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A Near-IR Reversible Fluorescent Probe Modulated by Selenium for Monitoring Peroxynitrite and Imaging in Living Cells

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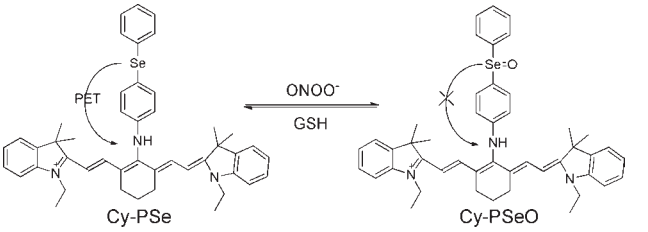
Supporting Information

ABSTRACT: We have developed a near-IR reversible fluorescent probe containing an organoselenium functional group that can be used for the highly sensitive and selective monitoring of peroxynitrite oxidation and reduction events under physiological conditions. The probe effectively avoids the influence of autofluorescence in biological systems and gave positive results when tested in both aqueous solution and living cells. Real-time images of cellular peroxynitrite were successfully acquired.

Peroxynitrite ($ONOO^-$) performs as a strong oxidizing agent in physiological and pathological processes.[1−3] In vivo, abnormally high concentrations of $ONOO^-$ are formed from the fast reaction between nitric oxide (NO) and superoxide anion (O_2^-), which requires no enzymatic catalysis.[4] The peroxynitrite anion is relatively stable, but the acid form (ONOOH) rapidly decays to nitrate. Although the half-life of ONOOH is ∼1 s at pH 7.40,[5] the oxidative species contributes to signal transduction, homeostasis regulation, and oxidative damage, which forms a unique biological oxidation−reduction cycle indicating human health and disease.[6] Therefore, the development of reversible detection technology for peroxynitrite would have important biomedical significance. In comparison with other technologies, fluorescence microscopy provides greater sensitivity, less invasiveness, and more convenience.[7] Especially the use of near-IR (NIR) light (650−900 nm) allows deep penetration into tissues and efficaciously avoids the influence of bioautofluorescence. However, there exists a major obstacle to the design of novel fluorescent probes for $ONOO^-$, namely, the nitro group, which is considered to be a strong quencher for fluorophores.[3] To date, only a few fluorescent probes for $ONOO^-$ detection have been reported.[8] Thus, we anticipate widespread interest in a redox-reversible NIR fluorescent probe, which would exhibit much more value for visualizing cycles of redox signaling and stress caused by peroxynitrite.[9] Here we report a redox-responsive NIR fluorescent probe for continuous monitoring of $ONOO^-$.

$ONOO^-$ is modulated by cellular antioxidant defense systems,[1,10] in which selenium (Se) plays an important role as the active site of the antioxidant enzyme glutathione peroxidase (GPx).[11] GPx can catalyze the reduction of $ONOO^-$ by glutathione (GSH) via a unique ping-pong mechanism.[12] Taking the advantage of this, we mimicked the catalytic cycle and developed an NIR fluorescent probe containing an organoselenium moiety that can be used for reversible peroxynitrite detection.

Scheme 1. Structures of Cy-PSe and Its Oxidized Product Cy-PSeO and the Fluorescence Enhancement Mechanism



As an overall strategy, cyanine (Cy), an NIR fluorescent dye with a high extinction coefficient,[13] was selected as a signal transducer, while 4-(phenylselenyl)aniline (PSe) was selected as a modulator because it can respond sensitively to $ONOO^-$.[11,14] Following the ping-pong mechanism,[12] we designed and synthesized a new NIR reversible fluorescent probe (Cy-PSe) for detection of $ONOO^-$ in living cells through a fast photoinduced electron transfer (PET) process. The fluorescence of Cy-PSe is quenched as a result of PET between the modulator and the transducer, but Se oxidation prevents the PET, causing the fluorescence emission to be "switched on" (Scheme 1). Notably, there is no significant change in the absorption spectrum in going from the "off" state to the "on" state (Figure 1a). These spectral properties indicate that the PET has been successfully regulated in the molecule.[15] On the basis of the enzymatic catalytic cycle, selenoxide can be effectively and rapidly reduced to selenide by GSH, at which point the probe begins to function.

