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Cerium oxide@metal−organic framework nanoenzyme as tandem catalyst for enhanced photodynamic therapy

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Experimental

Materials and reagents. Cerium (III) nitrate hexahydrate, polyvinyl pyrrolidone (PVP, Mw 30000), aminoterephthalic acid (NH_2-BDC), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), methylene blue (MB), 1,3-diphenylisobenzofuran (DPBF), methylene blue (MB), N-hydroxysuccinimide (NHS), HOOC-poly(ethylene glycol)-folate (PEG-FA, Mw 2000), and D-glucose were purchased from Sigma-Aldrich (U.S.A). Hydrogen peroxide, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ethanol, and ammonium hydroxide aqueous solution (30%) were obtained from Sinopharm Chemical Reagent Co. (China). Cell Counting Kit-8 (CCK-8) was provided by Dojindo (Japan). Tris (4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride ($[Ru(dpp)_3]Cl_2$) was acquired from J&K Chemical Technology Co., Ltd. (Beijing, China). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (U.S.A).

LysoTracker Green and Hoechst 33342 were got from Invitrogen (Carlsbad, CA, U.S.A). EDVEDG-Cy3 (Cy3-p) as Caspase-3 specific substrate peptide was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). Ultrapure water (≥ 18.2 MΩ; Millpore Co., U.S.A) was used throughout the experiment.

Apparatus. Dynamic light scattering (DLS) studies were carried out by a 90Plus instrument (Brook Haven, USA). UV-visible spectroscopic measurements were performed by using a UV-2600 spectrophotometer (Shimadzu Co., Japan). The Fourier Transform Infrared (FT-IR) spectra were detected with Nicolet iS5 FT-IR spectrometer (Thermo, USA). Powder X-ray diffraction (PXRD) was performed by a X'TRA diffractometer (ARL, Switzerland). The transmission electron microscopic (TEM) images were captured on a JEM-2100 high resolution transmission electron microscope (JEOL Ltd., Japan). Zeta potential experiments were done by a Zetasizer (Nano-Z, UK). The fluorescent measurement was carried out on a FluoroMax-4 spectrometer (HORIBA, Japan). Flow cytometric analysis was recorded on a Coulter FC-500 flow cytometer (Beckman-Coulter). CCK-8 assays were analyzed by Thermo Scientific Varioskan Flash (Thermo). The cell images were obtained from a TCS SP5 laser scanning confocal microscope (Leica, Germany).

Synthesis of CeO_x. CeO_x nanoparticles were synthesized by simple aqueous method with minor modifications.[S1] Briefly, cerium (III) nitrate hexahydrate (0.6 g) was mixes with 50 mL deionized aqueous solution containing 20 mM D-glucose. Then, aqueous ammonia (NH_4OH, 30%) was introduced into the solution slowly for homogeneity (pH 9). After stirred continuously for 12 h, the solution changed from colorless to yellow. The resultant mixture was applied on centrifugation at 3,000 rpm for 10 min to remove the oversized particles and agglomerates. The

supernatant was collected to centrifuge at 15,000 rpm for 0.5 h and washed with water thrice. The obtained CeO_x nanoparticles were resuspended in deionized water for storage.

Synthesis of CeO_x@MIL. For stepwise synthesis, 10 mg PVP (MW = 30,000) was added into 1 mL CeO_x solution before the growth of MIL shell on the CeO_x. The mixture of PVP and CeO_x nanoparticles was shaken overnight at room temperature, followed by centrifuged at 15,000 rpm for 30 min to obtain PVP-CeO_x. The PVP-CeO_x was redispersed in a 10 mL precursor ethanol solution consisted of 13.5 mg $FeCl_3 \cdot 6H_2O$ and 9 mg NH_2-BDC. The solution was heated at 40 ℃ for 1.5 h, centrifuged for 12000 rpm for 20 min, washed with ethanol twice, and redispersed in 10 ml ethanol to obtain the intermediate product. After adding additional 13.5 mg $FeCl_3 \cdot 6H_2O$ and 9 mg NH_2-BDC, the intermediate ethanol solution was transferred into the water bath 40 ℃ for another 1 h. Finally, the precipitate was washed by ethanol by centrifugation at 8,000 rpm for 8 min to remove the excess regents. The resulted CeO_x@MIL was redispersed in PBS for further use.

