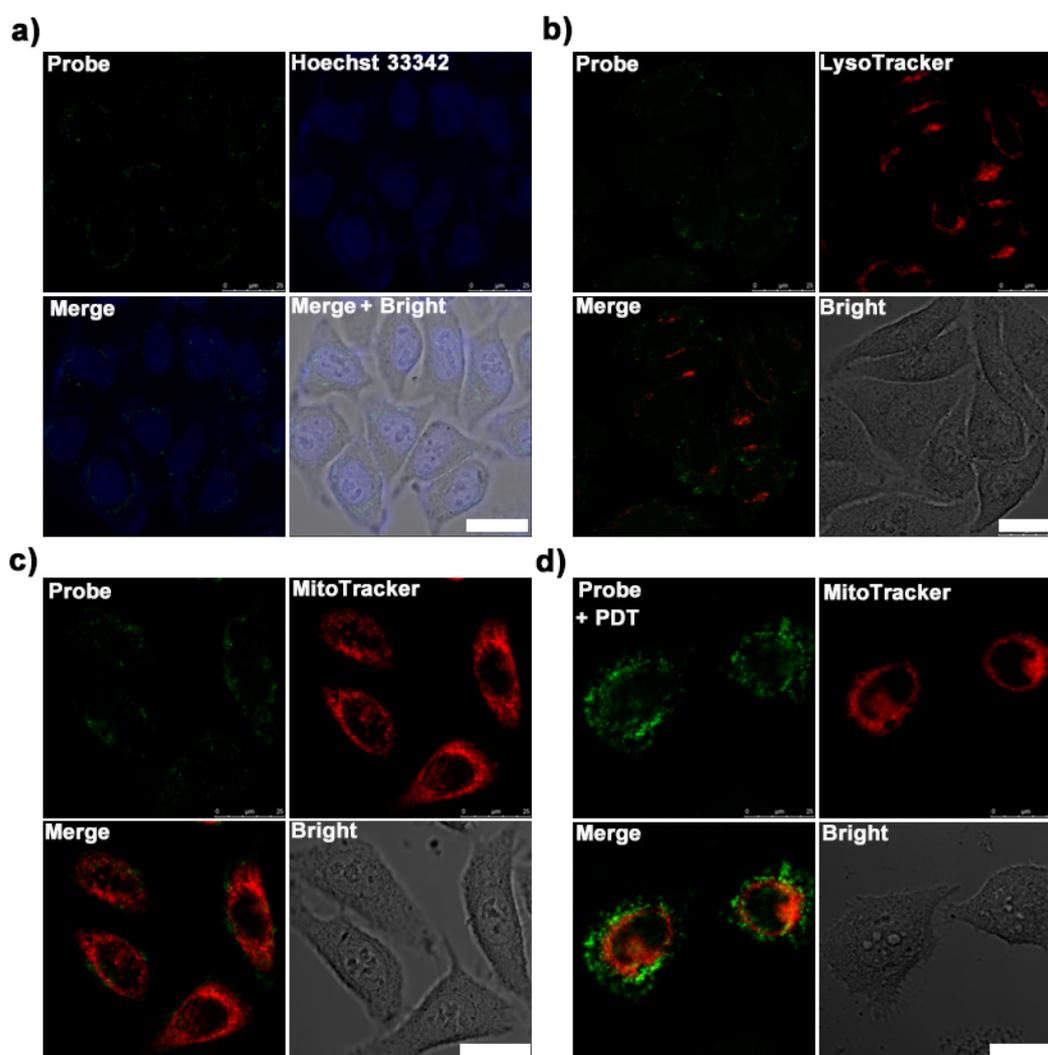


Fig. S10 Co-staining of PS@MOF probe-incubated HeLa cells with (a) Hoechst 33342 ($5.0 \mu\text{g mL}^{-1}$), (b) LysoTracker Deep Red (75 nM), and (c) MitoTracker Deep Red FM (500 nM) before and (d) after PDT. Scale bars, 25 μm .

Fluorescence Imaging of Cellular ROS

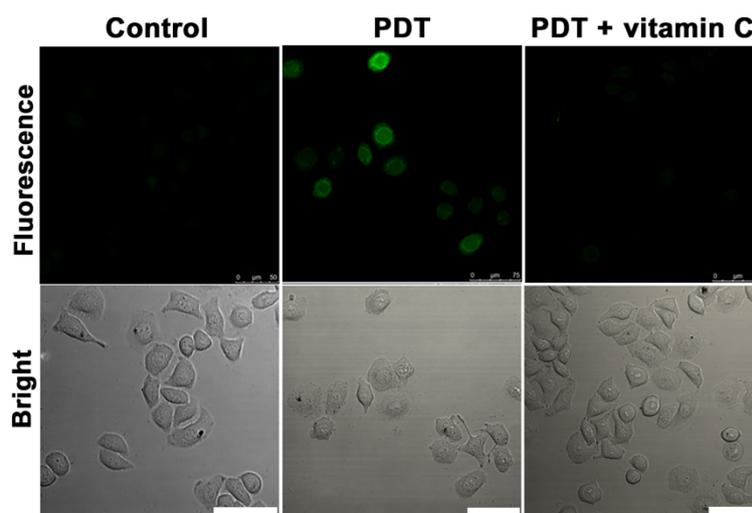


Fig. S11 Confocal fluorescence imaging of cellular ROS generated with PS@MOF probe-mediated PDT using DCFH-DA as ROS probe. Scale bars, 75 μm .

