Supporting Information

Multifunctional self-assembled polymeric nanoprobes for FRET-based ratiometric detection of mitochondrial H_2O_2 in living cells

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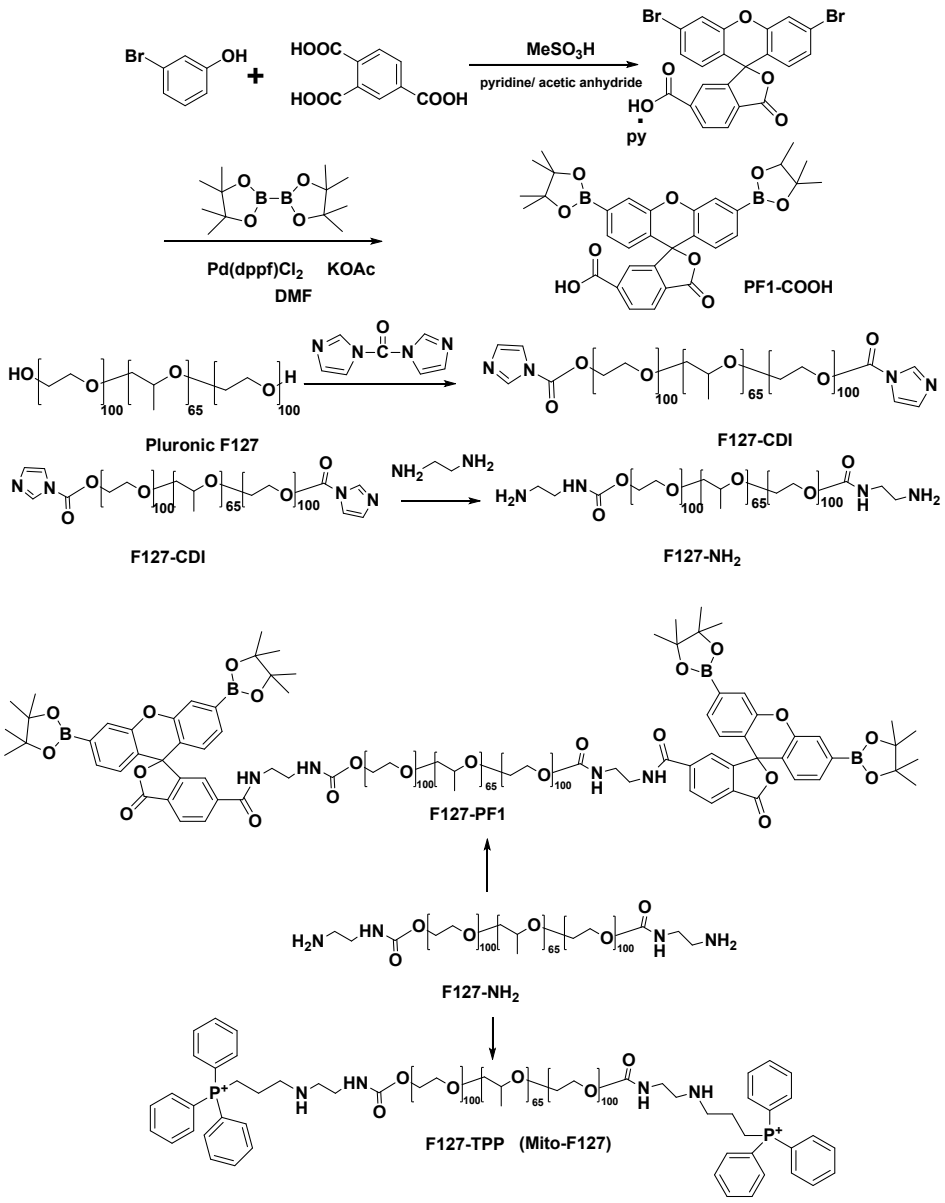
EXPERIMENTAL:

Materials and Instrumentation. Methanesulfonic acid, bis(pinacolato)diboron, 3-bromophenol, 1,2,4-benzenetricarboxylic acid, tert-butylhydroperoxide, Pd(dppf)Cl_2 were supplied by J & k chemical. Hypochlorite, H_2O_2 were supplied by Aladdin Industrial, Tetraphenylethylene (TPE), Glutathione, ascorbic acid, iron(III) chloride, N-hydroxysulfosuccinimide sodium salt, sodium salts of anions (NO_3^-, NO_2^-), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC HCl) and N,N'- Carbonyldiimidazole (CDI), 1,2-ethylenediamine and potassiumacetate solution were purchased from TCI (Shanghai), Plounic F127 and phorbol 12-myristate 13 acetate (PMA) were purchased from Sigma-Aldrich. MitoTracker® Deep Red and LysoTracker® Deep Red were supplied from Life Technologies. N,N-Dimethyl-formamide (DMF) was dried with CaH_2 and vacuum distilled. The water used in this study was the triple distilled water which was further treated by ion exchange columns and then by a Milli-Q water purification system.

Chemical structures were identified by a Bruker Avance 400 MHz NMR spectrometer and Mass spectra were obtained by Electrospray Ionization Mass Spectrometry (ESI-MS). DLS data was collected on a DynaProNanoStar instrument (Wyatt Technology) with a He-Ne laser (λ =

659 nm) operated at 10 mW and analyzed with Dynamics software v6.11. Experiments were run in triplicate, with 10 acquisitions per measurement. The visualized TEM images of the assemblies were obtained from a JEM-2100F electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 120 kV. The sample for TEM measurements was prepared by dropping the solution onto a copper grid coated with carbon. The grid was then freeze-dry without stain. UV–Vis spectra were acquired on a Hitachi U-3010 UV–Vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer at ambient temperature.



Scheme S1. Synthesis route for the PFl-COOH and the modifications of F127.

Synthesis of 3-Oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro [isobenzofuran-1,9'-xanthene]-6-carboxylic acid (PF1-COOH).

PF1-COOH was synthesized according to a procedure reported by Chang and coworkers.[S1] (200 mg, 42% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.29 (d, 1H), 8.09 (d, 1H), 7.75 (m, 3H), 7.45 (d, 2H), 6.84 (d, 2H), 1.35 (s, 24H). ESI-MS m/z $[MH]^+$= 597.2449.