Synthesis of CeO_x@fMIL. Before the surface functionalization of CeO_x@MIL with target element (PEG-FA) and signal reporter (Cy3-pep), EDC (2 mg) and NHS (3 mg) were added into 1 mL PBS buffer containing PEG-FA (2 mg) and Cy3-pep (5 μM). After shaken for 60 min, the mixture was injected into the prepared CeO_x@MIL solution (1 mL, 1 mg mL^{-1}) followed by vortex at 20 ℃ for another 4 h. The precipitates were separated by centrifuging and washed with PBS thrice to gain the functionalized CeO_x@MIL (CeO_x@fMIL).

Catalase Activity Assay. The relative enzymatic activity of CeO_x or CeO_x@fMIL was determined by the Góth method.[S2] 20 μL CeO_x or CeO_x@fMIL was added into PBS buffer at different pHs (4.5, 6.5 and 7.4), treated with H_2O_2 (1.0 mM) for the designed time, then

terminated by the addition of ammonium molybdate (1.0 mM) and cooled to room temperature. The enzymatic activity was calculated via UV−vis spectroscopy to detect the absorbance at 400 nm of a primrose stable complex, which formed by ammonium molybdate and residual hydrogen peroxide.

Oxygen Generation by CeO_x@fMIL. In order to monitor the ability of CeO_x and CeO_x@fMIL for transferring H_2O_2 to O_2, the quenched O_2 indicator $[(Ru(dpp)_3)]Cl_2$ was used in this assay. Briefly, 5 μM $[(Ru(dpp)_3)]Cl_2$ was introduced into the nitrogen-saturated CeO_x or CeO_x@fMIL solution at pH 7.4 with or without 100 μM H_2O_2. The fluorescence intensity of $[(Ru(dpp)_3)]Cl_2$ at 610 nm was recorded in different incubation times under the excitation at 488 nm. As for the intracellular assays, the method of oxygen generation measurement was similar with that in buffer. The confocal imaging photos were collected at the channel of 600-700 nm under the excitation at 488 nm.

Measurement of ROS Generation. To monitor the ROS generation by CeO_x or CeO_x@fMIL, a commercial UV-VIS indicator DPBF, whose absorbance of 410 nm could be decreased after incubated with ROS, was applied in the experiment via UV−vis spectroscopy. Typically, 20 μg mL^{-1} DPBF was mixed with CeO_x, CeO_x@fMIL, MIL or MB in the oxygen-saturated PBS and irradiated with 660 nm laser for different periods of time. The ROS quantum yield (Φ_Δ) was determined by the following eq. S1, in which MB (Φ_Δ= 0.52) was used as the standard.[S3]

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

where subscripted x and std referred to the sample and MB, respectively, S denoted the plot slope of the absorbance of DPBF (at 410 nm) vs. irradiation time, and F represented the

absorption correction factor, which was given by $F = 1-10^{-OD}$ (OD stood for the optical density of sample and MB at 660 nm).

The extra ROS generation of CeO_x or CeO_x@fMIL in air, nitrogen or 5% oxygen at different pHs was investigated by the similar protocol. 20 μL CeOx or CeO_x@fMIL was incubated with or without 100 μM H_2O_2 and 20 μg mL^{-1} DPBF in different atmosphere-saturated PBS buffer at different pHs (4.5, 6.5 and 7.4), and then the changes of the DPBF absorbance at 410 nm were recorded for comparison.

In Vitro Response to Caspase-3 by Fluorescence. Twenty μL CeO_x@fMIL was mixed with different amounts of caspase-3 (0 to 10.0 Unit mL^{-1}) in HEPES buffer (40 mM, pH 7.4) containing 1 mM EDTA, 0.1 M NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT at 37 °C for the optimized time. The fluorescent response at 565 nm was recorded under an excitation wavelength at 543 nm.

