Electronic Supplementary Information

Cyanine Fluorophore for Cellular Protection Against ROS in Stimulated Macrophages and Two-Photon ROS Detection

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Contents:

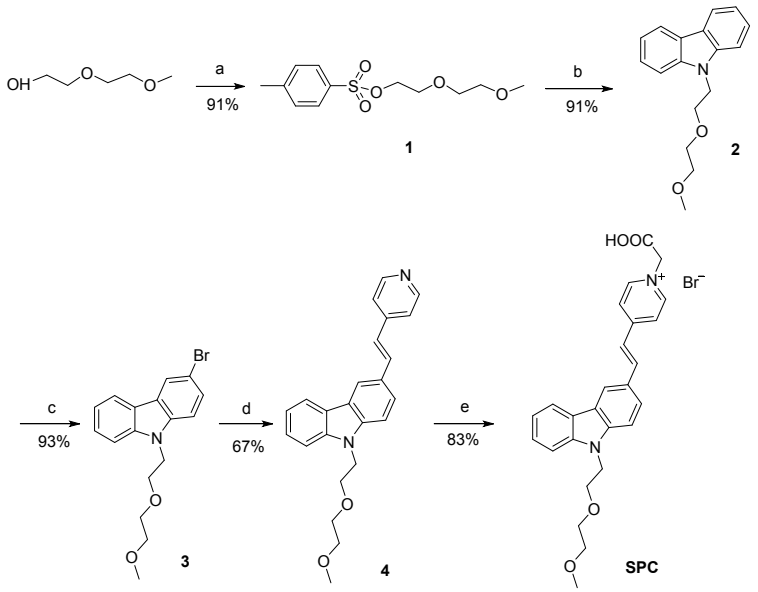
1. Materials and reagents

Lipopolysaccharide (LPS), phorbol-12 myristate-13 acetate (PMA), Tiron, diethylenetriamine pentaacetic acid, potassium superoxide and 12 M hydrochloric acid were used as purchased from Sigma- Aldrich. Sodium molybdate, sodium nitrite, sodium hydroxide, calcium chloride, sodium chloride, zine chloride, potassium chloride and barium chloride were purchased from J&K. 37% of hydrogen peroxide was purchased from UNI-CHEM. Sodium hypochlorite was purchased from Acros Organics. Fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM) and penicillin streptomycin solution were purchased from Invitrogen.

2. Instrumentation

Absorbance, emission, stability and quenching of SPC in different solutions were characterized by UV-VD (Agilent 8453) and Spectrofluorometer (Fluormax-4). Confocal fluorescence imaging and bright field imaging were performed on Laser Confocal Scanning Microscope (Leica TCS SP5) with magnification of 63X. The MTT experiment was conducted at Bio Tek Powerwave XS microplate reader. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker-400 NMR spectrometer. Mass spectroscopy (MS) measurements were carried out by fast atom bombardment on the API ASTER Pulser I Hybrid Mass Spectrometer or matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technique.

3. Synthesis and characterization of SPC



Scheme S1 Reagents and conditions：a) 4-methylbenzene-1-sulfonyl chloride, NaOH, H_2O; b) carbazole, NaH, THF, reflux; c) NBS, $CHCl_3$, 0°C to rt; d) 4-vinylpyridine, $Pd(OAc)_2$, $P(o\text{-}tol)_3$, Et_3N, DMF, 90°C; e) Ethanol, bromoacetic acid.

(*E*)-1-(carboxymethyl)-4-(2-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazol-3-yl)vinyl)pyridin-1-ium bromide (SPC) A solution of **4** (0.17 g, 0.5 mmol) and bromoacetic acid (0.28 g, 2.0 mmol) in ethanol was stirred overnight at room temperature. After removing the solvent, the residue was precipitated from methanol and ethyl acetate to afford SPC in 83% yield.

^{1}H NMR (400MHz, DMSO-d_6) δ 8.68 (d, *J*=5.2 Hz, 2H, Ar-CH), 8.56 (s, 1H, Ar-H), 8.11-8.19 (m, 4H, C=CH and Ar-H), 7.86 (dd, *J*=1.2 Hz, *J*=8.4 Hz, 1H, Ar-H), 7.72 (d, *J*=8.8 Hz, 1H, Ar-H), 7.67 (d, *J*=8.4 Hz, 1H, Ar-H), 7.53-7.47 (m, 2H, C=CH and Ar-H), 7.27(t, *J*=7.2, 1H, Ar-H), 4.84(s, 2H, CH_2COO), 4.58(t, *J*=5.2 Hz, 2H, N-CH_2), 3.81(t, J=5.2 Hz, 2H, CH_2), 3.44-3.47 (m, 2H, CH_2), 3.30-3.28 (m, 2H, CH_2), 3.10(s, 3H, OCH_3). ^{13}C NMR (400MHz, $CDCl_3$) **δ**170.4, 152.7, 144.8, 141.9, 141.7, 140.9, 126.4, 126.3, 126.1, 122.7, 122.2, 122.1, 120.9, 120.3, 120.1, 119.7, 110.4, 110.2, 71.3, 69.8, 68.8, 58.1, 42.9;HRMS (MALDI-TOF) m/z Calcd for $C_{26}H_{27}N_2O_4$ 431.1965 Found 431.1953[M^+]

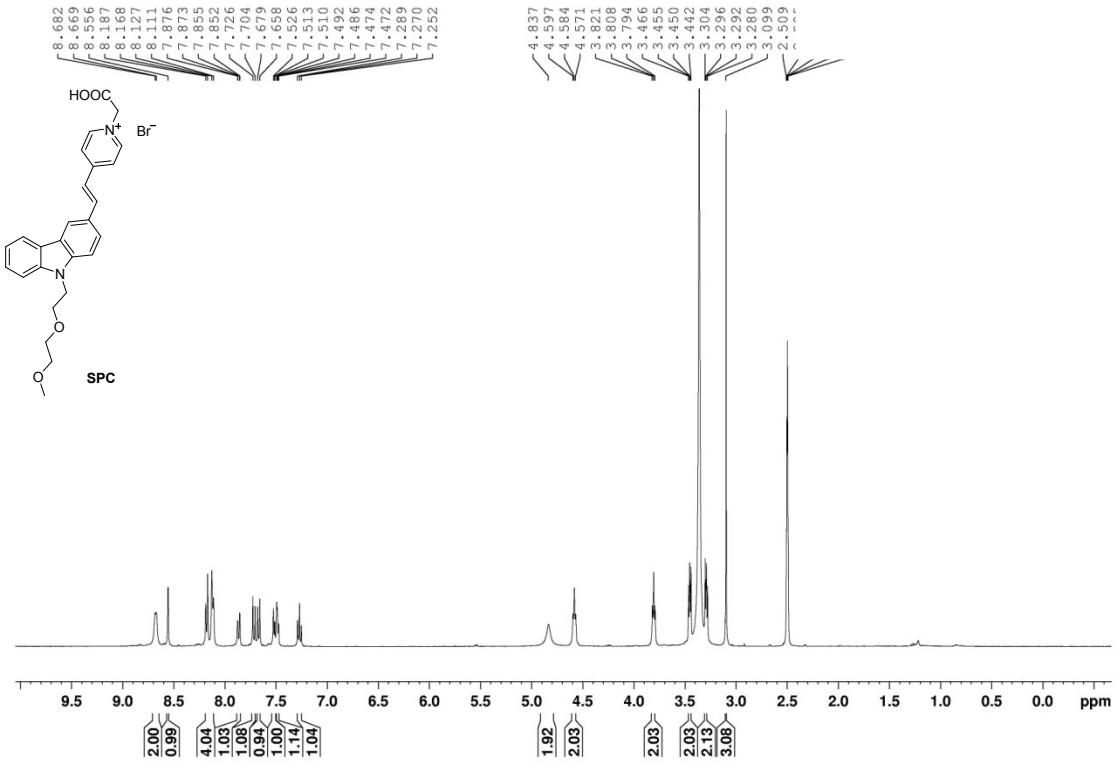


Fig. S1 ^{1}H NMR of SPC in DMSO.