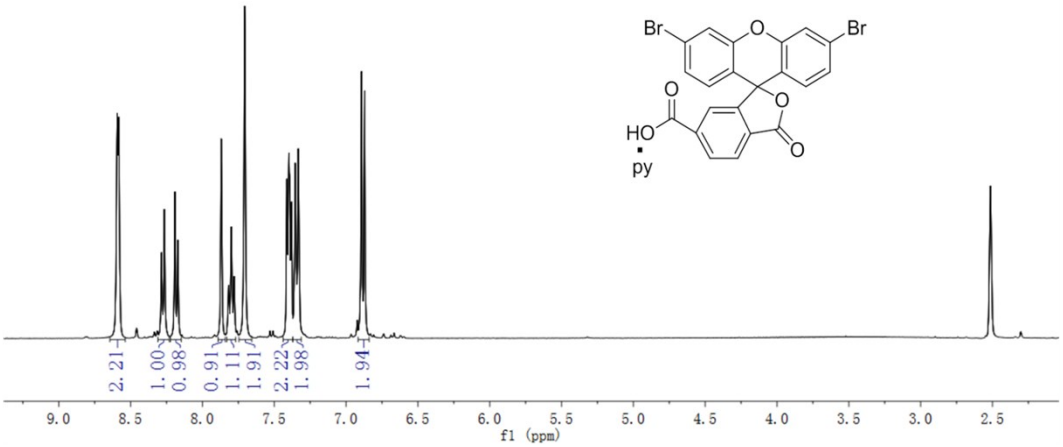


Fig. S1 ^{1}H NMR (DMSO-d6) spectrum of 3',6'-Dibromo-6-carboxy-fluoran pyridinium salt.

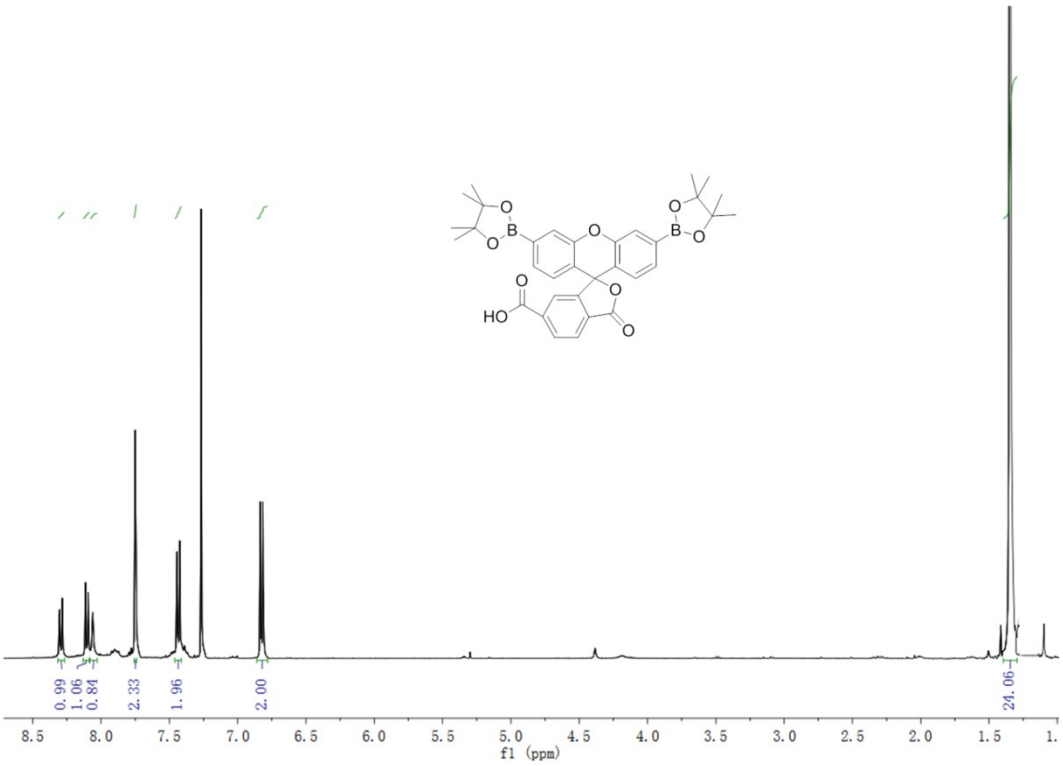


Fig. S2 ^{1}H NMR ($CDCl_3$) spectrum of 3-Oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxa-borolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (PF1-COOH).

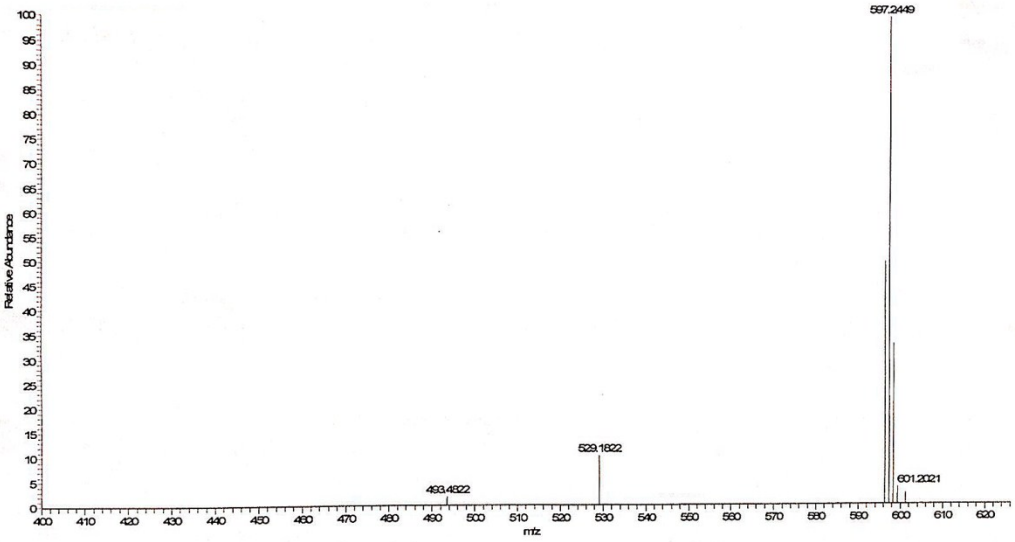


Fig.S3 ESI mass spectrumof PF1-COOH. MS-ESI: m/z =597.2499 for $[MH]^+$.

Synthesis of amino-terminated Pluronic F127.

The modification of Pluronic F127 was synthesized according to a reported literature.[S2]