Evaluation of Cytotoxicity. The cytotoxicity of CeO_x@fMIL on cells was assessed by CCK-8 analysis. Typically, HeLa or HaCaT cells (1.0×10^5) were seeded, cultured for 24 h, washed with PBS twice and incubated in Dulbecco's modified Eagle's culture medium containing increasing amount of CeO_x@fMIL for 6 h. The cells were washed with PBS and incubated with CCK-8 (10 μL) at 37 °C for 2 h. The cell viability was determined by monitoring the absorbance at 450 nm and using control group with PBS for normalization. The cytotoxicity of CeO_x@fMIL against the incubation time was analyzed using the method similar to the mentioned above with the CeO_x@fMIL at 20 μL.

Confocal Imaging Assays. The HeLa cells (5.0×10^4) were seeded in the confocal dishes and incubated at 37 °C overnight. Then fresh culture containing CeO_x@fMIL substitute for the

previous culture medium. After 6-h incubation, the cells were treated with LysoTracker Green (1.0 μM) and Hoechst 33342 (1.0 μM) for 20 min. The cellular confocal fluorescence images were collected at $\lambda_{ex/em}$ of 488/510-550 nm for LysoTracker Green, $\lambda_{ex/em}$ of 543/560-600 nm for CeO_x@fMIL, and $\lambda_{ex/em}$ of 400/430-480 nm Hoechst 33342 for on the stack mode of a Leica confocal laser scanning microscope.

Enhanced Therapy Efficacy. HeLa cells (5.0×10^4) were cultured for 12 h in normoxic or hypoxic atmosphere (21% or 5% oxygen). The medium was replaced with f-CeO_x or CeO_x@fMIL in PBS buffer. The cells were cultured for another 6 h and treated with a 660-nm at 200 mW cm^{-2} laser irradiation for 5 min. Thereafter, the cell fluorescent images were captured at $\lambda_{ex/em}$ of 543/560-600 nm on the confocal laser scanning microscope. In the flow cytometry analysis, the HeLa cells were treated with the similar procedure and collected for further staining with Annexin V-FITC (5.0 μL) and PI (5.0 μL) for 15 min.

Supplementary Data

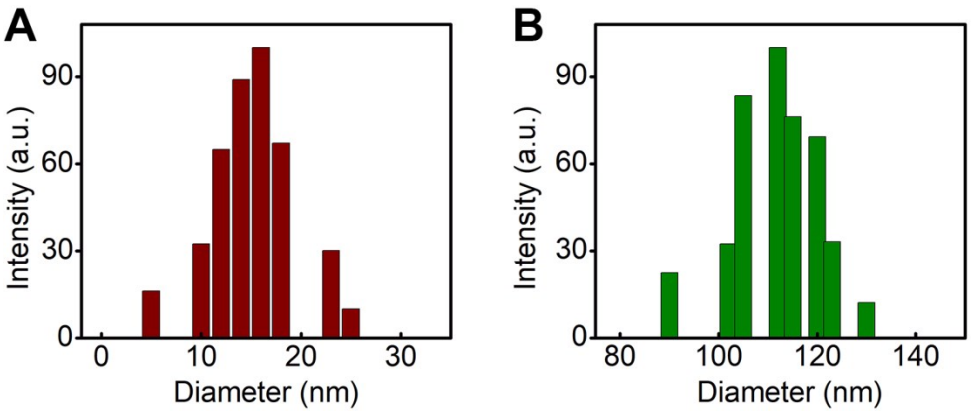

Fig. S1 DLS assays of (A) CeO_x and (B) CeO_x@MIL.