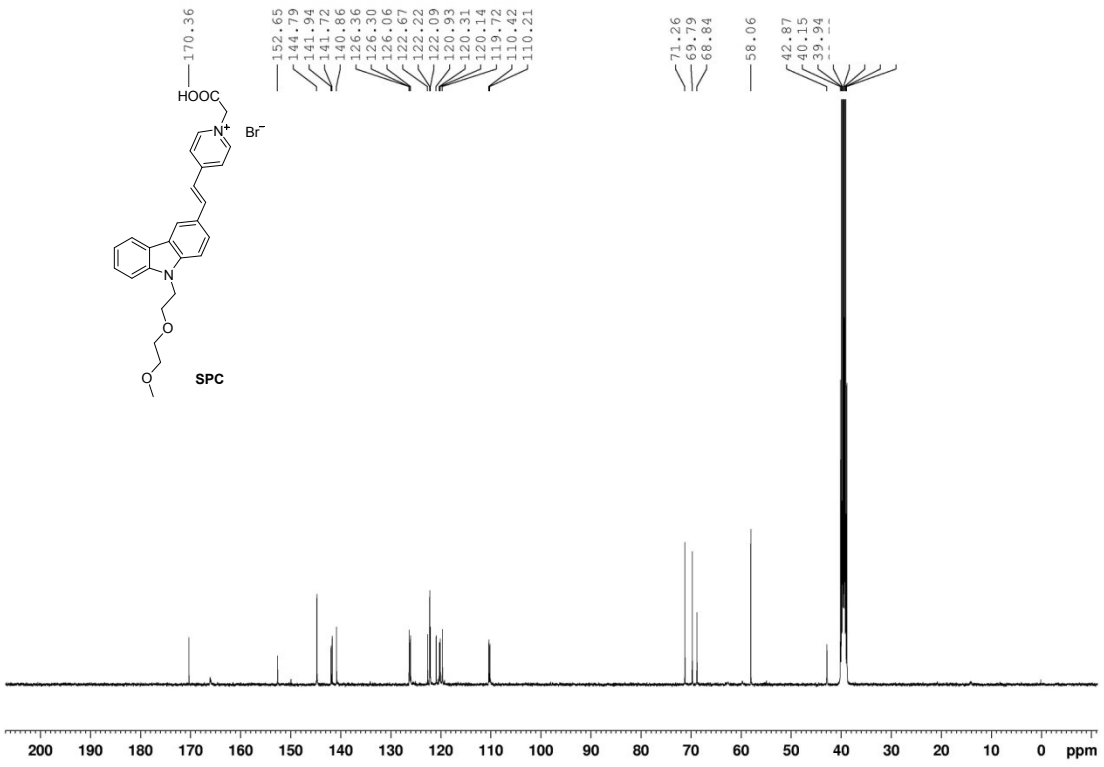


Fig. S2 ^{13}C NMR of SPC in DMSO.

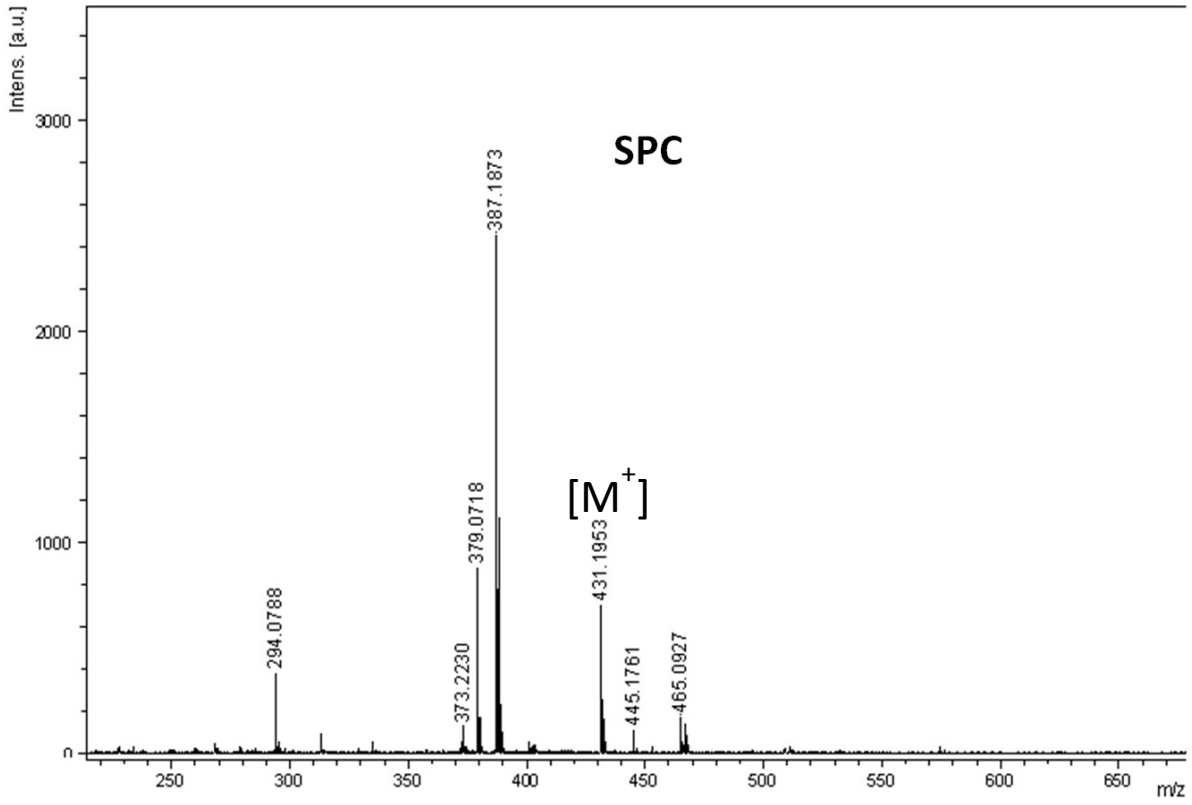


Fig. S3 HRMS (MALDI-TOF) of SPC**.**

4. Physical properties of SPC

UV-vis absorption spectra were measured by preparing 0.01 mM SPC in DMSO or in phosphate buffer (PB). Fluorescence emission spectra were measured by preparing 0.001 mM SPC in DMSO or in PB. SPC samples in DMSO and in PB were excited at 430 and 423 nm respectively, and then collected their emission signals from 450 nm to 800 nm.

	Solvent	λ^{abs}_{max}/nm ε ($\times 10^4/M^{-1}cm$)	λ^{em}_{max}/nm (stoke shift）/nm	Φ_{PL}
SPC	DMSO	431 (2.48)	563 (132)	0.15
	PB	423 (3.26)	575 (151)	0.012

Table S1. Physical measurements of SPC in DMSO and in phosphate buffer.