Furthermore, the photophysical properties of Cy-PSe and Cy-PSeO were investigated using time-dependent density functional theory calculations (BP86 functional with TZVP basis sets as implemented in the Gaussian 09 package[16]) to confirm the PET mechanism. To evaluate the influence of the oxidation reaction on the fluorescence properties, excitation energies and oscillator strengths (f) were defined for Cy-PSe and Cy-PSeO using the COSMO solvent model for water. The results showed that the process of PET from the PSe moiety to the cyanine moiety occurs in the S_1 state of Cy-PSe [Figure S5 in the Supporting Information (SI)]. The small oscillator strength of the $S_0 \rightarrow S_1$ excitation ($f = 0.013$) implies a forbidden transition, indicating that the S_1 state is a dark state rather than an emissive state. In other words, this cannot be accessed directly by excitation from the S_0 state. However, it can be populated by internal conversion

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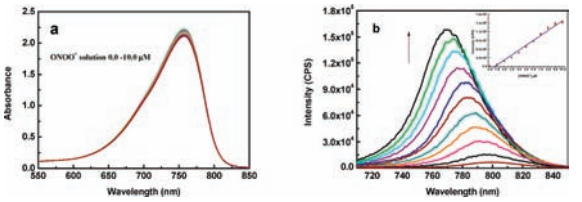

Figure 1. (a) Absorption spectra of Cy-PSe buffer solution treated with various amounts of 10.0 μM peroxynitrite in 0.1 M NaOH solution. (b) Fluorescence response of 10.0 μM Cy-PSe to $ONOO^-$ concentrations of 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 μM. Spectra were acquired in 0.1 M PBS (pH 7.40) with excitation at 758 nm and emission ranging from 710 to 850 nm. Inset: Relationship between the fluorescence intensity at 775 nm and $[ONOO^-]$.

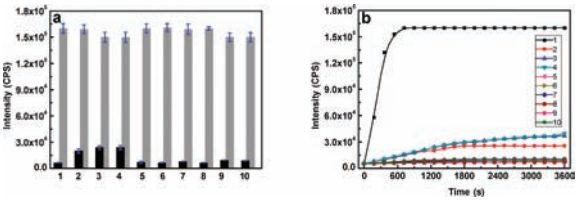

Figure 2. (a) Fluorescence responses of 10.0 μM Cy-PSe in 0.1 M PBS (pH 7.40) to diverse reactive oxygen species (ROS). Bars represent fluorescence responses to various compounds. In each group, the gray bar shows the response to 10.0 μM $ONOO^-$ and the black bar the response to another ROS: 1, blank; 2, NO (100 μM); 3, H_2O_2 (200 μM); 4, $\cdot$OH (200 μM); 5, ClO^- (500 μM); 6, $^1O_2$ (200 μM); 7, O_2^- (200 μM); 8, methyl linoleate hydroperoxide (MeLOOH, 200 μM); 9, *tert*-butyl hydroperoxide (t-BuOOH, 200 μM); 10, cumene hydroperoxide (CuOOH, 200 μM). The probe was incubated with the various ROS for 20 min before measurement. (b) Time courses of the responses of Cy-PSe to the ROS in (a) for 60 min.

from the S_3 state, in view of the maximum oscillator strength of the $S_0 \rightarrow S_3$ excitation (f = 1.617). In contrast to Cy-PSe, Cy-PSeO is a more strongly fluorescent molecule because of the absence of the PET process. The calculated results revealed that the low-lying transition-dipole-allowed $S_0 \rightarrow S_1$ excitation with an oscillator strength f = 1.014 corresponds to the orbital transition from the HOMO to the LUMO, both of which reside on the cyanine moiety. These calculations are consistent with the experimental results and rationalize the PET process (for details of the calculations, see the SI).

