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Design of a Phosphinate-Based Fluorescent Probe for Superoxide Detection in Mouse Peritoneal Macrophages

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Abstract: 3′,6′-Bis(diphenylphosphinyl)fluorescein (PF-1) was synthesized as a highly selective and sensitive fluorescent probe for imaging $O_2^{\bullet-}$ in living cells. The design strategy for the probe was based on the nucleophilic mechanism of $O_2^{\bullet-}$ to mediate deprotection of this probe to give fluorescein. Upon reaction with $O_2^{\bullet-}$, the probe exhibits a strong fluorescence response and high selectivity for $O_2^{\bullet-}$ over other reactive oxygen species and some biological compounds. The phosphinate-based probe, as a new fluorescent reagent, is cell-permeable and can detect micromolar changes of $O_2^{\bullet-}$ concentrations by using confocal microscopy in living cells. The unique combination of good selectivity, high sensitivity, good water solubility, and rapid reactivity establishes the potential value of the probe for facilitating investigations of the generation, metabolism, and mechanisms of superoxide-mediated cellular homeostasis and injury.

Keywords: analytical methods · fluorescein · fluorescent probes · nucleophilic substitution · superoxide

Introduction

Reactive oxygen species (ROS), such as a superoxide anion radical, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite, have been associated with many diseases and the aging process,[1–3] as well as many normal physiological processes.[4,5] The generation and reaction of superoxide ($O_2^{\bullet-}$), the precursor of other ROS, are of considerable interest in studies of cellular metabolism and the pathogenesis of diverse cytotoxic phenomena.[6] However, the most difficult obstacle has been the identification and quantification of $O_2^{\bullet-}$ with high selectivity and sensitivity.

Although several types of molecular probes for superoxide detection based on electron spin resonance spectroscopy,[7] SOD-inhibiting nitroblue tetrazolium,[8] chemiluminescence,[9,10] and fluorescence[11] have been described in previous reports, the limitations include low selectivity, low water solubility, and low biological compatibility. In fact, methods for detecting cell-derived $O_2^{\bullet-}$ with high selectivity, sensitivity and practicality are rarely available. For instance, hydroethidine (HE) is being used as a fluorescent probe for $O_2^{\bullet-}$.[11,12] However, the major drawback of HE is its poor selectivity toward $O_2^{\bullet-}$,[13] which is a result of its oxidative fluorescing mechanism.

Recently, a fluorescent probe for detecting $O_2^{\bullet-}$, based on a nonredox mechanism, was reported,[14] which has obvious advantages of improved detection limit and selectivity. However, in order to design an applicable probe, it is of great importance to improve the water solubility, increase reaction rate and avoid potential side reactions with native biological compounds within cells.

Among those molecular probes mentioned above, we believe that fluorescent probes are well suited to meet the need for mapping the spatial and temporal distribution of superoxide within living cells, because fluorescence assay can easily be detected by confocal laser scanning microscopy, microplate reader, and cell sorters. Thus, the aim of our research is to design practical fluorescent probes in order to make $O_2^{\bullet-}$ "visible" in living cells with high selectivity, good sensitivity, good solubility, fast response and light stability.

We recently found that superoxide possesses a "superb" characteristic, which is its "super-nucleophilicity". Superoxide based on a nucleophile substitution mechanism enhances