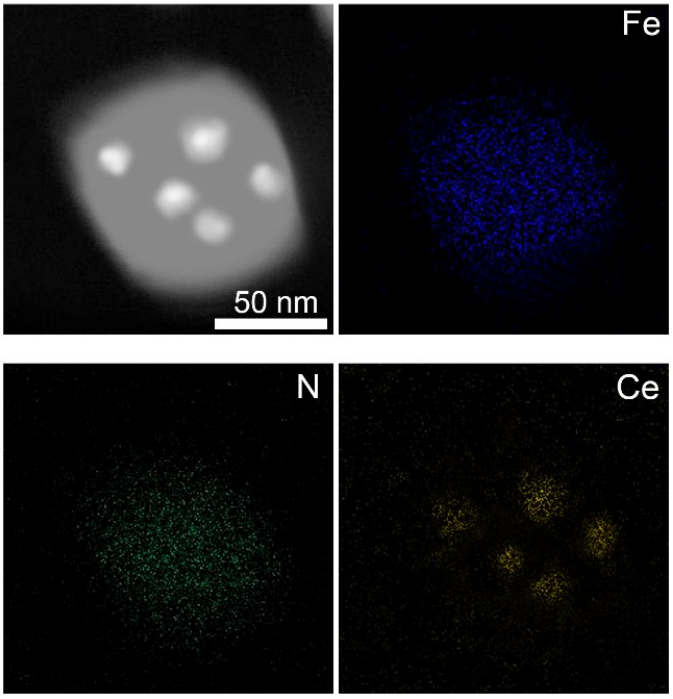

Fig. S2 STEM image and energy dispersive X-ray spectroscopy (EDX) elemental mapping of CeO_x@MIL heterostructure.

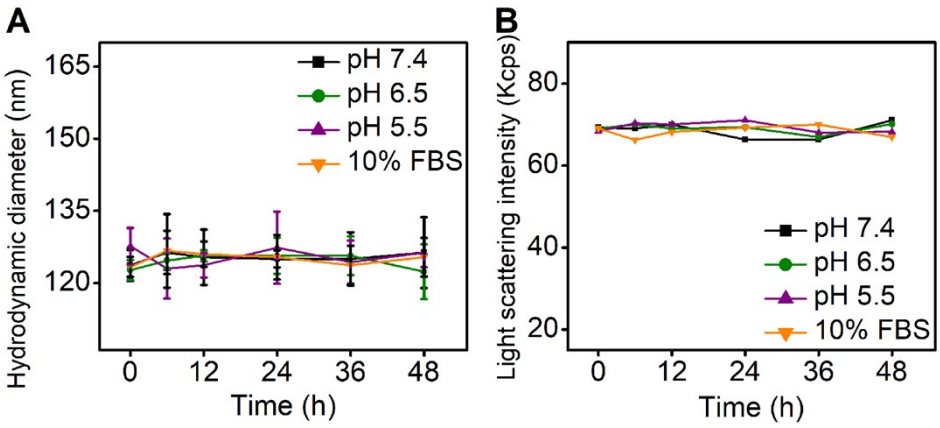

Fig. S3 The stability of CeO_x@fMIL in PBS at different pHs or at pH 7.4 with 10% fetal bovine serum by the measurements of (A) hydrodynamic diameter and (B) light scattering intensity.

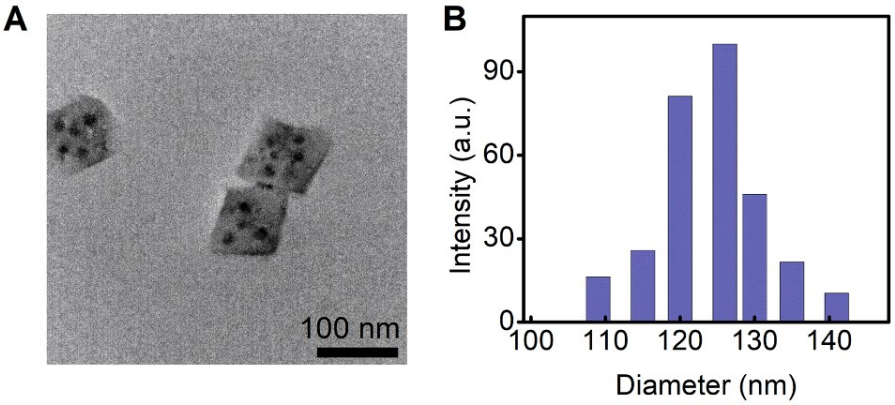

Fig. S4 TEM image and DLS assay of CeO_x@fMIL.