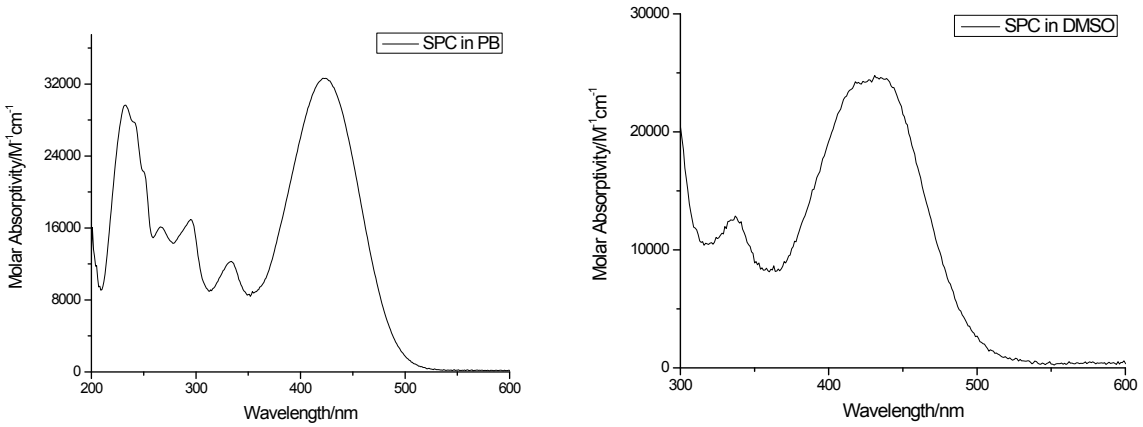


Fig. S4 Absorption spectra of SPC in different media.

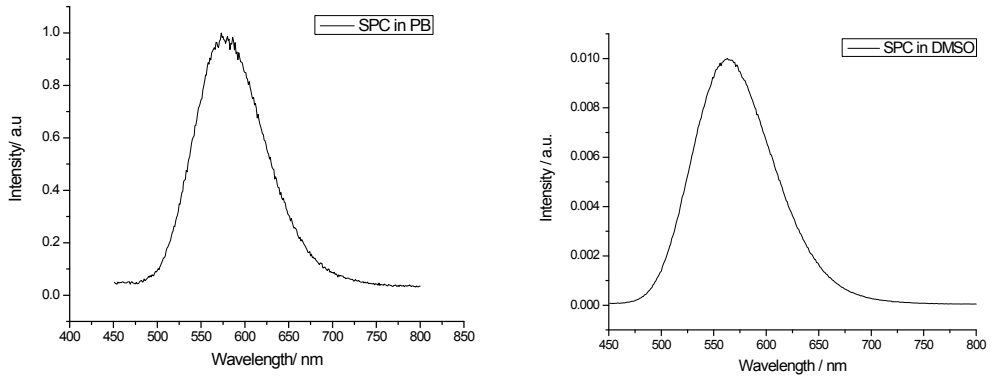


Fig. S5 Emission spectra of SPC in different media.

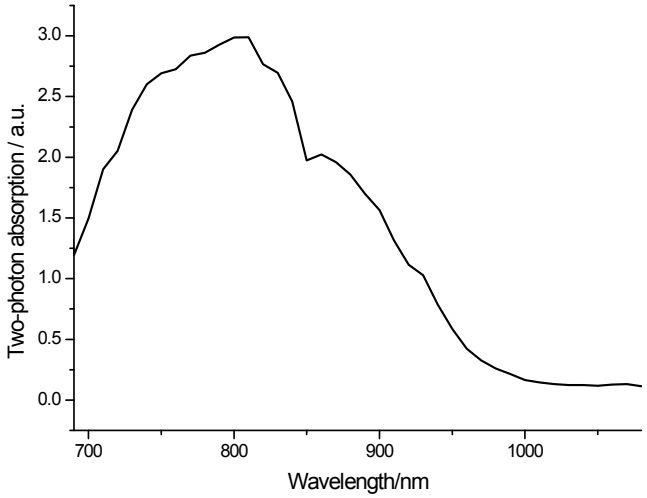
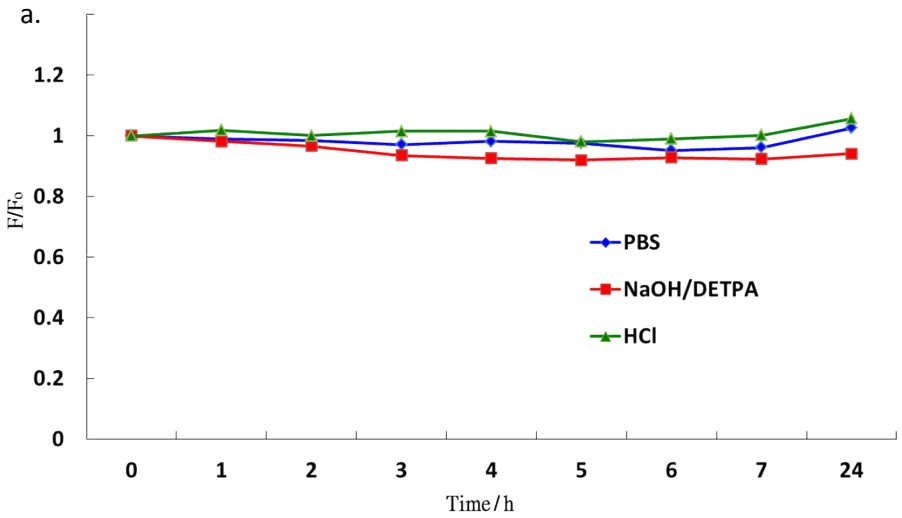


Fig. S6 The two-photon excitation spectrum of SPC.

5. Stability of SPC

The stability of SPC is measured as a function of time under particular pH condition and as a function of pH for 24 h. Fluorescence emission of SPC at 575 nm was obtained by measuring 23 μM of SPC at different time points being exposed to air (e.g 0, 1, 2, 3, 4, 5, 6, 7, and 24 h) in PBS, NaOH and HCl solutions individually (Fig. S7a) and in solutions under various pH values (Fig. S7b). The SPC samples were excited at 423 nm and then collected the emission data at 575 nm.



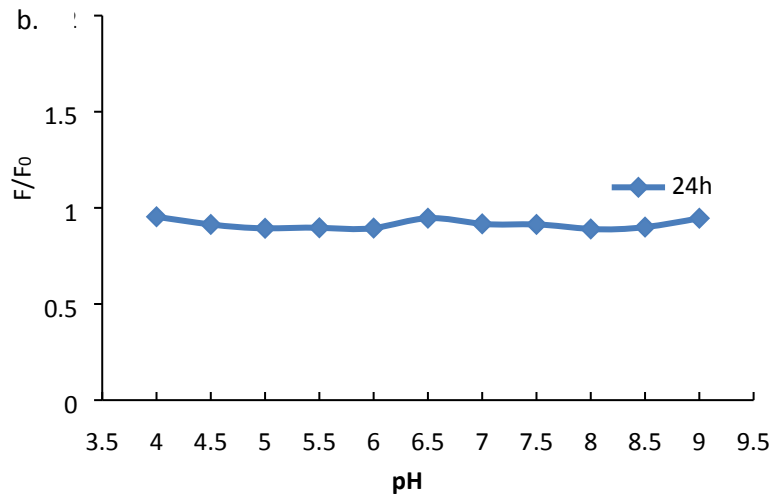


Fig. S7 (a) Fluorescence responses of SPC probe to (a) different solutions under pH 4, 7.4 and 10 as a function of time, (b) various pH from 4.0 to 9.0 with intervals of 0.5 after standing in air for t = 24 h. F_0 represented the measured fluorescence intensity of 23 μM SPC probe at initial stage t = 0. F represents the measured fluorescence intensity of 23 μM SPC after standing in air. Data were acquired at 25 °C in aqueous media, λ_{ex} = 423 nm, λ_{em} = 575 nm.