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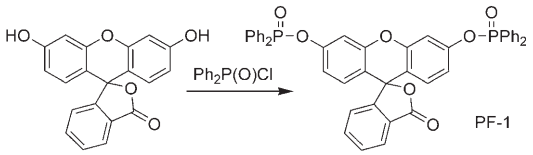
the hydrolysis of phosphatidylinositol to inositol 1,4,5-triphosphate in rat aortic smooth muscle cells.[15,16] Therefore, we assumed that a phosphinate compound might be a useful reagent that could be used as an indicator for $O_2^{\bullet-}$ assay. If so, it will have two obvious advantages: 1) a nucleophile-based mechanism could impart higher specificity toward such fluorescent probes for the detection of $O_2^{\bullet-}$ compared with a redox mechanism; 2) the miscibility of probe solutions in organic solvents with aqueous systems could be improved markedly, because the bond polarity of phosphor–oxygen double bond in phosphinate compounds is stronger than that of sulphur–oxygen double bond in sulfonylated fluorescein. In addition, if diarylphosphinate fluorescein acts as a probe, a large steric effect in the phosphinate moiety could effectively hinder potential side reactions with native biological compounds within cells, such as glutathione (GSH) which is a large bulk nucleophile. On the other hand, though many organophosphorus compounds, including those used as drugs for the treatment of Alzheimer's disease and glaucoma,[17,18] exhibit significantly acute toxicity, a series of O-aryl-diarylphosphinate compounds are not only non-toxic to living cells, but can also effectively inhibit neurotoxic esterase.[19] Thus, in this paper, we propose a novel fluorescencing reaction to design a fluorescent probe, diphenylphosphinate fluorescein: 3′,6′-bis(diphenylphosphinyl)-fluorescein (PF-1). The probe shows excellent selectivity toward $O_2^{\bullet-}$ over competing ROS and biological compounds in aqueous solution, and is capable of imaging micromolar changes in $O_2^{\bullet-}$ concentrations in mouse peritoneal macrophages using fluorescence microscopy. Our results establish the value of the probe for imaging $O_2^{\bullet-}$ in living biological samples and provide a basis for further developments of fluorescent probes to facilitate investigations of the generation, metabolism, and mechanisms of superoxide-mediated cellular homeostasis and injury.

Results and Discussion

Design and synthesis of PF-1 for superoxide: Our strategy for designing probes is based on the nucleophilic mechanism of $O_2^{\bullet-}$ to mediate deprotection of diphenylphosphinate fluorescein to fluorescein. It is well known that fluorescein, a fluorescent group, possesses good biological compatibility, near-unity quantum yield, and sizable extinction coeffient.[20] Additionally, its visible excitation and emission profiles limit photodamage to biological samples, avoid autofluorescence from native cellular species, and offer compatibility with common optical filter sets for fluorescence microscopy.

The probe PF-1 was synthesized and characterized with elemental analysis, IR, ^{1}H NMR, ^{31}P NMR spectroscopy, and chemical ionization mass spectrometry (CIMS). The synthesis method is simple and original. Upon treatment with $O_2^{\bullet-}$, PF-1, a closed, colorless, and non-fluorescent lactone, was transformed into an open, colored, and fluorescent product (Scheme 1).



Scheme 1. Synthesis of PF-1.

Spectroscopic properties and optical responses to $O_2^{\bullet-}$: PF-1 was evaluated under simulated physiological conditions (0.1 M phosphate buffered saline, PBS, pH 7.4). As expected, the probe PF-1 showed low blank fluorescence, while additions of different concentrations of xanthine plus xanthine oxidase (X/XO, generating $O_2^{\bullet-}$[21]) triggered promptly fluorescent increases ($\lambda_{em}=530$ nm) (Figure 1) with concomitant

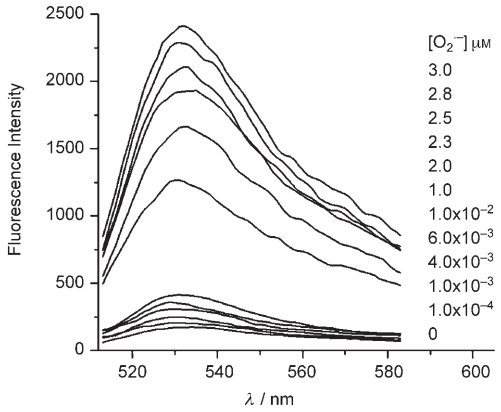


Figure 1. Fluorescence responses of 10 μM PF-1 in the absence and the presence of $O_2^{\bullet-}$ (from 0 to 3 μM) generated by adding different concentrations of X/XO (final concentration: 0/0, $3.0\times10^{-4}/3\times10^{-3}$, $3.0\times10^{-3}/3\times10^{-2}$, $1.2\times10^{-2}/0.12$, $1.8\times10^{-2}/0.18$, $3.0\times10^{-2}/0.3$, 3.0/30, 6.0/60, 7.0/70, 7.5/75, 8.5/85 and 9μM/90 mU) at 25 °C for 5 min (theoretically 1μM xanthine transforms to $^1/_3$ μM superoxide under physiological conditions,[21] $\lambda_{ex}=490$ nm).