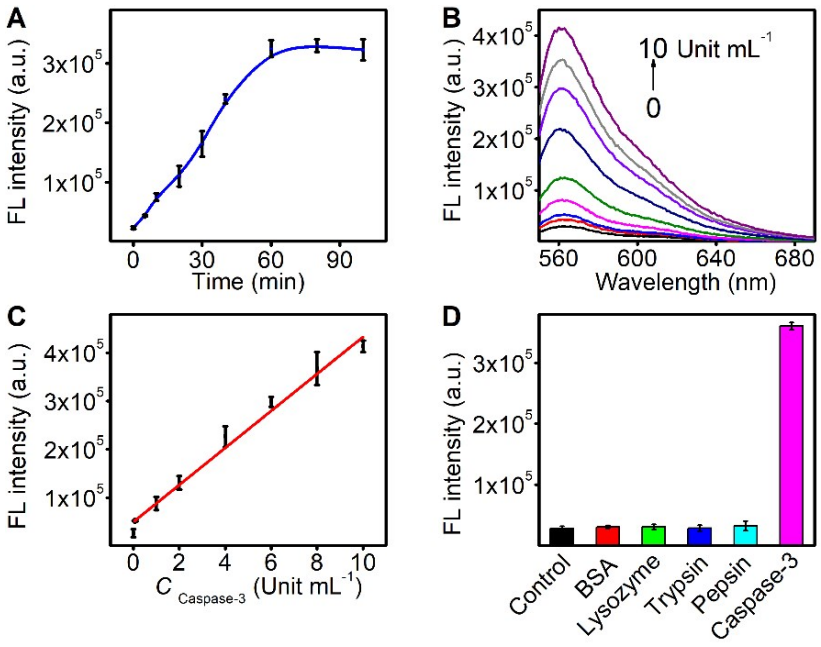

Fig. S5 (A) Plot of fluorescence intensities of CeO_x@fMIL at 565 nm *vs*. different incubation time with of caspase-3. (B) Fluorescence response of CeO_x@fMIL to caspase-3 at the concentrations of 0, 0.05, 0.1, 1, 2, 4, 6, 8 and 10 Unit mL^{-1}. (C) Plot of fluorescence intensities *vs*. caspase-3 concentration. (D) Fluorescent response of CeO_x@fMIL to caspase-3 (6.0 Unit mL^{-1}) and other proteins (1.0 μM).

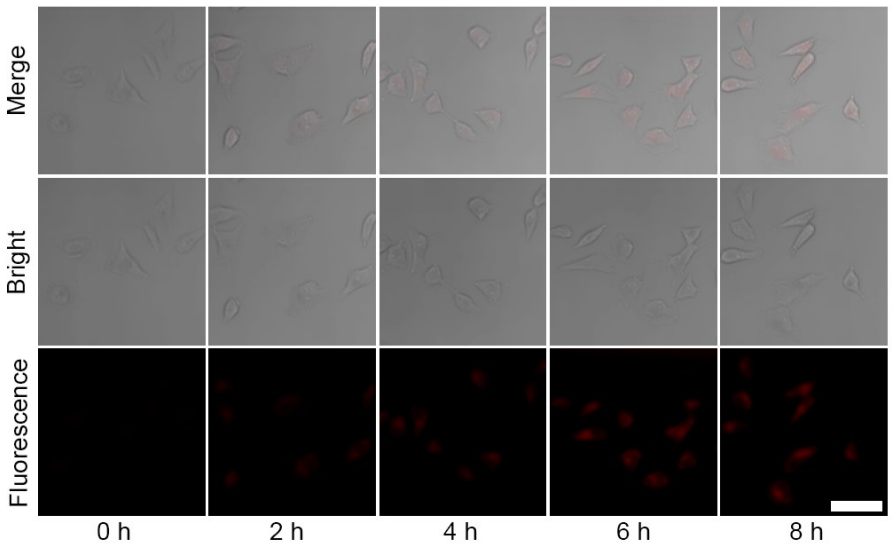

Fig. S6 Cy3 fluorescence microscopic images of HeLa cells taken at different time points after being incubated with CeOx@fMIL at 37 °C. Scale bar: 50 μm.