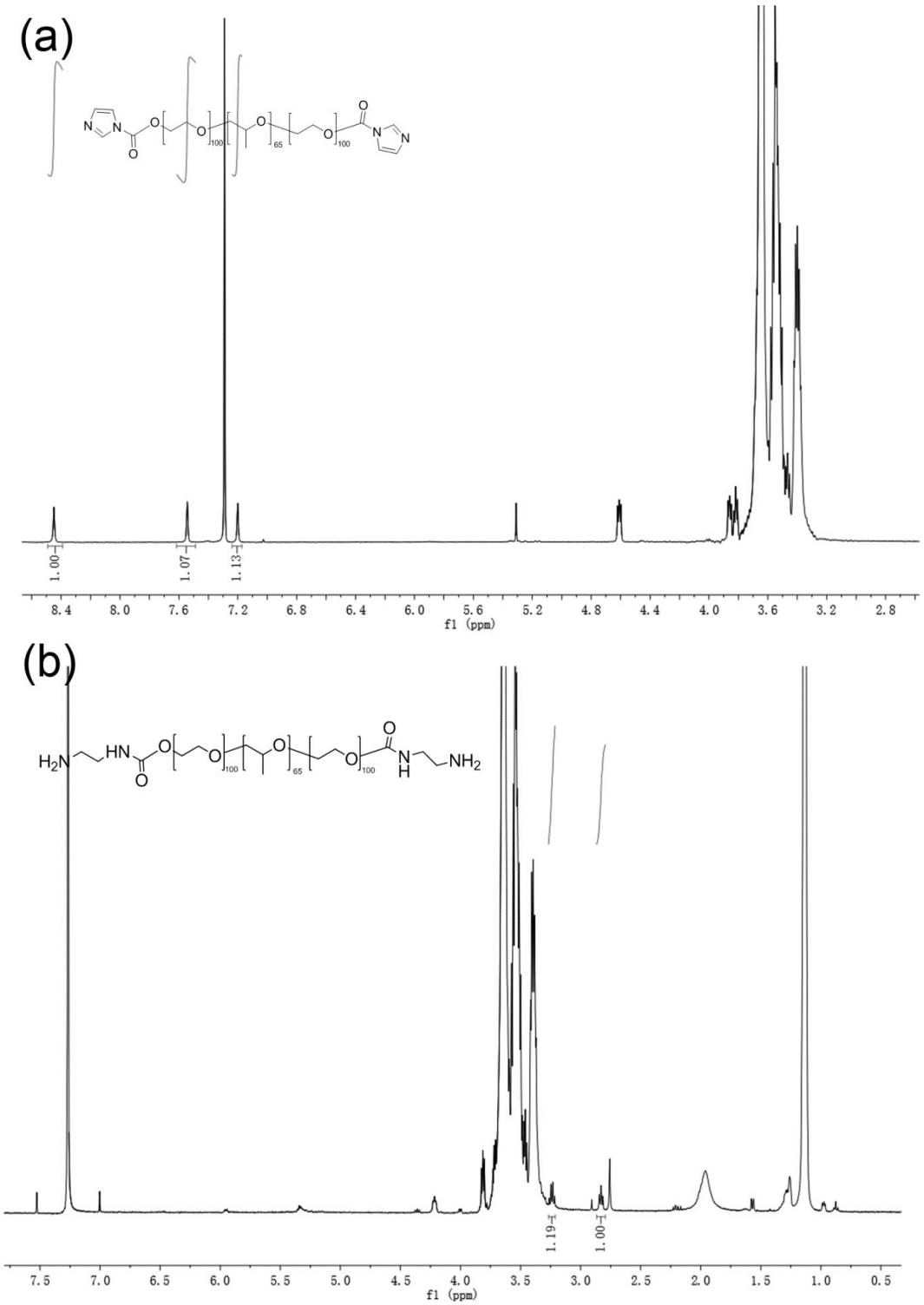


Fig.S4 ^{1}H NMR spectra of F127-CDI (a) and F127-NH_2 (b).

Synthesis of PF1COOH-conjugated Pluronic F127 (PF1-F127).

The F127-NH_2 (1 g, 0.079 mmol), PF1COOH (142 mg, 0.237mmol), NHS (60 mg, 0.522 mmol) and DCC (108 mg, 0.522 mmol) were dissolved in 10 mL DMSO. The mixture was stirred in a nitrogen atmosphere at room temperature in the dark for 24 h. Then, the mixture was diluted with 20 mL deionized water and centrifuged in order to separate DCU. The supernatant was further purified by dialysis against deionized water for 2 days using a dialysis membrane (with molecular weight cut–off of 3600 Da) under nitrogen bubbling followed by lyophilization.

Synthesis of (3-bromopropyl)triphenylphosphonium bromide modified Pluronic F127 (Mito-F127).

The F127-NH_2 (1 g, 0.079 mmol), (3-Bromopropyl) triphenylphosphonium bromide (147 mg, 0.316 mmol) and K_2CO_3 (160 mg, 1.2 mmol) were dissolved in 10mL DMSO. The mixture was stirred under a nitrogen atmosphere at 60 °C for 12 h, cooled to room temperature. The mixture was purified by dialysis against deionized water for 2 days using a dialysis membrane (with molecular weight cut−off of 3600 Da), followed by lyophilization.