growth of a long wavelength absorption band characteristic of fluorescein ($\lambda_{ex}=490$ nm). Absorption and emission spectra confirm that fluorescein is the product generated from the reaction between PF-1 and $O_2^{\bullet-}$. After concentrating the reaction solution, a crystal byproduct was collected. ^{31}P, ^{1}H NMR spectroscopy, and melting point determination confirm that the byproduct is diphenylphosphinite acid. Thus, it was proposed that PF-1 is transformed into fluorescein by reaction with superoxide as a nucleophile rather than an oxidant. Additionally, the probe can promptly capture $O_2^{\bullet-}$ within 5 min according to our experimental conditions, which is of importance to an applicable probe owing to the short life time of superoxide.

The reactivity of PF-1 toward various ROS and reductants was studied in detail. The fluorescence response from the solution of PF-1 (10 μM, in PBS) with $O_2^{\bullet-}$ generated by the enzymatic reaction of X/XO (9 μM/90 mU) or with KO_2 at 530 nm with excitation at 490 nm after incubation at 25 °C for 5 min was compared with those of reactions with reduc-

tants (5 μM for 1,4-hydroquinone (HQ) and glutathione (GSH), respectively), and other ROS (5 μM for each). Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*BuOOH), and hypochlorite ($^-$OCl) were delivered from 30, 70, and 5% aqueous solutions, respectively. Hydroxyl radical ($^{\bullet}$OH) was generated by reaction of H_2O_2 (5 μM) with Co^{2+} (50 μM), and single oxygen ($^1O_2$) was obtained by addition NaOCl (50 μM) to H_2O_2 (5 μM). Peroxynitrite ($ONOO^-$) and nitric oxide (NO) were delivered using 3-morpholinosydnonimine (SIN-1), and 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), respectively. The results are summarized in Figure 2. The experiments

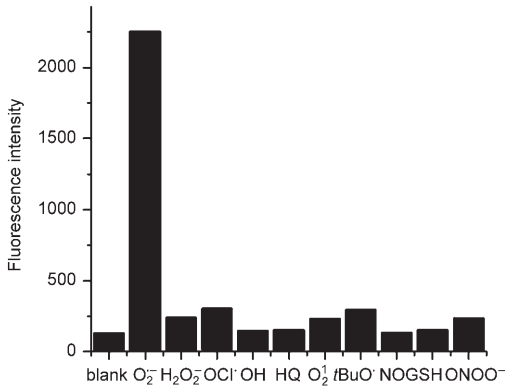


Figure 2. Fluorescence responses of 10 μM PF-1 in PBS to various ROS (5 μM for each) and reductants (5 μM for each). All data were obtained after the probe solutions were incubated with ROS or reductant at 25°C for 5 min (λ_{ex} = 490 nm).

show that PF-1 provided highly specific fluorescence response toward $O_2^{\bullet-}$, while gave only slight response to H_2O_2, $^-$OCl, $^1O_2$, *t*BuO$^{\bullet}$ and 1,4-hydroquinone, especially to hydroxyl radical, $ONOO^-$, GSH and NO. We believe that the observed high selectivity of PF-1 for $O_2^{\bullet-}$ over more oxidizing ROS is due to the reaction of PF-1 with $O_2^{\bullet-}$ based on a simple nucleophile deprotection, rather than redox mechanism.

The fluorescence responses of the PF-1 were characterized over a $O_2^{\bullet-}$ concentration range of 0–8 μM with 10 μM PF-1. Figure 3a and b display two calibration plots for PF-1. Figure 3a shows a linear correlation between low $O_2^{\bullet-}$ level (1×10^{-10}–1×10^{-8} M) and fluorescence responses after 5 min with a correlation coefficient 0.9997. The detection limit was 4.6 pM (relative standard deviation, n = 11; 4.1%) Figure 3b gives the other linear calibration curve in higher $O_2^{\bullet-}$ level (1×10^{-6}–8×10^{-6} M). Under these conditions, the probe can reliably detect $O_2^{\bullet-}$ concentration down to 0.1 nM in aqueous solution. In vivo, superoxide concentrations are usually considered to be in the lower nanomolar range, while quantities of superoxide will rapidly increase up to a higher micromolar range when an "oxidative stress" exists. Therefore, the probe should be applicable to biological samples. Figure 4 shows the time courses for the fluorescence intensity of 10 μM PF-1 (in 0.1 M PBS, pH 7.4) treated with 1 μM $O_2^{\bullet-}$ at