Mitochondrial Pathway of Apoptosis

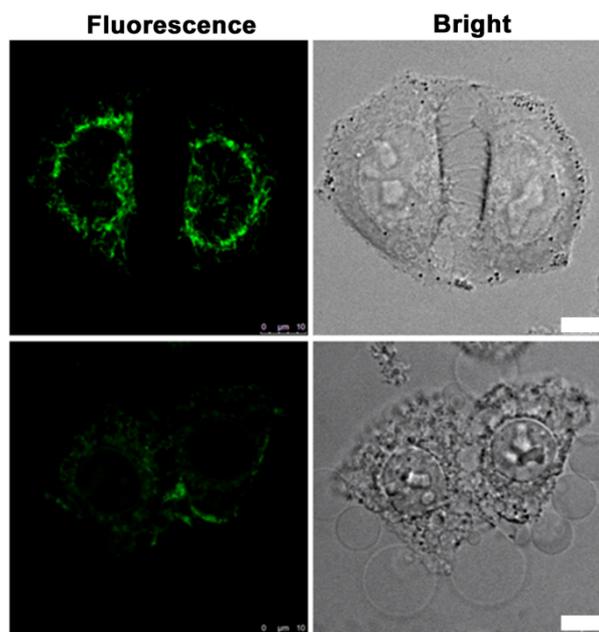


Fig. S12 Confocal fluorescence imaging of HeLa cells incubated with PS@MOF probe (1.0 μM PS equiv.) and then stained for living mitochondria in cells with Rhodamine 123 (5 $\mu\text{g mL}^{-1}$) before (top) and after (down) photodynamic therapy. $\lambda_{\text{ex/em}}$, 488/500–550 nm. Scale bars, 10 μm .

PS@MOF Probe-Treated HaCat Cells

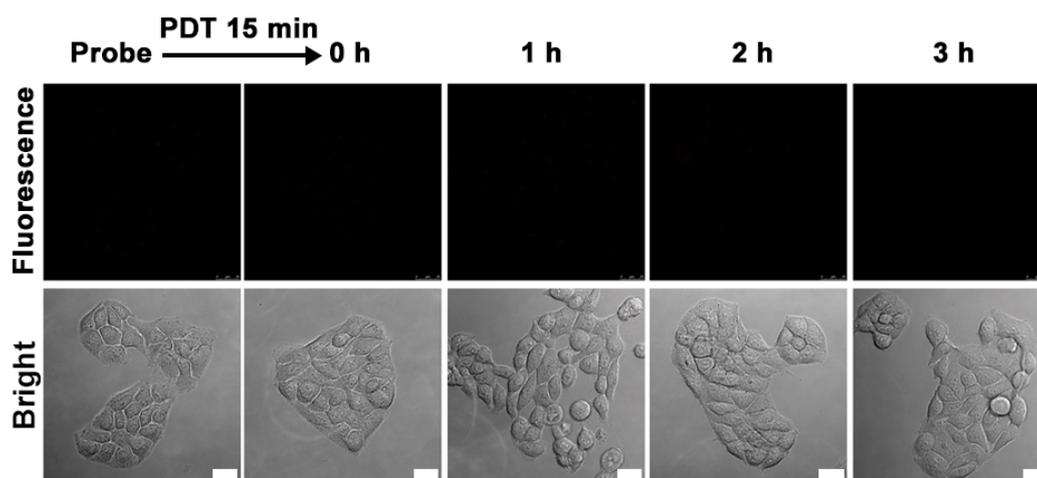


Fig. S13 Confocal monitoring of fluorescence and bright-field images of PS@MOF probe-incubated HaCat cells after different postirradiation times upon irradiation with a 660 nm laser at 100 mW cm^{-2} for 15 min. Scale bars, $25 \mu\text{m}$.