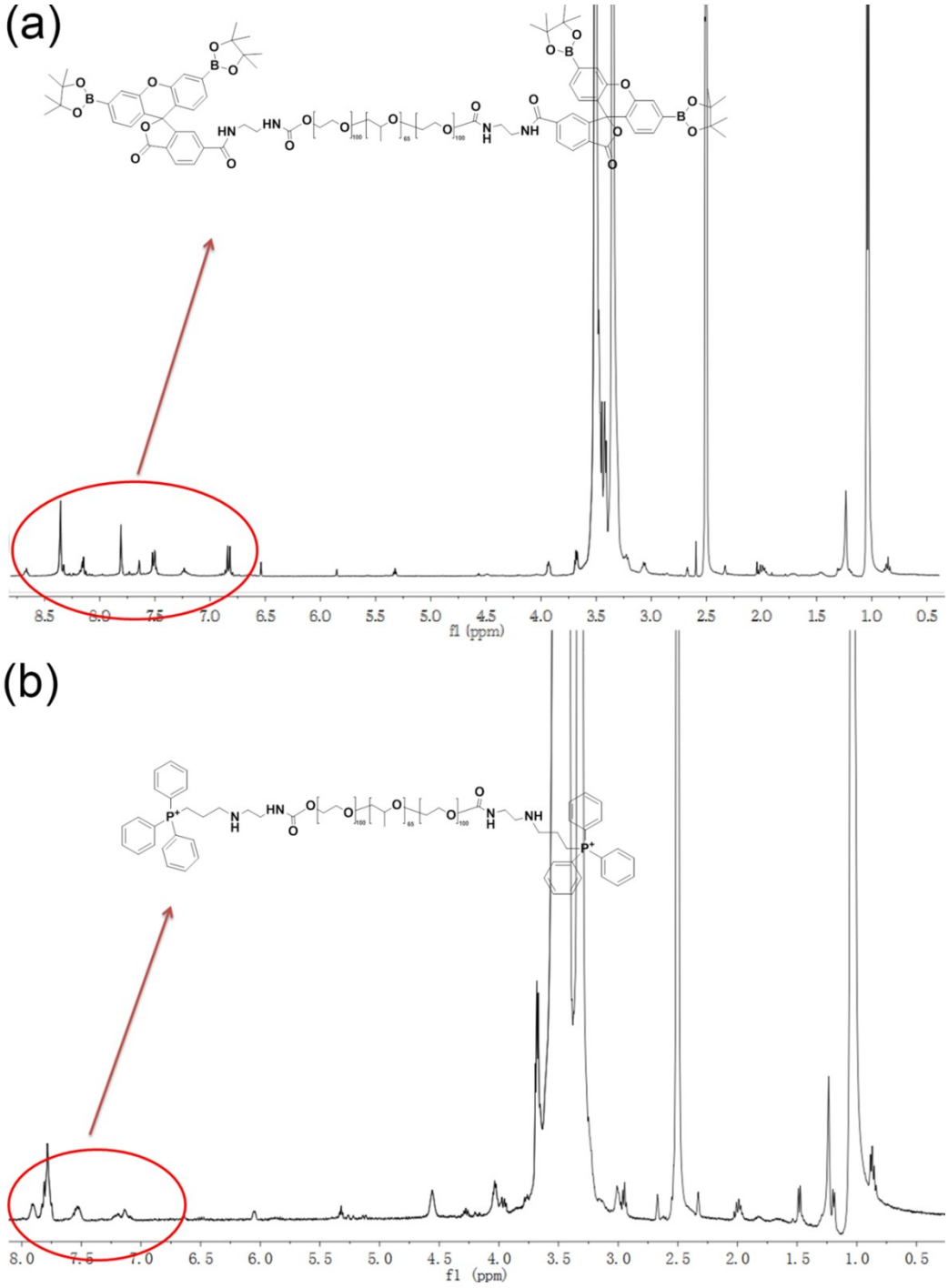


Fig.S5 ^{1}H NMR spectra of PF1-F127 (a) and Mito-F127 (b).

Preparation of TPE-loaded micelles (TPE/F127).

TPE-loaded Pluronic F127 micelles were prepared by thin-film hydration methods. Briefly, 10 mg of TPE, and 270 mg of F127 were dissolved in 20 mL dichloromethane in a round bottom

flask. The solvent was evaporated by rotary evaporation to obtain a solid TPE/F127 matrix. Residual dichloromethane remaining in the film was removed under vacuum overnight at room temperature. The resultant thin film was hydrated with 10 mL HEPES buffer (20 mM, pH=7.0) for 30 min to obtain a micelle solution, which was then filtrated through 0.22 μm filter membrane to remove the uncorporated TPE. PF1COOH and Mito-modified polymeric micelles encapsulating TPE (PMT/F127) were prepared as described above except that Pluronic F127 was substituted by adding a mixture of 200 mg F127, 50 mg PF1-F127 and 20 mg Mito-F127. For leakage rate study, TPE/F127 micelle solutions were transferred to a dialysis bag (molecular weight cut–off of 3600 Da) and dialyzed against HEPES (20 mM, pH 7.0) for one day.

Fluorescence Spectra Measurement in Aqueous Media.

The fluorescence spectra for the probe (PMT/F127) in the presence of H_2O_2, other ROS or some non-ROS analytes (such as ascorbic acid, GSH, Fe^{3+}, NO_3^-, etc.) in HEPES buffered (20 mM, pH 7.0) water were recorded at 25 °C. Various analytes were respectively added to the HEPES buffered solutions containing the probe PMT/F127; and then the fluorescence spectra were recorded with the excitation wavelength at 360 nm. The preparation of various ROS for the fluorescence measurement is described as follows. hypochlorite (NaClO), H_2O_2 and tert-butyl hydroperoxide (TBHP) were delivered from their 10%, 30% and 70% aqueous solutions respectively. Hydroxyl radicals (·OH) and tert-butoxy radicals (·OtBu) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or 200 μM TBHP, respectively. Nitric oxide (NO) was used from its stock solution (1.0 mM), which was prepared by dissolving DEA/NONOate in 0.01 M NaOH aqueous solution.

Determination of Fluorescence Lifetime.

The fluorescence lifetime wasmeasured by time-correlated single-photon counting on PicoQuantFluoTime 200 avalanche photodiode using 340 nm light for excitation of TPE donor. Intensity decay curves obtained were fitted as follows:

$$I(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_i)$$

Where A_i is a preexponential factor representing the functional contribution to the time-resolved decay of the component with a lifetime τ_i.

Calculation of the concentration of PMT/F127 micelles and PFl:

Calculation of the concentration of PMT/F127 micelles and PFlCOOH were according to the literature[S3] simply based on the absorbance increment of the PMT/F127 micelles and PFl moieties on the nanoprobe. TPE loaded hybrid F127 micelles exhibit their characteristic absorption band at 365 nm. PF1COOH exhibit a weak absorption band in the absence of H_2O_2, but turns to 6-carboxyfluorescein (FAM) when reaction with H_2O_2, displaying a strong absorption at 490 nm. Thus the concentration of the PMT/F127 and PF1COOH can be calculated by using TPE/F127 micelles and 6-carboxyfluorescein as the standards measuring their extinction coefficient at 365 nm and 490 nm in water. On the basis of Beer's law, we can use the following equations to calculate the amount PMT/F127 and PFlCOOH:

$$A_{365nm} = \varepsilon_{TPE/F127_{365}} \times C_{TPE/F127} \times b + \varepsilon_{6-carboxyfluorescent_{365}} \times C_{6-carboxyfluorescent_{365}} \times b$$
$$A_{490nm} = \varepsilon_{TPE/F127_{490}} \times C_{TPE/F127} \times b + \varepsilon_{6-carboxyfluorescent_{490}} \times C_{6-carboxyfluorescent_{365}} \times b$$

The co-efficients $\varepsilon_{TPE/F127_{365}}$, $\varepsilon_{TPE/F127_{490}}$ were determined as Figure S6. $\varepsilon_{6-FAM\,365}$, $\varepsilon_{6-FAM\,490}$ were obtained from literature[S3].

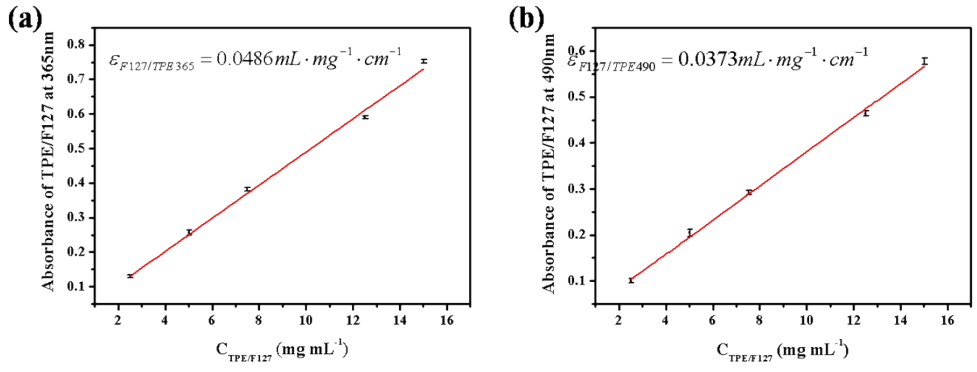


Fig. S6 Plot of absorbance to concentrations of TPE/F127 at 365 nm and 490 nm, all of the measurements were conducted in triplicate.