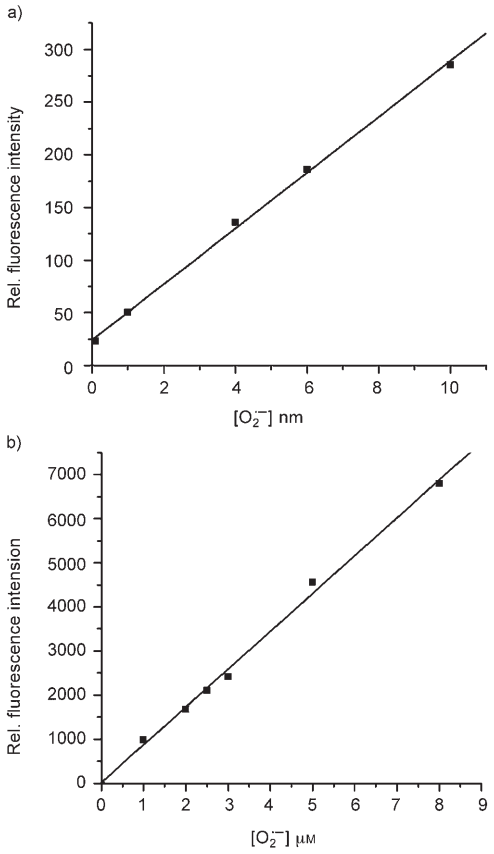


Figure 3. Fluorescence responses of 10 μM PF-1 (in 0.1 M PBS, pH 7.4) to various concentrations of $O_2^{\bullet-}$. a) 0.1–10 nM $O_2^{\bullet-}$ concentrations. b) 1–8 μM $O_2^{\bullet-}$ concentrations. Data were collected after incubation of PF-1 with X/XO at 25°C for 5 min. Total emission was integrated between 510 and 580 nm (λ_{ex} = 490 nm).

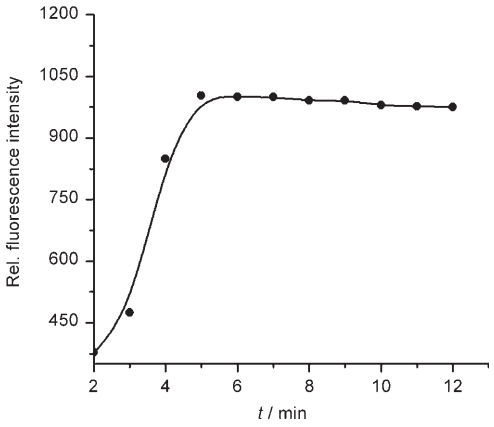


Figure 4. Time course of relative fluorescence intensity of 10 μM PF-1 in 0.1 M PBS before and after the addition of 1 μM $O_2^{\bullet-}$ at room temperature ($\lambda_{ex/em}$ = 490/530 nm).

room temperature. The experiment indicates that the reaction of PF-1 with $O_2^{\bullet-}$ can be carried out within 5 min under these reaction conditions.

Effect of SOD on fluorescence intensity of the reaction of PF-1 with superoxide: To confirm that the fluorescent changes of the probe solution were caused by $O_2^{\bullet-}$, superoxide dismutase (SOD), a scavenger of $O_2^{\bullet-}$ which catalyzes the disproportionation of $O_2^{\bullet-}$ to yield H_2O_2, was used in the reactive system. After the reaction of SOD with X/XO in PBS buffer was carried out for 30 min, PF-1 was added and the reaction solution was immediately diluted to the volume with doubly distilled water. The mixture was equilibrated and was allowed to react at 25 °C for 5 min before measurement. As can be seen from Figure 5, the fluorescence intensity was markedly suppressed by SOD addition.