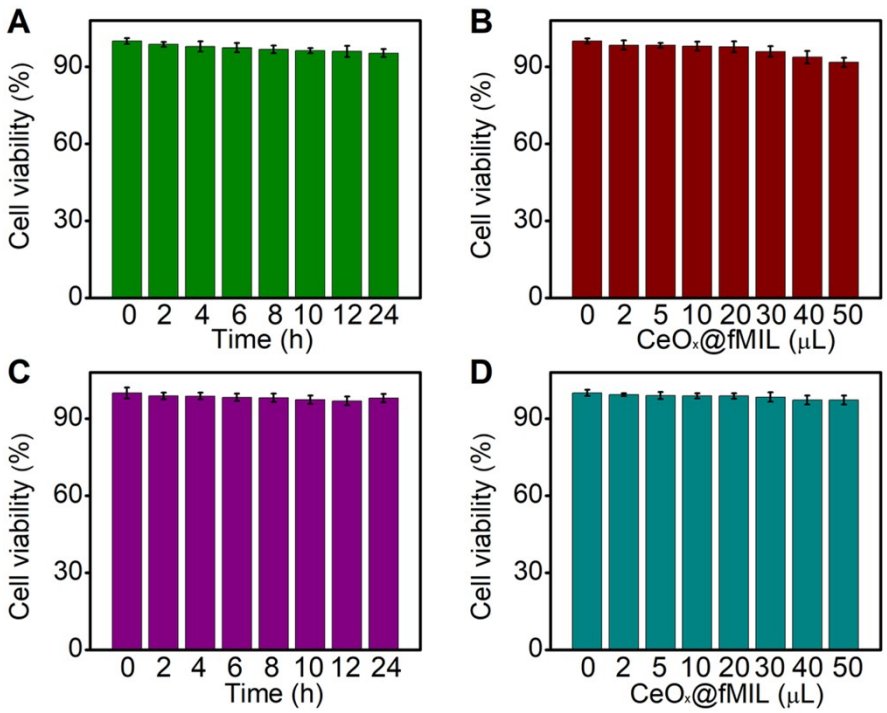

Fig. S7 CCK-8 assays for (A) HeLa cells and (C) normal HaCaT cells after incubated with CeO_x@fMIL for different time, and (B) HeLa cells and (D) normal HaCaT cells with increasing amounts of CeO_x@fMIL for 6 h.

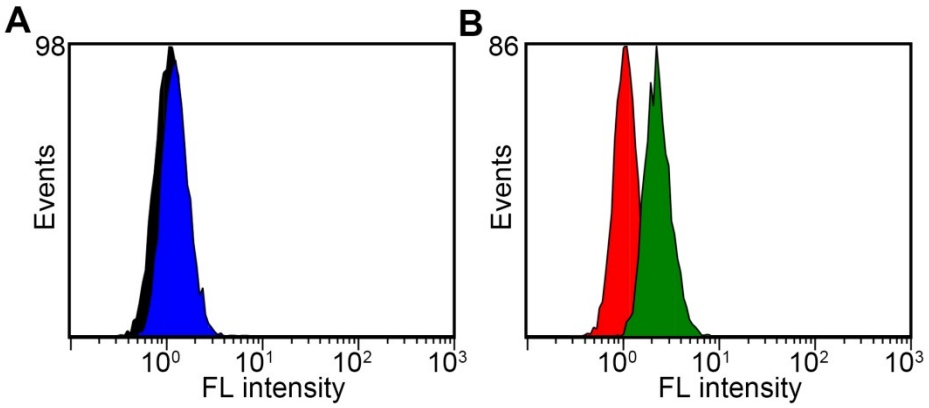

Fig. S8 Flow cytometric assay of (A) HeLa cells and (B) HaCaT cells incubated with CeO_x@fMIL for 6 h at 37 °C.

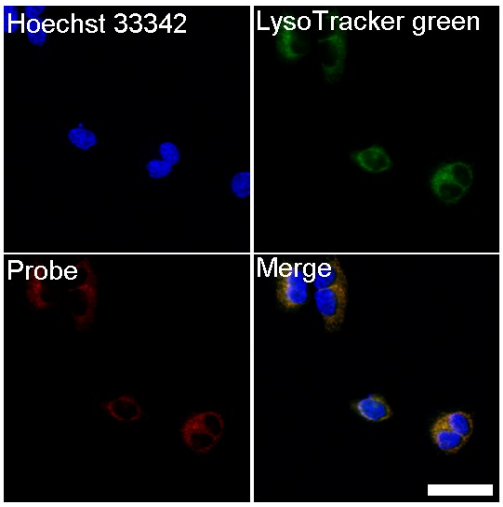

Fig. S9 Colocalization images of CeO_x@fMIL-incubated HeLa cells with LysoTracker green (1.0 μM) and Hoechst 33342 (1.0 μM). Scale bar, 50 μm.