6. Preparation of ROS and metal ion solutions

Various ROSs and metal ion solutions have been prepared according to the previously reported methods.[i] Detail preparation information is the following:

Various metal ion solutions: 8mM of different types of cations including Ca^{2+}, Na^{+}, K^{+}, Ba^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} were prepared from commercially available metals chloride. **Singlet oxygen**: Singlet oxygen was generated by mixing sodium molybdate dehydrate with H_2O_2 at molar ratio of 1: 2 under pH range from 9.5 to 11.5. **Hydrogen peroxide:** 8 mM hydrogen peroxide was generated by diluting 37% H_2O_2. The final concentration of H_2O_2 was determined by its absorbance at 240 nm (ε = 43.6 $M^{-1}cm^{-1}$) **Hypochlorite anions**: 8 mM OCl^- was prepared by diluting concentrated commercially available NaOCl. The concentration of OCl^- was determined by its absorbance at 292 nm (ε = 350 $M^{-1}cm^{-1}$). **Superoxide**: 8mM superoxide was initially prepared by dissolving KO_2 in 50 mM ice-cold NaOH solution and then mixing with 0.5 mM diethylenetriamine pentaacetic acid. **Peroxynitrite**: 8 mM nitrite and nitrate anions were prepared from sodium nitrite and sodium nitrate. ***Tert*-butyl hydroperoxide** 100 μM of *Tert*-butyl hydroperoxide was mixed with 0.1 M sodium phosphate buffer at 37°C, pH 7.4 followed by adding 0.1% DMF as co-solvent. **Cysteine**: 8 mM cysteine solution was prepared from commercially available cysteine. **Hydroxide radical:** 8 mM of Iron(II) perchlorate and 37% hydrogen peroxide were mixed and

added up to total volume of 5 mL with D. I. water. The ratio of H_2O_2 to Fe^{2+} is 10:1 through the Fenton reaction.

7. Spectroscopic changes of SPC after reacting with superoxide or hypochlorite

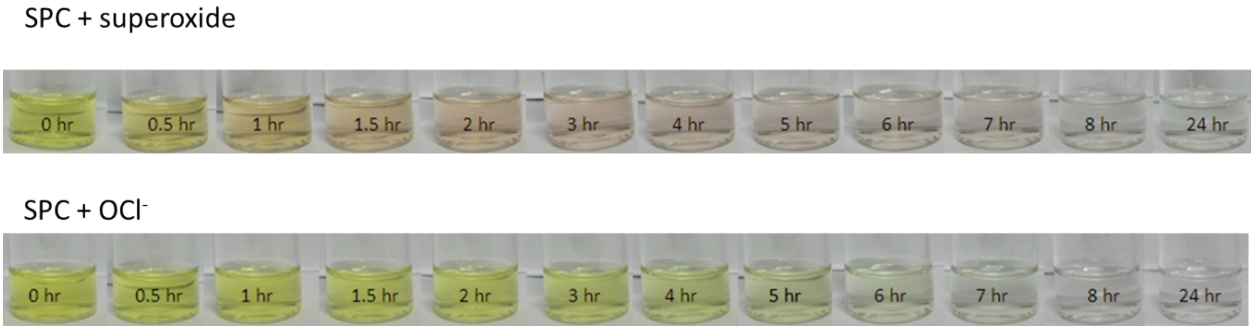
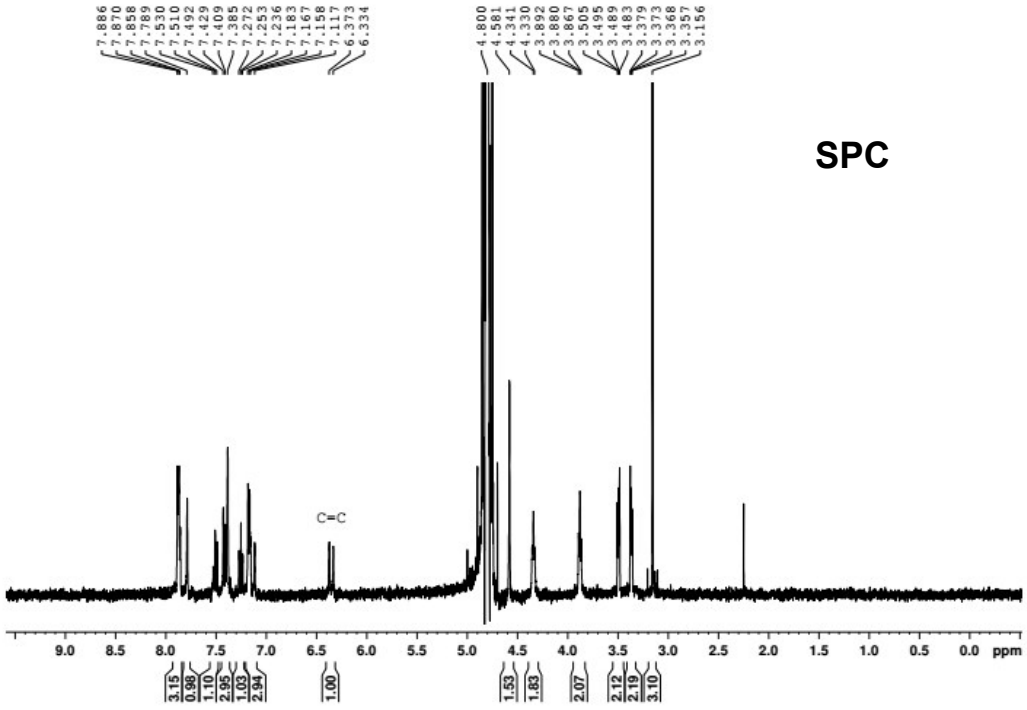


Fig. S8 A color change of 23 μM SPC solution as a function of time after reacting with superoxide or hypochlorite anions.