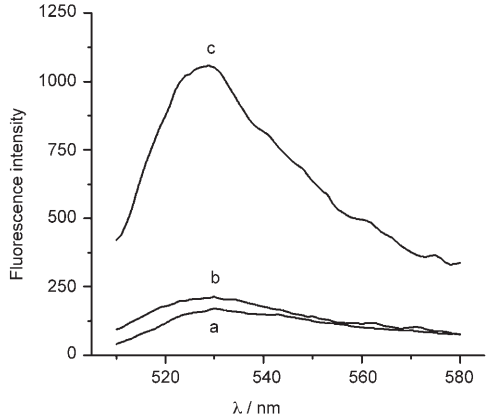
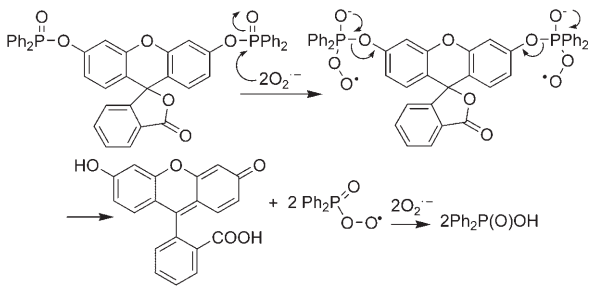


Figure 5. Effect of SOD on fluorescence intensity of the reaction of PF-1 with superoxide: a) 10 μM PF-1 in PBS; b) after the reaction of SOD (150 U) with X/XO (final 3 μM/30 mU) in PBS was carried out for 30 min, PF-1 (final 10 μM) was added into the reactive solution; c) PF-1 (final 10 μM) was added into a solution of X/XO (final 3 μM/30 mU) in PBS. All reactions were kept at 25 °C for 5 min (λ_{ex} = 490 nm).

Proposed mechanism for the reaction of PF-1 with superoxide anion radical: According to product analysis, fluorescence properties and articles reported,[14,15] we propose that the mechanism of the reaction of $O_2^{\bullet-}$ with probe PF-1 was as follows (Scheme 2). The phosphinyl phosphorus is electron deficient since the neighboring oxygen is more polarized through the electrons in the double bond, so the superoxide generated from xanthine plus xanthine oxidase uses the pair of electrons to form a new bond to the electron deficient phosphorus. As this new bond is formed, the relatively weak π bond of the phosphinyl group breaks and the two electrons move onto the oxygen to give a negative charge, while a single electron remains in superoxide moiety. Then the phosphor–oxygen double bond is reformed, the P–O σ bond breaks with both eletrons ending up on the departing fluorescein ion, which transforms into a conjugating form to give off fluorescence.



Scheme 2. Proposed mechanism for transformation of PF-1 to fluorescein by $O_2^{\bullet-}$.

Fluorescence detection of $O_2^{\bullet-}$ in living cells: With a good understanding of $O_2^{\bullet-}$-specific fluorescence responses of PF-1, we assessed the reaction of PF-1 with $O_2^{\bullet-}$ generated by mouse peritoneal macrophages (PM). Probe-loaded macrophages showed weak intracellular background fluorescence in Figure 6a and the phase contrast image was shown in Figure 6b. A strong fluorescence signal was observed (Figure 6c) from the cells which were stimulated with phorbol myristate acetate[22] (PMA, a stimulator of cell respiratory burst to give rise to ROS) for 12 h, then were incubated with PF-1 for 10 min; a brightfield image of these cells was