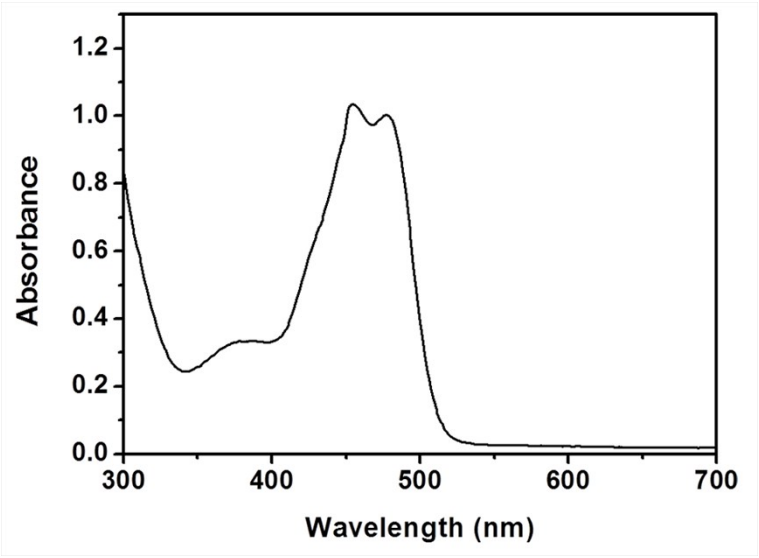


Fig. S7 Absorption spectrum of PMT/F127 (dilute for 4 times) upon addition of excessive H_2O_2 in HEPES (pH 7.0).

By using the extinction coefficient values shown in Fig. S6a and the absorbance values at 365 nm and 490 nm from Figure S7b, we can obtain the following equations:

$$A_{365nm} = 0.309 = 0.0486 \times C_{TPE/F127} + 2210 \times C_{6-carboxyfluorescent_{365}}$$
$$A_{490nm} = 0.781 = 0.0373 \times C_{TPE/F127} + 73530 \times C_{6-carboxyfluorescent_{365}}$$

Therefore, $C_{PMT/F127}$ can be calculated as 24 mg mL^{-1} and $C_{6\text{-carboxyfluorescein}}$ (= $C_{PF1COOH}$) be calculated as 14.8 μM in water.

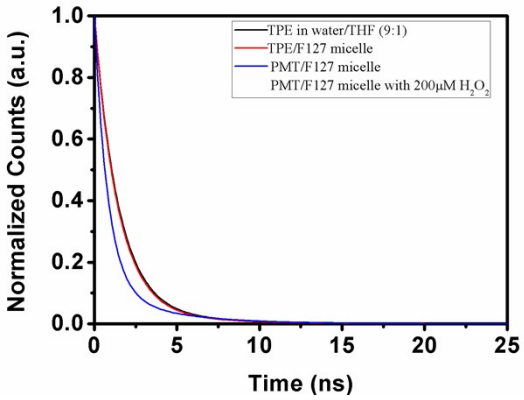


Fig. S8 The fluorescence decay of PMT/F127 micelle with 200 μM H_2O_2.

Table S1. Fluorescence lifetime of TPE in differrent environments.

	A_1/A_2	τ_1(ns)	τ_2 (ns)	τ_{avg} (ns)
TPE in H_2O/THF (9:1)	0.41/0.59	2.07	5.78	4.26
TPE/F127 in H_2O	0.83/0.17	1.44	5.30	2.09
PMT/F127 in H_2O	0.85/0.15	1.41	5.12	1.97
PMT/F127 in H_2O with 200 μM H_2O_2	0.92/0.08	0.82	3.82	1.03

A_1 andA_2 are pre-exponential factors representing the functional contributions to the time resolved decay of the components with a lifetime τ_1 and τ_2 respectively.

Determination of Quantum Yields:

The Fluorescence quantum yield φ of TPE/F127 was measured using Hitachi U-3010 UV–Vis spectrophotometer and Hitachi F-4600 fluorescence spectrophotometer. Diethylaminocoumarin excited at 380 nm were used as standard to determine the fluorescence quantum yields of TPE loaded in F127 micelle in aqueous solutions. The Quantum yield φ was calculated according to the following equation :

$$\varphi_s = \varphi_r \left(\frac{A_r}{A_s}\right)\left(\frac{I_s}{I_r}\right)\left(\frac{n_s}{n_r}\right)^2$$

where φ_s and φ_r are the fluorescence quantum yield of standards and the samples, respectively; A_r andA_s are the absorbance of the standards and measured samples at the excitation wavelength, respectively. I_r and I_s are the integrated emission intensity of standards and samples, and n_r and n_s

are the refractive indices of the corresponding solvents of the solutions, respectively. The final value of quantum yield was obtained from the average of four measurements with different absorbance in the range between 0.02 and 0.09. The standard deviation is less than 10%.

The measurement of H_2O_2 concentration

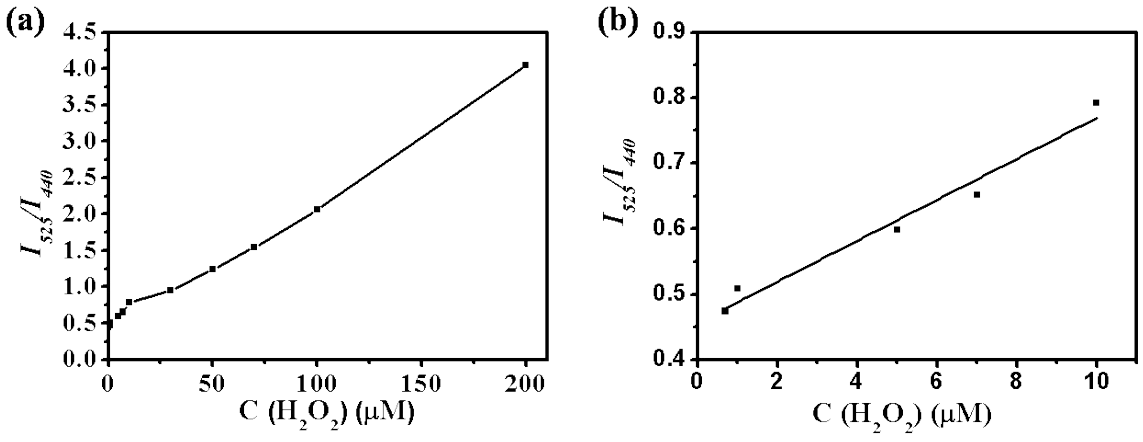


Fig. S9 The fluorescent intensity ratio of the nanoprobe (24 mg mL^{-1}) at different H_2O_2 concentration in pH 7.0 HEPES (λ_{exc} = 360 nm).

The detection limit was calculated as following:

The detection limit = 3 × S.D./ k

where k is the slope of the curve equation, and S.D. represents the standard deviation for the PMT/F127 probe solution's fluorescence intensity ratio in the absence of H_2O_2.