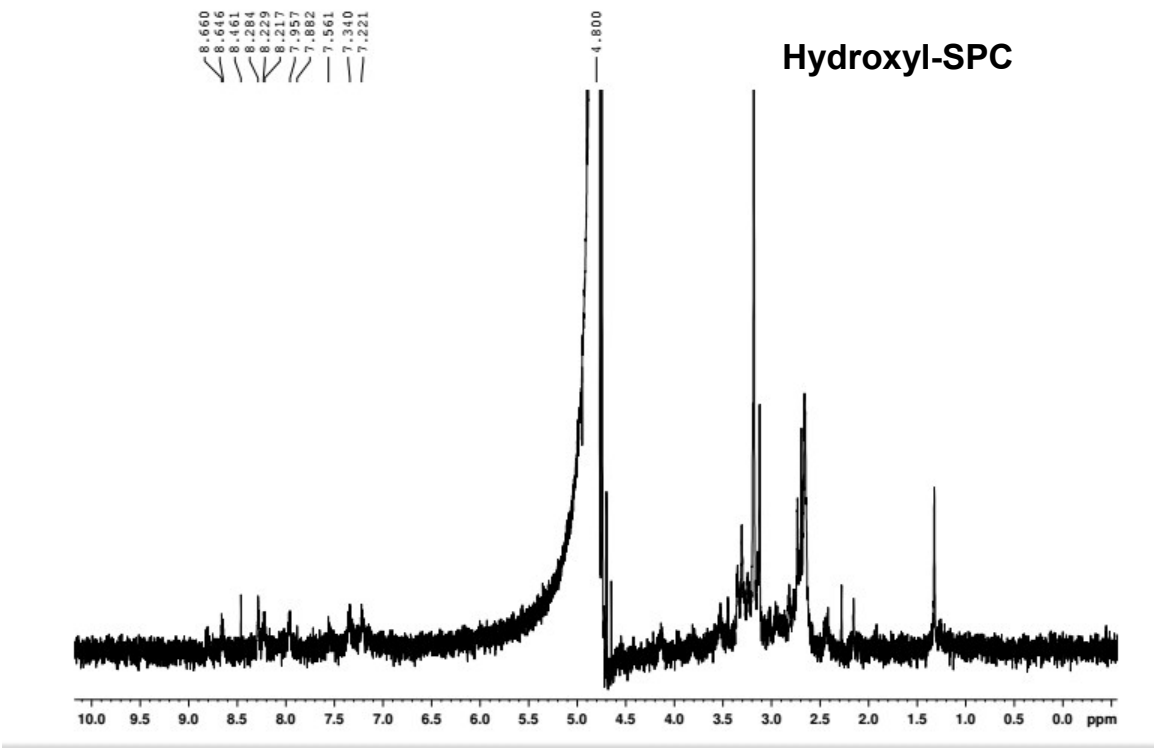


Fig. S9 ^{1}H NMR spectra of SPC before and after reacting with superoxide in a mixture of DETPA. The double bond peaks at 6.3 ppm was disappeared after the reaction.

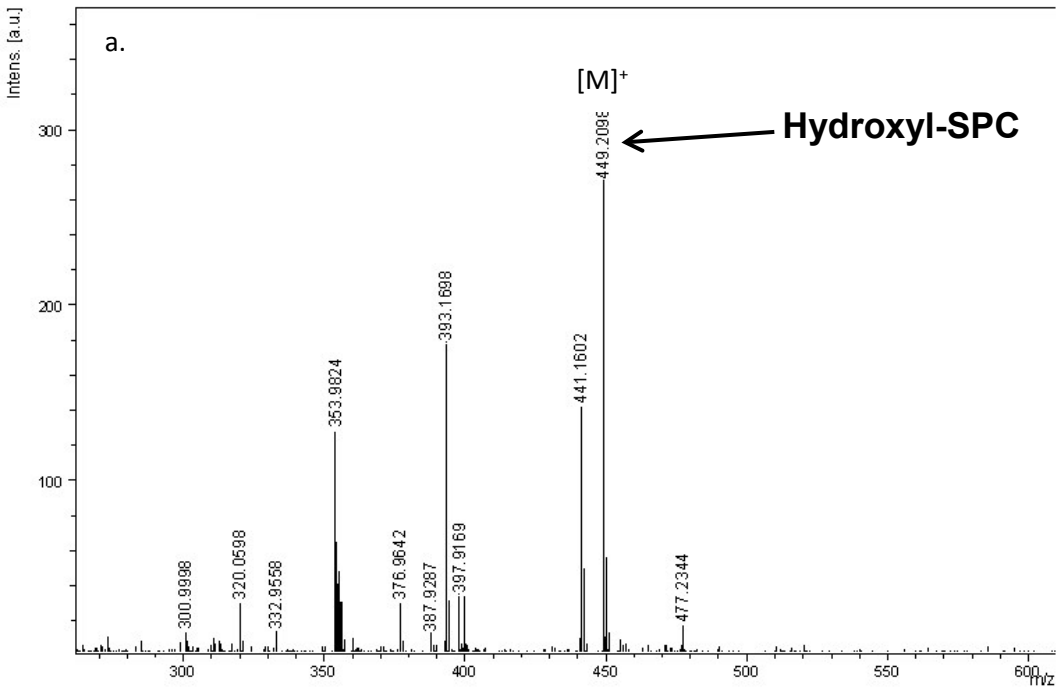


Fig. S10 HRMS spectra of SPC after reacting with superoxide.

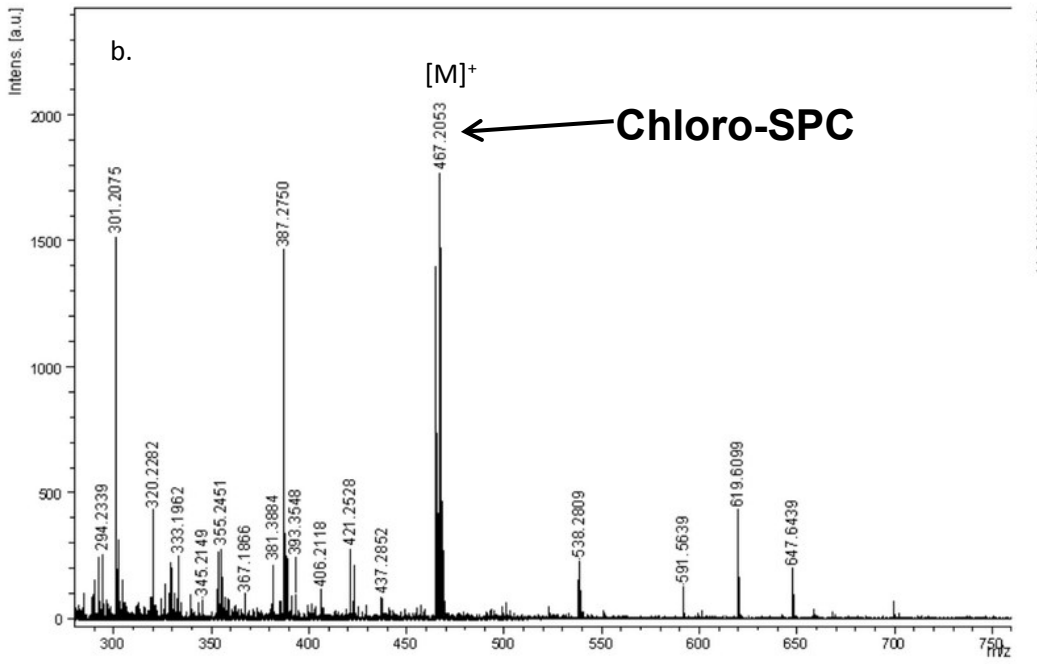


Fig. S11 HRMS spectra of SPC after reacting with hypochlorite.