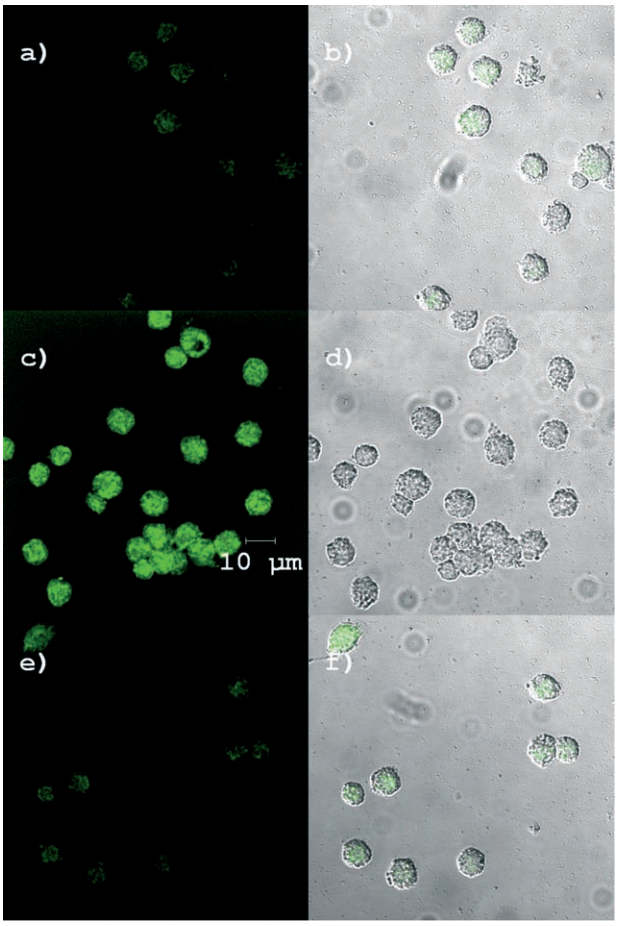


Figure 6. Confocal fluorescence (a, c, e), corresponding phase contrast (b, f) and brightfield images (d) of live mouse peritoneal macrophages. a,b) Cells incubated with 10 μM PF-1 at 37 °C for 10 min. c,d) Cells incubated with 10 μM PF-1 at 37 °C for 10 min after PMA stimulation for 12 h. e,f) Cells incubated with 100 μM Tiron at 37 °C for 1 h after PMA stimulation for 12 h, followed by loading with 10 μM PF-1 at 37 °C for 10 min.

shown in Figure 6d. The cells, which were PMA-stimulated for 12 h, were further treated with a solution of Tiron (Tiron, 1,2-dihydroxy-3,5-benzenedisulfonic acid disodium salt, a cell-permeable $O_2^{\bullet-}$ scavenger[23]) for 1 h and incubated with PF-1 for 10 min, the fluorescence intensity was markedly suppressed in Figure 6e; the phase contrast image is given in Figure 6f. The specificity of the measurement is confirmed by adding a nonenzymatic superoxide scavenger, Tiron. Brightfield transmission light measurements in Figure 6d confirms that the cells are viable throughout the imaging experiments. These results establish that PF-1 is membrane-permeable, and can specially respond to the micromolar change of $O_2^{\bullet-}$ concentrations within living cells, which demonstrate that the probe is an excellent sensor to superoxide anion radical.

Conclusion

In summary, we describe the chemical synthesis, property analysis and biological application of the diphenylphosphinate fluorescein (PF-1), a new type of phosphinate-based fluorescent probe for imaging $O_2^{\bullet-}$ in biological environments. The synthesis method is simple and original. Photometric and fluorimetric experiments of the probe show that the sensitive fluorogenic reagent features a high selectivity for $O_2^{\bullet-}$ over other intracellular ROS and biological compounds, a wide response range and low detection limit owing to its nucleophilic mechanism. Using fluorescence microscope, cell-derived $O_2^{\bullet-}$ were located in living cells. The specific response of PF-1 to $O_2^{\bullet-}$ was confirmed by adding a nonenzymatic superoxide scavenger. These experimental results show that PF-1 is an excellent fluorescent probe, which possesses good selectivity, high sensitivity, good water solubility, and prompt reactivity. Current efforts are devoted toward applying PF-1 to different biological systems to explore its potential applications. We believe that such a phosphinate-based fluorescent dye will have a great application in detecting oxidative stress through direct intracellular imaging. The ability to detect superoxide by intact cells will facilitate investigations of the generation, metabolism, and mechanisms of superoxide-mediated cellular homeostasis and injury.