I_{525}/ I_{440}= 0.456 + 3.1 E4 [H_2O_2]

LOD = 3 × 0.01 / 3.1 E4 = 0.97 × 10^{-6}(M)

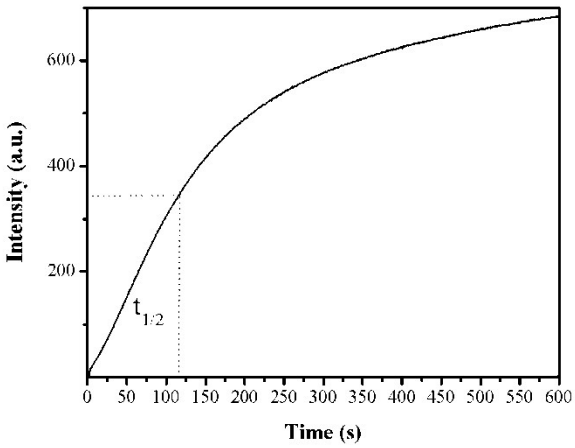


Fig. S10. Time-course kinetic measurement of the fluorescent intensity changes toward 1000 equiv of H_2O_2 excited at 490 nm in HEPES (20 mM, pH 7.0), slits 2.5/2.5 nm. Data were collected under pseudo-first-order conditions.

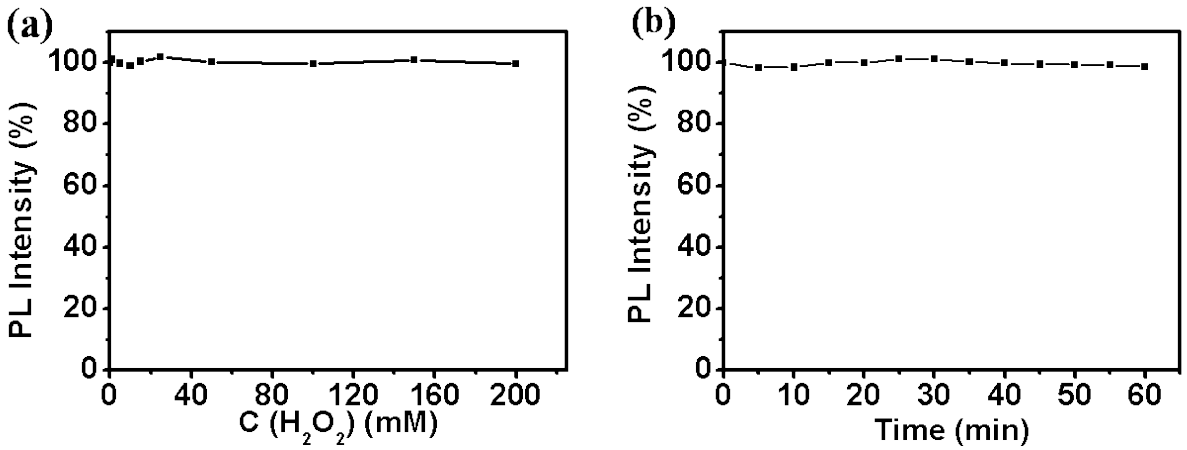


Fig. S11 The response of florescent intensity of TPE/F127 micelles (24 mg mL^{-1}) (a) upon the addition of different concentrations of H_2O_2 and recorded after 40 min, and (b) under different time of continued UV treatment in the presence of 200 μM H_2O_2 in pH 7.0 HEPES.

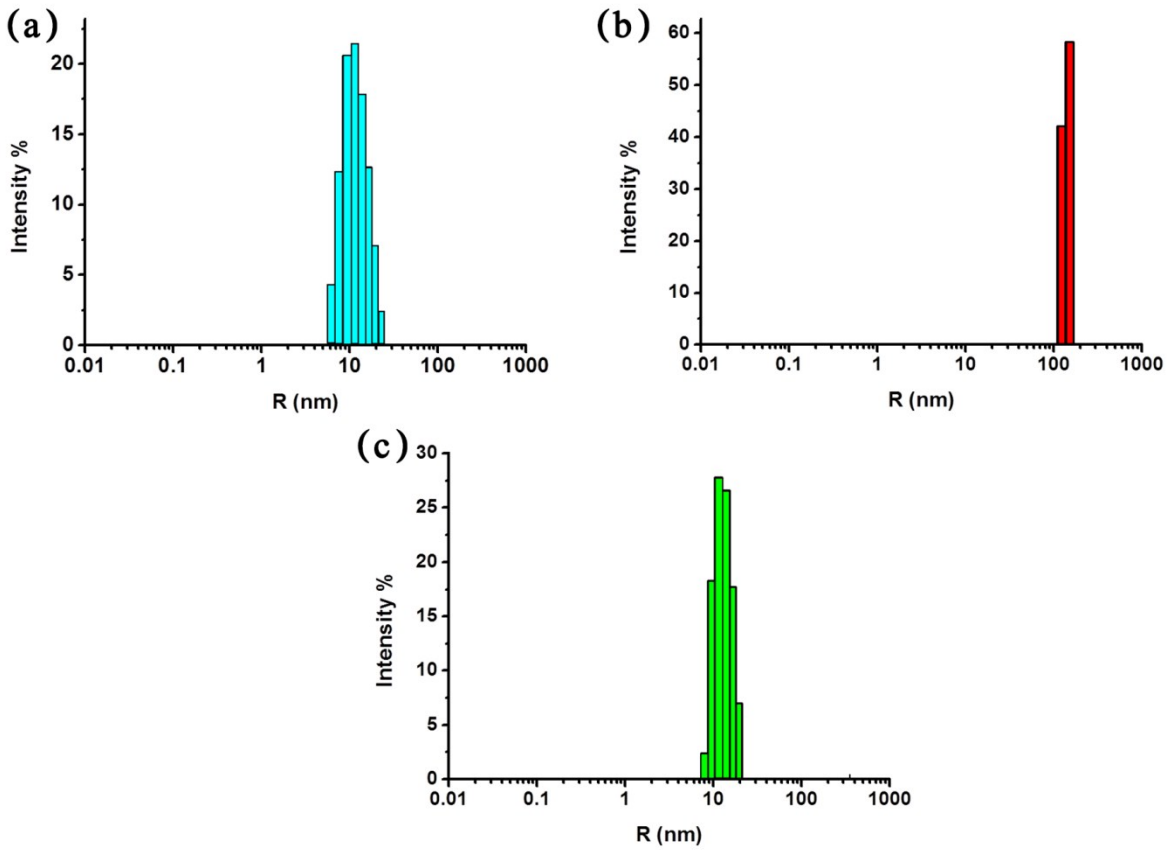


Fig. S12 Hydrodynamic radius measured by DLS of (a) PMT/F127 (24mg mL^{-1}) in pH 7.0 HEPES buffered water, (b) PMT/F127 (24 mg mL^{-1}) in DMEM (filtrated through 0.22 μm filter membrane), and (c) PMT/F127 micelles (0.24 mg mL^{-1}) in DMEM. All the data were obtained at 37 °C.