To determine the detection mechanism of the SPC probe to ROS, UV-vis measurements of 23 μM SPC in the presence of one of the following ROS species including H_2O_2, $^1O_2$, OCl^-, O_2^-, or ·OH were performed accordingly in addition to the NMR studies. Their absorption spectra were collected as shown in Fig. S13. It is found that the maximum absorption peak of SPC at 420 nm corresponds to the donor-acceptor π-conjugated system of SPC molecule. It is clearly shown that this absorption maximum is disappeared after mixing SPC with OCl^-, O_2^-, or ·OH suggesting the disruption of the π-conjugated system. On the contrary, the same absorption peak still remains intact after mixing SPC with H_2O_2, or $^1O_2$ even after 24 h which is attributed to the poor reactivity under reaction conditions. In addition, according to the disappearance of ^{1}H NMR signals of the double bond of SPC and the appearance of new mass spectroscopic peak in the HRMS studies, it is strongly convinced that the double bond of the SPC probe is cleaved by OCl^-, O_2^-, or ·OH providing the detection mechanism of this probe.

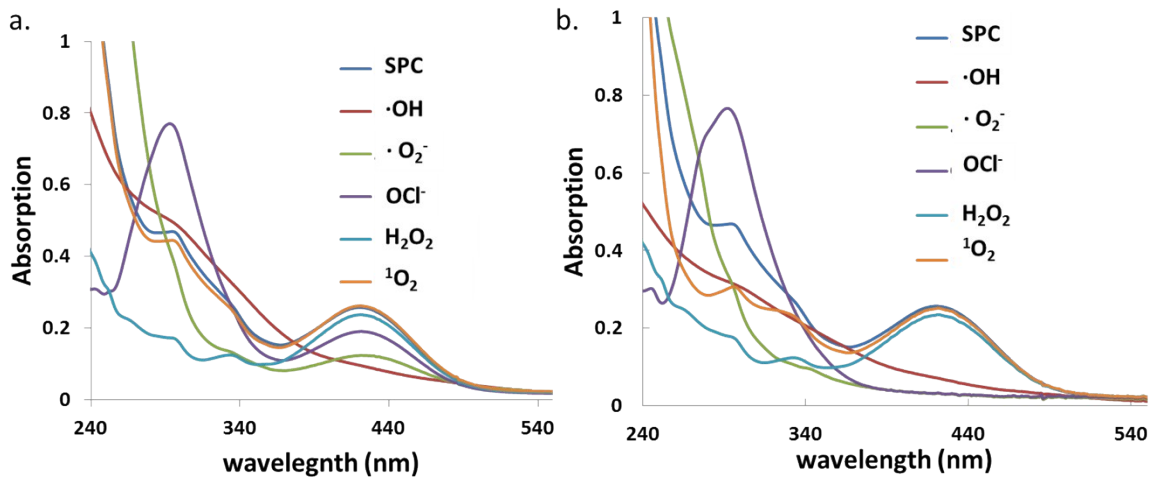


Fig. S12 UV-vis absorption spectra of 23 μM SPC in the presence of H_2O_2, $^1O_2$, OCl^-, O_2^-, or ·OH after 30 min and 24 h.

8. Cellular properties of SPC

Cytotoxicity test of SPC by MTT assay

Cells (1 x 10^5) were seeded on 96 wells plate, cell samples were incubated with different concentrations of SPC (0 μM, 5.73 μM, 11.45 μM, 23 μM and 45.8 μM) at 37℃ under 5% CO_2 for overnight. MTT reagent (50 ug) was then added to each sample after refreshing the media. The absorbance at 570 nm of each sample was measured by BioTek Powerwave XS microplate reader after dissolving with ethanol/DMSO (1:1).

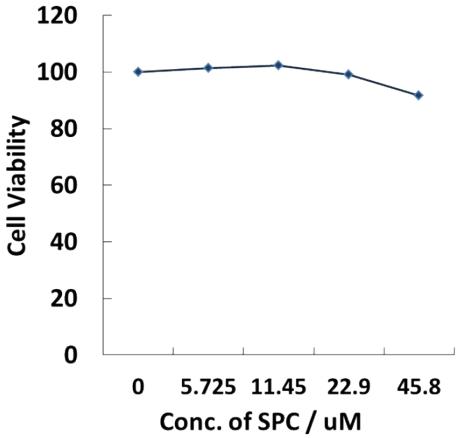


Fig. S13 The cell viability of RAW 264.7 in terms of percentage under different concentrations of SPC.

Internalization efficiency of SPC and SPM in different cell lines

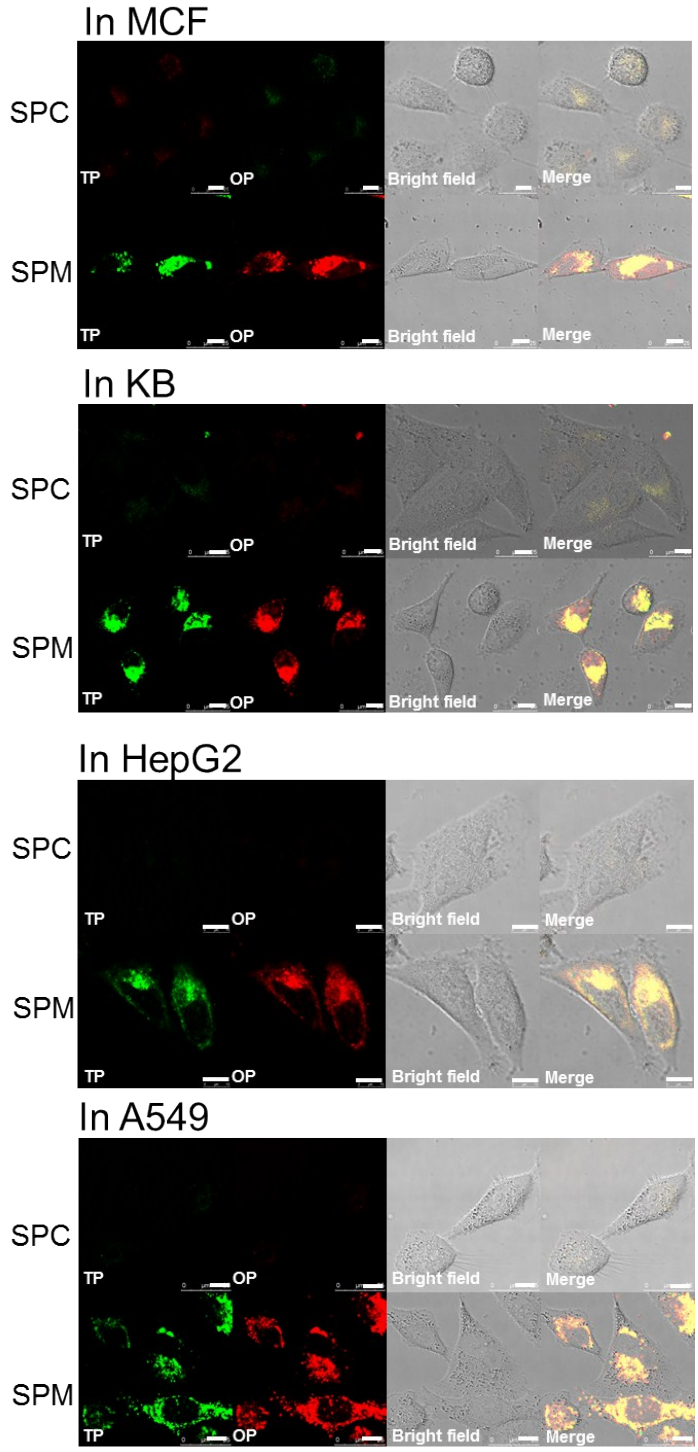
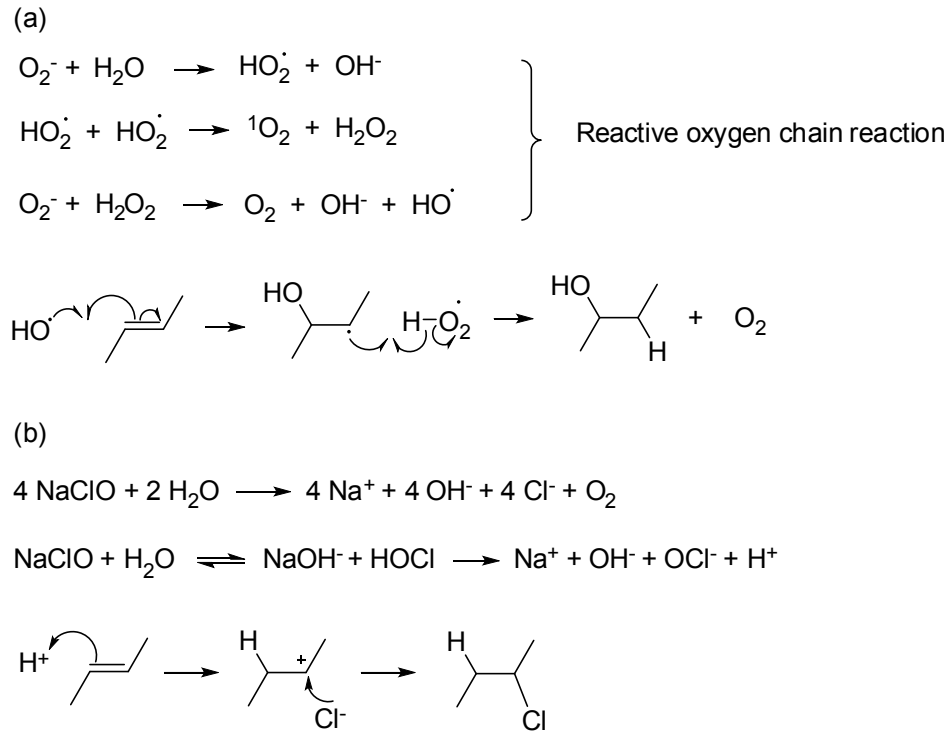