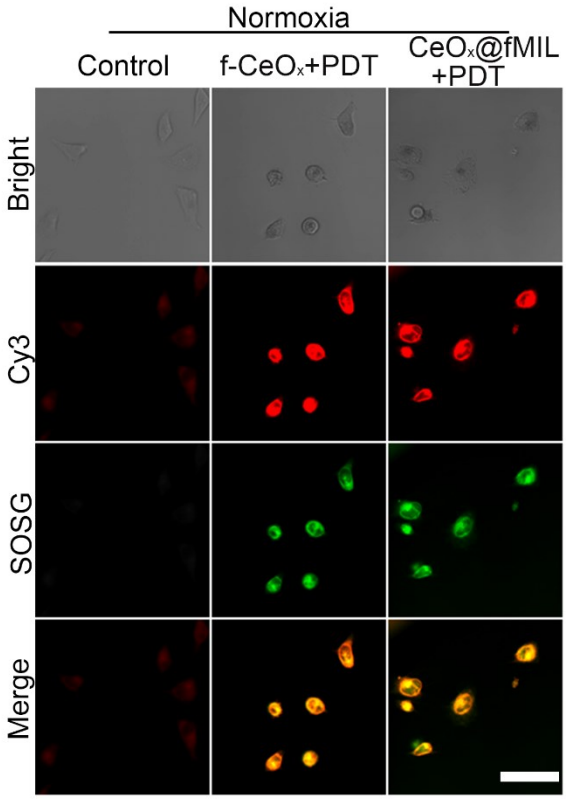

Fig. S10 Confocal fluorescence images of HeLa cells treated with f-CeO_x and CeO_x@fMIL under normoxic conditions by dual-channel of Cy3 for the CeO_x@fMIL or f-CeO_x and SOSG for the monitoring of ROS after PDT. Scale bar: 50 μm.

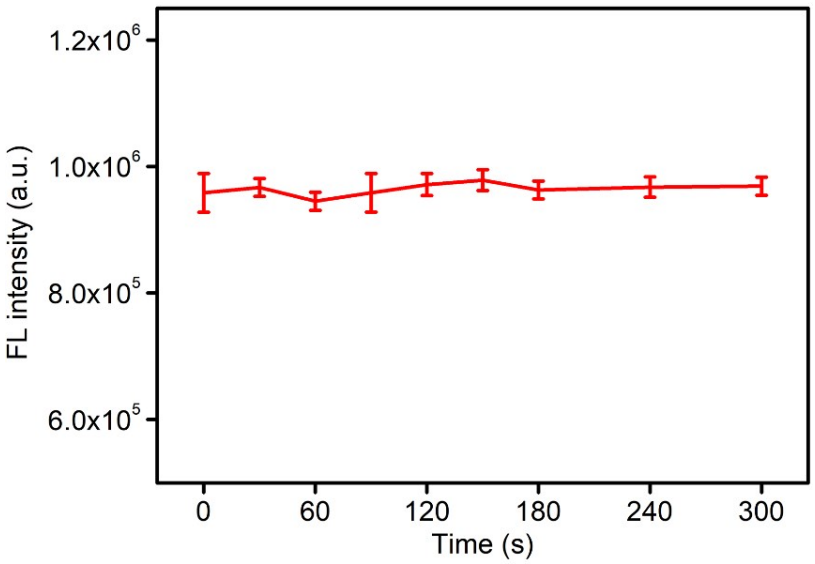

Fig. S11 Dependence of fluorescence intensity of Cy3 in the presence of ROS generated by CeO_x@MIL on the irradiation time by laser at 200 mW cm^{-2}.

Supporting References

(S1) M. Li, P. Shi, C. Xu, J. S. Ren, and X. G. Qu, *Chem. Sci.,* 2013, **4,** 2536.

(S2) G. S. Song, Y. Y. Chen, C. Liang, X. Yi, J. J. Liu, X. Q. Sun, S. D. Shen, K. Yang and Z. Liu, *Adv. Mater.,* 2016, **28,** 7143.

(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.