Experimental Section

Synthetic materials and methods: Silica gel (100–200 mesh, Qingdao Haiyang Chemical Co.) was used for column chromatography. Analytical thin-layer chromatography was performed using GF254 silica gel (precoated sheets, 0.77 mm thick, Taizhou Si-Jia Biochemical Plastic Company). Diphenylphosphinyl chloride ($Ph_2P(O)Cl$) and fluorescein were purchased from Sigma Chemical Co. All the other chemicals were purchased from Chinese Chemical Co. ^1H NMR spectra were recorded on a Bruker Avance 300 MHz. Mass spectra were measured with a Agilent HP 1100 LC-MSD (U.S). The IR spectrum was obtained on a Bruker Tensor-27. Elemental analysis was performed on Perkin Elmer Series CHNS/O Analyzer. ^{31}P NMR spectra were measured on a Bruker DPX-400 with a working frequency of 161.9 MHz using DMSO as the solvent and 85% H_3PO_4 as the external standard. All melting points were measured on a Yanaco micro-melting point apparatus (Yanagimoto MFG Company), and are given (uncorrected).

3′,6′-Bis(diphenylphosphinyl)fluorescein (PF-1): Freshly distilled triethylamine (2.0 mL) was added to a solution of $Ph_2P(O)Cl$ (2.37 g, 10 mmol) in anhydrous THF (25 mL) and then, dropwise with stirring, a solution of fluorescein (1.66 g, 5 mmol) in anhydrous THF (50 mL). The reaction mixture was heated for 30 min with mild boiling of the solvent. Stirring and cooling to room temperature, the precipitated triethylamine hydrochloride was separated by filtration. The solvent of the resulting solution was removed by evaporation, and the residue was dissolved in anhydrous benzene (50 mL). The solution was washed with 5% ammonia, water and saturated aqueous NaCl solution. The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was dissolved in benzene, and insoluble solid was filtered at room temperature. Then hexane was dropped slowly to the filtrate until a precipitate formed, which was filtered and dried in vacuum. The solid powder obtained was subjected to silica gel chromatography eluted with ethyl acetate/hexane 3:1 to afford PF-1 (2.56 g, 70%) as a white solid. M.p. 118–120 °C; ^1H NMR (300 MHz, [D$_6$]DMSO/$CDCl_3$ 20:1, 25 °C, TMS): $\delta = 8.01$ (d, ^3J(H,H) = 7.86 Hz, 1H; CH), 7.96–7.90 (m, 8H; CH), 7.72 (m, 2H; CH), 7.67–7.56 (m, 12H; CH), 7.36 (s, 2H; CH), 7.28 (d, ^3J(H,H) = 7.80 Hz, 1H; CH), 7.08 (d, ^3J(H,H) = 8.76 Hz, 2H; CH), 6.78 ppm (d, ^3J(H,H) = 8.76 Hz, 2H; CH); ^{31}P NMR (400 MHz, DMSO, 85% H_3PO_4): δ = 31.384 ppm; ^1H NMR and MS spectra were shown in Figures S7 and S8 (see Supporting Information); IR (KBr): $\tilde{\nu} = 1768$ (lactone, C=O), 1606, 1486 cm^{-1} (phenyl); elemental analysis calcd (%) for $C_{44}H_{30}O_7P_2$ (732.2): C 72.14, H 4.13; found: C 72.26, H 4.15; MS (APCI): m/z: calcd 732.2, found 732.2.

Product analysis of the reaction of PF-1 with $O_2^{\bullet-}$: KO_2 (71.0 mg, 1.0 mmol) was added to a solution of the probe PF-1 (73.2 mg, 0.1 mmol) in 10% DMSO-PBS (20 mL). The reaction solution was stirred for 10 min and cooled to room temperature. Excitation and emission spectra of the solution diluted with PBS buffer (0.1 M, pH 7.4) confirm that fluorescein is the main product generated from the reaction between PF-1 and $O_2^{\bullet-}$ ($\lambda_{ex}/\lambda_{em} = 490/530$ nm), while the byproduct, a white acicular solid, can be obtained by vaporizing automatically the reactive solution. M.p. 191–192 °C; ^1H NMR (300 MHz, [D$_6$]DMSO, 25 °C, TMS): $\delta = 7.75$–7.72 (m, 8H; CH), 7.52–7.43 (m, 12H; CH), 5.56 ppm (s, 1H; OH); ^{31}P NMR: 34.193 ppm. The solid should be diphenylphosphinic acid.