Fig. S14 Confocal fluorescence microscopy images of different cells after incubating with 23 μM of SPC or 23 μM of SPM for 24 h at 37°C. Scale bar is 10 μm.

Detection mechanism

Based on the absorption spectra, HRMS and the ^{1}H NMR spectra, the double bond of SPC was definitely cleaved after the ROS reactions. By comparing mass spectroscopic peak of SPC (M^+ = 431.1953) and the new mass spectroscopic peaks of hydroxyl-SPC (M^+ = 449.2098 (based peak)) and chloro-SPC (M^+ = 467.2053 (based peak)) after the ROS reactions in the HRMS studies, it was obvious to us that there was a H-OH and H-Cl adding across the double bond of SPC upon reacting with O_2^- and OCl^-, respectively, resulting in a fragment/product. Based on this information, we have proposed the reaction mechanisms of the probe with superoxide and hypochlorite, respectively which are shown below:



Scheme S2. The proposed mechanism of reaction of the SPC probe with (a) superoxide and (b) hypochlorite. (The reactive oxygen chain reaction is documented in the following reference: X. Dou, Z. Lin, H. Chen, Y. Zheng, C. Lua and J.-M. Lin, *Chem. Commun.*, **2013**, 49, 5871-5873.)

Photostability test of SPC

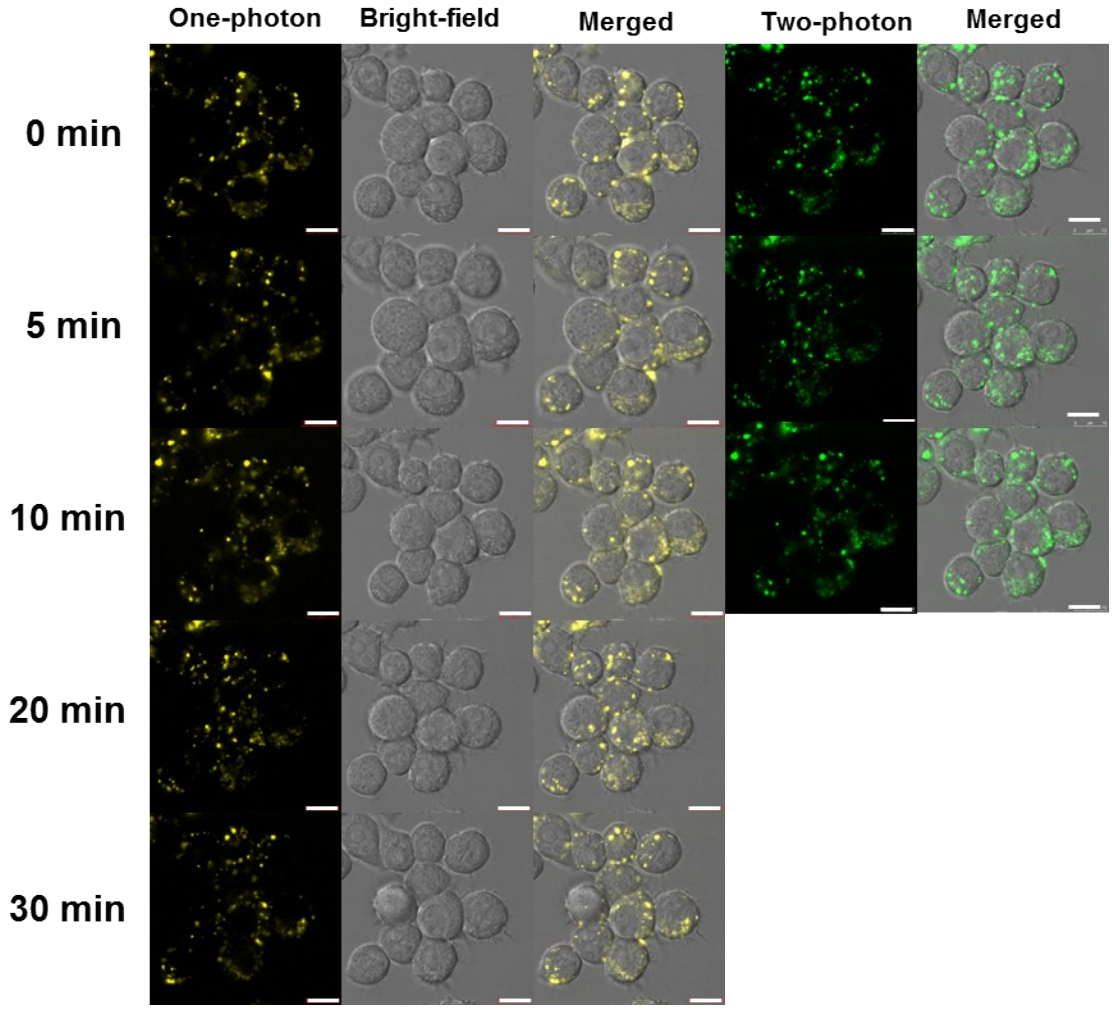


Fig. S15 Confocal one/two-photon fluorescence microscopy images showing high photostability of SPC in RAW 264.7 cells after exciting the samples with 548 nm laser for different time points.