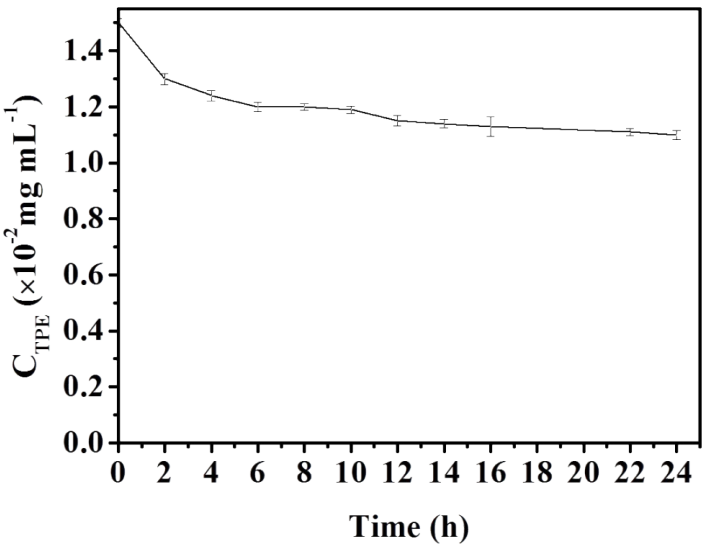


Fig. S13 Leakage rate study of TPE/F127 micelles in 20 mM HEPES (pH 7.0), all of the measurements were conducted in triplicate.

Cell Viability Assay.

The cytotoxicity of the probe against Hela cells was assessed by using a Cell Counting Kit-8 (CCK-8) method. The cells harvested in a logarithmic growth phase were seeded in 96-well plates with a density of 5000 cells per well. Cells were incubated in DMEM (Solarbio, China) supplemented with 10% Fetal bovine serum (FBS, Gibco®) and 1% penicillin/streptomycin (P/S) at 37 °C in 5% CO_2. Then the cells were incubated with the growth medium containing micelles of different concentrations. The micelles concentrations of each formulation were prepared by serial dilution with DMEM medium. After a treatment for 24 h, 10 μL of the CCK-8 solution was added to each well of the plate, followed by incubation for another 3 h. Then, cell viability was determined by measuring absorbance at 450 nm with a microplate reader. By recording the absorbance at 450 nm using EnSpire Multimode Plate Reader (PerkinElmer), the cell viability was determined as followed:

$$\text{Cell viability (\%)} = \frac{I_{sample}}{I_{control}} \times 100\%$$

Where I_{sample} and $I_{control}$ represent the intensity determined for cells treated with different samples and for control cells (untreated), respectively. All of the measurements were conducted in triplicate.

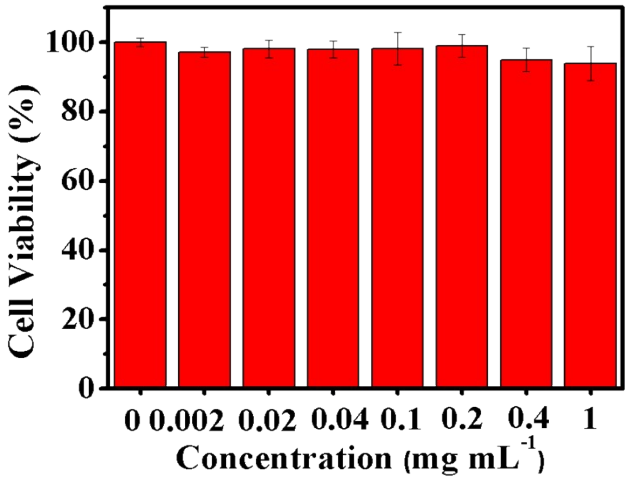


Fig. S14 Viability of HeLa cells incubated with varied concentration of PMT/F127 micelles for 24 h. The viability was calculated relative to the control group.

***In Vitro* Cell Labeling and Imaging.**

HeLa cells and Raw 264.7 cells were incubated in Dulbecco's modified Eaglemedium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (P/S) at 37 °C in 5% CO_2. Forintracellularimaging, thecellswereseeded in glass-bottomcellculturedishes (NEST Biotechnology Co., LTD.) andculturedovernight.

For co-localization study, HeLa and Raw 264.7 cells were incubated with the probe (0.24 mg mL^{-1}) for 24 h after which MitoTracker® Deep Red (50 nM) or LysoTracker® Deep Red (50 nM) were added for another 0.5 h. After replacing the loading solution with fresh growth medium, the cells were imaged directly on Nikon Eclipse Ticonfocal laser scanning microscopy (CLSM) with

a TDKAI HIT live cell imaging system, no further fixation and staining needed. With excitation at 405 nmand 640 nm, cells were imaged by collecting fluorescence channel of 425−475 nm (TPE), and 650−690 nm (MitoTracker® Deep Red or LysoTracker® Deep Red). In order to compare clearly, we changed the blue color to green.

To measure the exogenous H_2O_2 ratiometricly, HeLa cells were treated with PMT/F127 (0.24 mg mL^{-1}) for 1 h, during the last 40 min of which the cells were co-incubated with 0 μM, 50 μM and 200 μM H_2O_2. After replacing the loading solution with fresh growth medium, the cells were placed on the confocal microscope for imaging. The fluorescence was then collected in the ranges of 425–475 nm (blue channel) and 500–550 nm (green channel), respectively.

For the endogenous H_2O_2 detection, Raw 264.7 macrophages were pre-treated with PMT/F127 (0.24 mg mL^{-1}) for 1 h, during the last 40 min of which the cells were added 0 (for control), 1 μg mL^{-1} and 2 μg mL^{-1} PMA. Other processes were the same as HeLa cells.

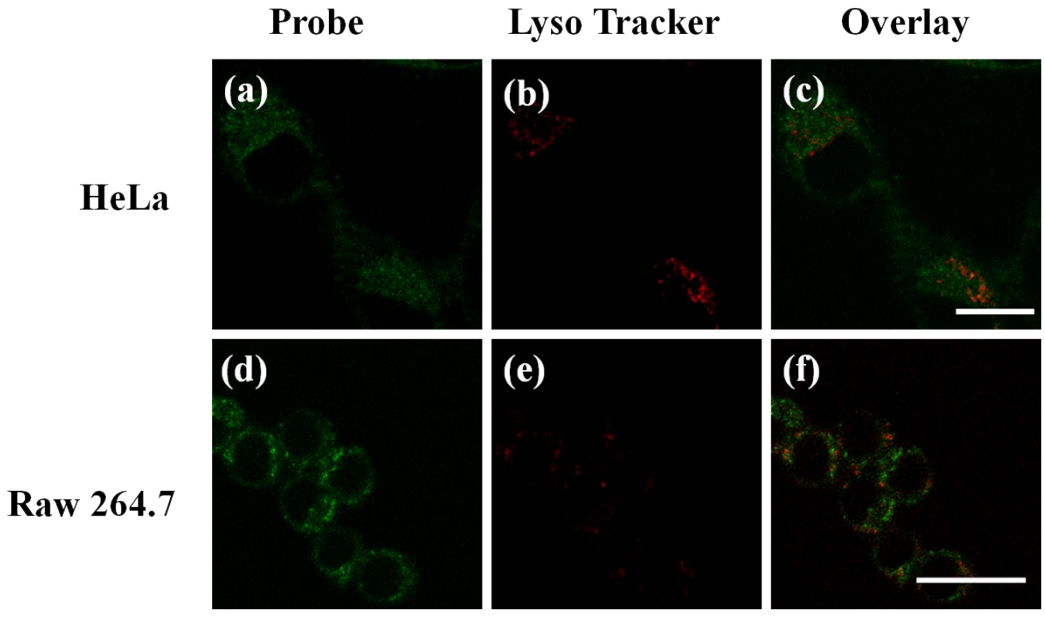


Fig. S15. Confocal laser scanning microscopy (CLSM) images ofHeLa cells (a–c) and Raw 264.7 (d–f) cells co-stained with PMT/F127 (0.24 mg mL^{-1}) and LysoTracker® Deep Red (50