Spectroscopic materials and methods: Water was purified using a arium 611 VF system with ultrafilter and UV lamp (Sartorius, Germany). Xanthine oxidase (XO), xanthine (X), L-glutathione reduced (GSH), *t*BuOOH (aq 70%), H_2O_2 (aq 30%), NaOCl (aq 5%), DMSO, KO_2, 3-morpholinosydnonimine hydrochloride (SIN-1), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), superoxide dismutase (SOD), phorbol 12-myristate 13-acetate (PMA), phosphate buffered saline (PBS), and RPMI 1640 medium were purchased from Sigma Chemical Co. 1,4-Hydroquinone was purchased from Fluka Chemical Co. Kunming Balb/c mouse were purchased from the animal experimental center of the Shandong University. Mice used in this study were 6–12 weeks old. 1,2-Dihydroxy-3,5-benzenedisulfonic acid disodium salt (Tiron) were purchased from Shanghai sss reagent Co. LTD.

Sample preparation: Probe solutions (0.1 mM) were prepared immediately before use by diluting a stock solution of PF-1 (1 mM) in DMSO with pH 7.4, 0.1 M PBS buffer (the miscibility of probe solution in organic solvents with aqueous systems was markedly improved compared with that reported[24]). Solutions of XO (0.5 U mL^{-1} for evaluating specificity and obtaining calibration curves), xanthine (1.0 mM), SOD (1500 U mL^{-1}), GSH (1.0 mM), H_2O_2 (1.0 mM), $CoSO_4$ (1.0 mM), NaClO (1.0 mM), *t*BuOOH (1.0 mM), SIN-1 (1.0 mM), 4-hydroquinone (HQ, 1.0 mM) were prepared with H_2O. NOC-5 was used as a solution (1.0 mM) in aqueous 10 mM NaOH. KO_2 was used as a solution (1.0 mM) in DMSO.

Fluorescence analysis: Fluorescence emission spectra were obtained with FLS-920 Edingburgh Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cells. Into a 10 mL color comparison tube were added PBS buffer (2.0 mL, 0.1 M), PF-1 (1.0 mL, 0.1 mM), and different concentrations of xanthine plus xanthine oxidase or KO_2. After diluted to the

volume with doubly distilled water, the mixture was equilibrated and was allowed to react at 25 °C for 5 min before measurement. Then the fluorescence intensity was measured at $\lambda_{ex/em} = 490/530$ nm against blank at the same time. The excitation and emission slit were set to 0.5 and 0.5 nm, respectively.

Preparation and staining of cell cultures: Macrophage monolayers were prepared as described previously.[25] Peritoneal exudate cells were harvested from peritoneal lavage using chilled serum-free RPMI 1640 medium, were centrifuged at 1200 rpm at 4 °C for 5 min, and suspended with PBS. Adjust the concentration of counted cells as 2×10^5 cells mL^{-1} and add to glass coverslips in the culture plates. After 2 h of incubation at 37 °C in an atmosphere of 5 % CO_2 in a CO_2 incubator, the nonadherent cells were removed by vigorous washing (3×) with warm serum-free medium, and the adherent cells were incubated overnight in complete medium to form macrophage monolayers. A set of cells was stimulated with PMA (2 ng mL^{-1}) at 37 °C for 12 h. Then part of cells stimulated for 12 h were washed with serum-free RPMI 1640 medium, and incubated with a Tiron solution (100 μM in serum-free RPMI 1640 medium, 2.5 mL) for 1 h. Finally, the cells were washed with RPMI 1640, and a DMSO solution of PF-1 (0.5 mM, 50 μL) was added to each dish loaded 2.5 mL serum-free RPMI 1640 medium in turn, incubated for 10 min. Before imaging, each coverslip was washed with PBS (0.1 M, pH 7.4).

Fluorescence imaging experiments: Confocal fluorescence imaging was performed with a Zeiss LSM510 laser scanning microscope containing an objective lens (×40). Excitation of probe-loaded cells at 488 nm was carried out with an argon ion laser, and emission was collected in a window from 505 to 550 nm using a META detection system.

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