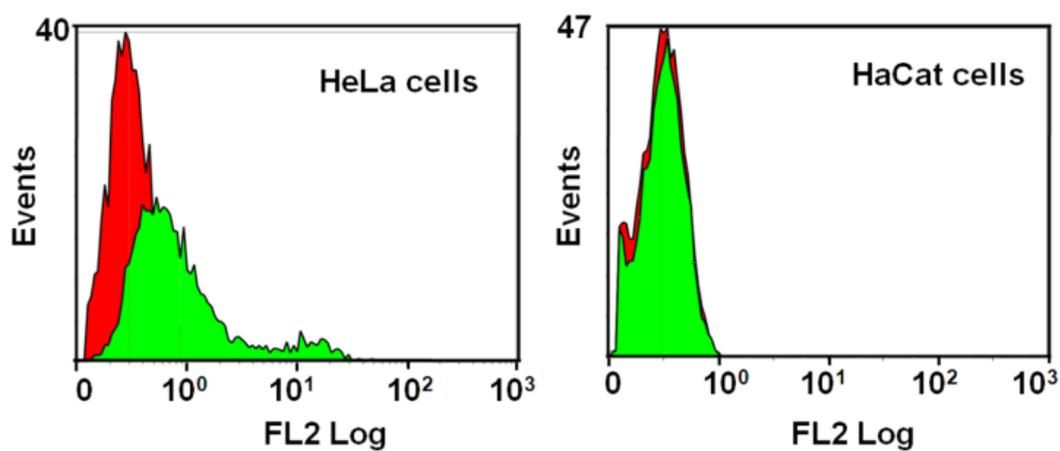


Fig. S14 Flow cytometric assays of HeLa and HaCaT cells incubated with PS@MOF probe ($1.0 \mu\text{M}$ PS equiv.) for 8 h before (red) and after (green) laser irradiation for 15 min.

TUNEL Assay

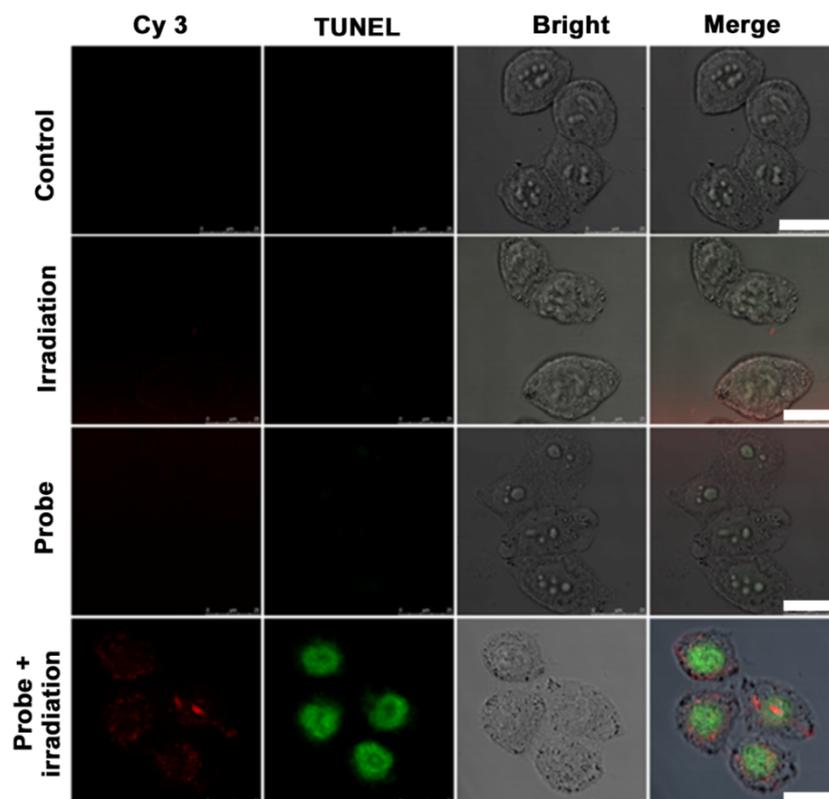


Fig. S15 TUNEL assay of HeLa cells treated with PS@MOF probe-mediated PDT. Scale bars, 25 μm .

Table S1. Excited singlet-state and triplet-state properties of TMPyP and PS@MOF.

	Singlet-state		Triplet-state	
	Φ_F	τ_F (ns)	Φ_P	τ_P (μs)
TMPyP	0.016	5.48	0.73	1.40
PS@MOF	0.019	1.63	0.25	1.71

Supporting Reference

[S1] Z. J. Zhang, L. P. Zhang, L. Wojtas, M. Eddaoudi and M. J. Zaworotko, *J. Am. Chem. Soc.*, 2012, **134**, 928.

[S2] A. Chiaviello, I. Postiglione and G. Palumbo, *Cancers*, 2011, **3**, 1014.

[S3] J. W. Tian, L. Ding, H. J. Xu, Z. Shen, H. X. Ju, L. Jia, L. Bao and J. S. Yu, *J. Am. Chem. Soc.*, 2013, **135**, 18850.

[S4] N. Adarsh, R. R. Avirah and D. Ramaiah, *Org. Lett.*, 2010, **12**, 5720.

[S5] G. A. Crosby and J. N. Demas, *J. Phys. Chem.*, 1971, **75**, 991.

[S6] E. Reddi, M. Ceccon, G. Valduga, G. Jori, J. C. Bommer, F. Elisei, L. Latterini and U. Mazzucato, *Photochem. Photobiol.*, 2002, **75**, 462.