This SPC probe also shows a remarkable photostability in macrophages under one-photon excitation as its fluorescence intensity remains fairly stable over a period of 30 min. As the NIR laser is a powerful laser, the cells will get burnt when they are excited more than 10 mins under the confocal fluorescence microscopy. Therefore, it is impossible to have a two-photon excitation for the photostability test more than 10 mins.

9. Characterizations of RAW 264.7 after a treatment of PMA or LPS in the presence or absence of SPC

In the treatment of PMA:

RAW 264.7 cells were incubated with 23 μM of SPC for 6 h, and then followed by a treatment of different concentrations of PMA for 3 h. The samples were then extracted with DMSO. Their emission signals were measured by spectrofluorometer.

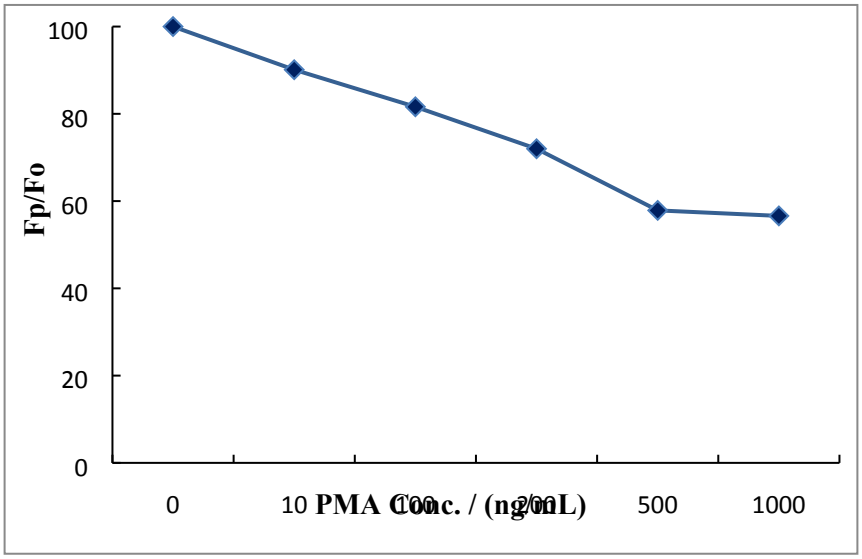


Fig. S16 Fluorescence responses of SPC in RAW 264.7 cells as a function of PMA concentrations. F_0 and F_p represent the measured fluorescence intensity of SPC before and after an addition of PMA for 3 h respectively.

Cells (1 x 10^5) were seeded on 96 wells plate, cell samples were incubated with different concentrations of PMA at 37℃ under 5% CO_2 for overnight. MTT reagent (50 ug) was then added to each sample after refreshing the media. The absorbance at 570 nm of each sample was measured by BioTek Powerwave XS microplate reader after dissolving with ethanol/DMSO (1:1).

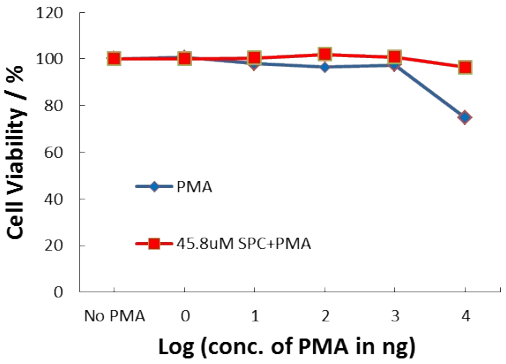


Fig. S17 The cell viability of RAW 264.7 in terms of percentage under different concentrations of PMA for 14 h at 37°C.

According to the MTT results and the bright field image shown in Fig. 5a in the manuscript, PMA-treated RAW 264.7 cells only exhibited morphology changes as a function of time. No obvious decrease in cell viability was observed. In the presence of SPC molecules, they showed remarkably suppressing capability to prevent the morphological alternation of these PMA-stimulated cells (see Fig. 5b in the manuscript). Therefore, we concluded that SPC exhibited cellular protection in stimulated macrophages in terms of PMA-induced morphological change by quenching of excessive production of O_2-, leading to a distinct fluorescence intensity change (see Fig. S4b and 5b in the manuscript).

In the treatment of LPS:

Cells (1 x 10^5) were seeded on 96 wells plate, cell samples were incubated with different concentrations of LPS together with/without 45.8 μg SPC at 37℃ under 5% CO_2 for overnight. MTT reagent (50 ug) was then added to each sample after refreshing the media. The absorbance at 570 nm of each sample was measured by BioTek Powerwave XS microplate reader after dissolving with ethanol/DMSO (1:1).

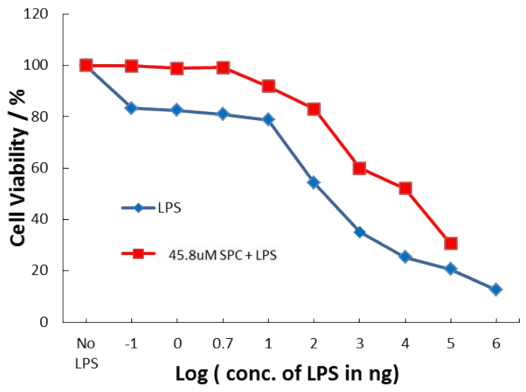


Fig. S18 The cell viability of RAW 264.7 in terms of percentage under different concentrations of LPS. Blue curve represents the % of cell viability of RAW 264.7 cells after a treatment of LPS under different concentrations for 14 h at 37°C. Red curve represents the % of cell viability of RAW 264.7 cells after an incubation of 23 μM of SPC for 6 h at 37°C followed by a treatment of LPS under different concentrations for 14 h at 37°C.

According to the MTT results shown in Fig. S18 and the bright field image shown in Fig. 6b in the manuscript, LPS-treated RAW 264.7 cells induced high cytotoxicity and simultaneously morphological change to polygonal shape with numerous pseudopodi and vesicles upon increasing the LPS concentrations. To further investigate the survival rate of LPS-stimulated RAW 264.7 cells and to evaluate the protection capability of SPC, live-dead assay was carried by staining LPS-treated RAW 264.7 cells with (i) proidium iodide (PI) and (ii) trypan blue (Fig. 6c in the mansucript). In the absence of SPC, a very high cell death rate was observed. On the other hand, a significant decrease in cell death was obtained after adding SPC. Therefore, we strongly believe that SPC showed protection effect to mitigate the LPS-induced cytotoxicity of RAW 264.7 cells in a dose-dependent manner by quenching of excessive production of OCl^-, leading to a distinct fluorescence intensity change (see Fig. S 6a in the manuscript).

References:

[i] (a) W. Zhang, P. Li, F. Yang, X. Hu, C.Sun, W. Zhang, D. Chen, B. Tang. *J. Am. Chem. Soc.* **2013**, *135*, 14956-14959. (b) G. Kim, Y. K. Lee, H. Xu, M. A. Philbert, R. Kopelman. *Anal Chem.* **2010** *82*, 2165–2169. (c) J. M. Aubry. *J. Org. Chem.* **1989**, 54, 726-728. (d) S. Marklund. *J. Biol. Chem.* **1976**, 251, 7504-7507.