nM).The images were collected at 425–475 nm for PMT/F127 (a and d, color changed to green by the software for better observation); 650–690 nm for LysoTracker® Deep Red (b and e); (c) and (f) are the merge images from the two channels (scale bars: 20 μm).

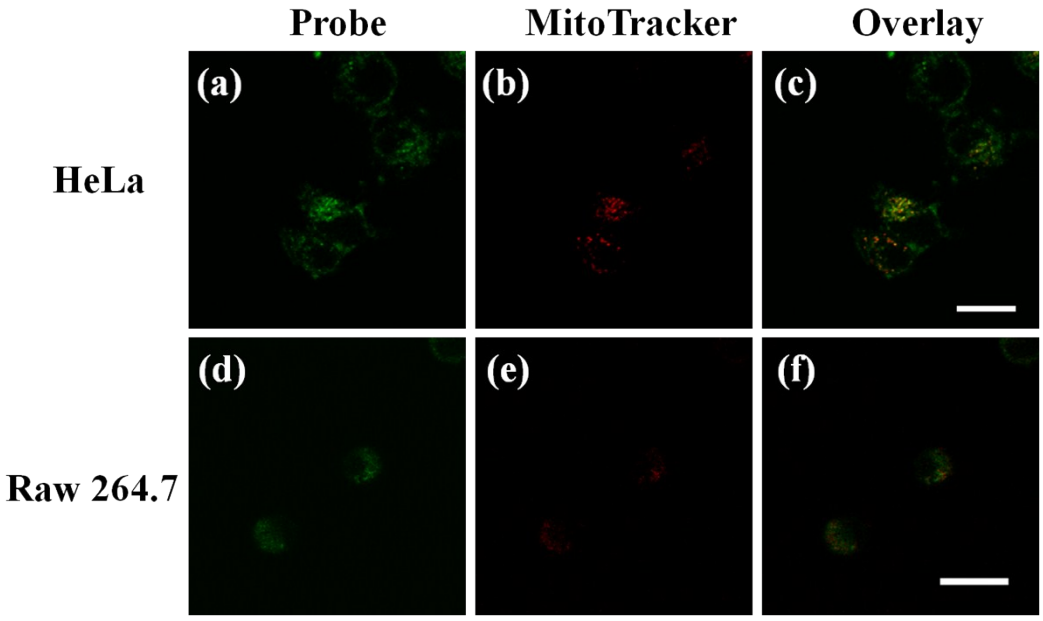


Fig. S16. CLSM images of HeLa cells (a–c) and Raw 264.7 (d–f) cells co-stained with TPE/F127 (0.24 mg mL^{-1})and MitoTracker® Deep Red(50 nM).The images were collected at 425–475 nm for PMT/F127 (a and d, color changed to green by the software for better observation); 650–690 nm for MitoTracker® Deep Red (b and e); (c) and (f) are the merge images from the two channels (scale bars: 20 μm).

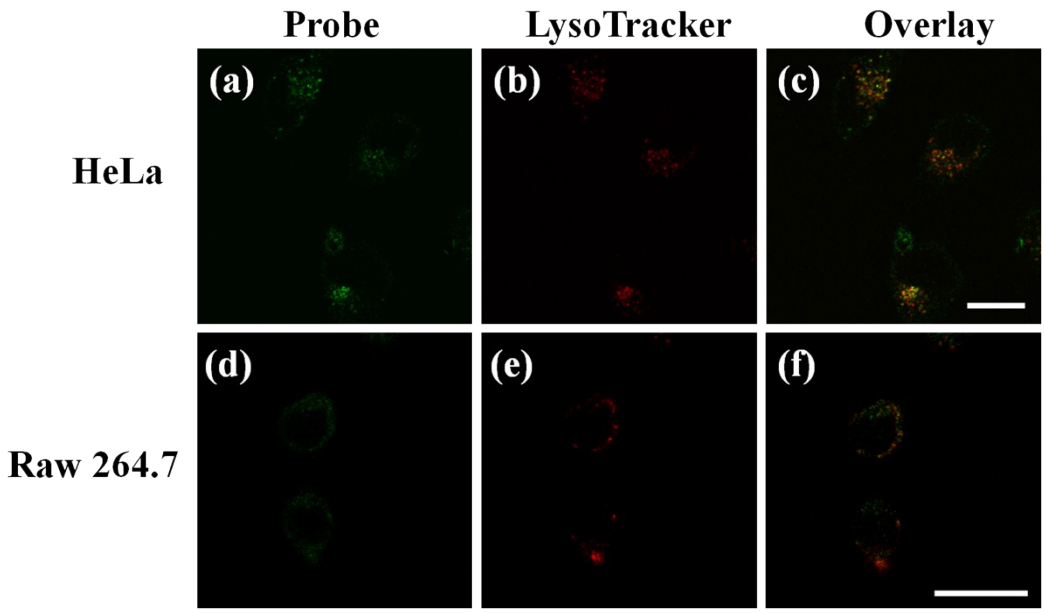


Figure S17. CLSM images of HeLa cells (a–c) and Raw 264.7 (d–f) cells co-stained with TPE/F127 (0.24 mg mL^{-1})and LysoTracker® Deep Red (50 nM).The images were collected at 425–475 nm for TPE/F127 (a and d, color changed to green by the software for better observation); 650–690 nm for LysoTracker® Deep Red (b and e); (c) and (f) are the merge images from the two channels (scale bars: 20 μm).

Table S2. The Pearson's colocalization coefficient of nanoprobe with MitoTracker® and LysoTracker®.

	HeLa	Raw 264.7
PMT/F127 and MitoTracker®	0.76	0.65
PMT/F127 and LysoTracker®	0.37	0.23
TPE/F127 and MitoTracker®	0.46	0.37
TPE/F127 and LysoTracker®	0.52	0.46

References.

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2. W. Zhang, Y. A. Shi, Y. Z. Chen, J. A. Ye, X. Y. Sha and X. L. Fang, *Biomaterials*. 2011, **32**, 2894.
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