We also evaluated the effect of pH on the fluorescence. The fluorescence of the probe was quenched at pH above 8.7. However, as shown in Figure S1, the pH of the medium had hardly any effect on the fluorescence intensity over the pH range from 4.0 to 8.6. Thus, the probe was expected to work well under physiological conditions (pH 7.40, 0.1 M PBS).

The fluorescence and absorbance spectral properties of the probe were examined under simulated physiological conditions (pH 7.40, 0.1 M PBS, 10.0 μM Cy-PSe). The probe showed λ_{max} for absorption and emission at 758 nm (ε_{758nm} = 203 700 M^{-1} cm^{-1}) and 800 nm, respectively, both of which lie in the NIR region. Upon addition of 1 equiv of $ONOO^-$ to the buffer solution containing Cy-PSe, the fluorescence intensity increased by ~23.3-fold and the quantum yield increased from 0.05 to 0.12. Interestingly, the emission profile showed a blue shift in λ_{max} from 800 to 775 nm (Figure 1b). This phenomenon was induced by the

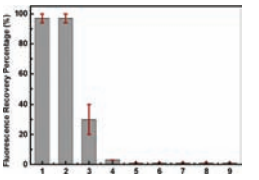

Figure 3. Fluorescence recovery rate with various reducing materials. Cy-PSe (10.0 μM) was oxidized by addition of 1 equiv of $ONOO^-$, and then the solution was treated with various reducing materials for 20 min: 1. L-cysteine (20.0 μM); 2, glutathione (GSH, 20.0 μM); 3, metallothionein (100.0 μM); 4, vitamin C (100 μM); 5, vitamin E (100 μM); 6, uric acid (100 μM); 7, tyrosine (100 μM); 8, histidine (100 μM); 9, hydroquinone (100 μM). The fluorescence recovery percentage is defined as $(F - F_0)/F \times 100\%$, where F is the fluorescence intensity of Cy-PSeO (10.0 μM) and F_0 is the fluorescence intensity of the probe after addition of the reducing material.

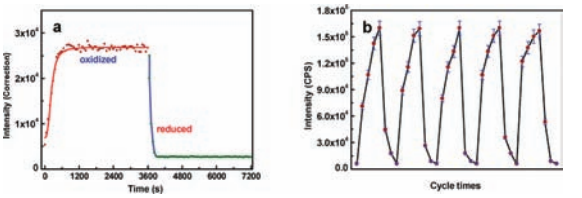

Figure 4. (a) Time course of Cy-PSe (10.0 μM) as measured by a spectrofluorometer. Cy-PSe was oxidized for 1 h by 1 equiv of added $ONOO^-$, after which the solution was treated with 2 equiv of GSH for another hour. (b) Fluorescence responses of Cy-PSe (10.0 μM) to redox cycles. Cy-PSe was oxidized by 1 equiv of added $ONOO^-$, and 8 min later the solution was treated with 2 equiv of GSH. When the fluorescence returned to the baseline level, another 1 equiv of $ONOO^-$ was added to the mixture. The redox cycle was repeated five times without an obvious change in fluorescence. All of the spectra were acquired in 0.1 M PBS (pH 7.4) at λ_{ex} = 758 nm. $ONOO^-$ was obtained from a peroxynitrite donor, 3-morpholinosydnonimine (SIN-1).

push−pull electronic effect of the modulator moiety.[17] Upon oxidization by $ONOO^-$, the probe contained an electron-withdrawing group (the PSeO moiety) instead of an electron-donating group (the PSe moiety). Therefore, the fluorescence spectrum underwent a slight blue shift. We studied the ability of the probe to quantify peroxynitrite in water solution. Figure 1b demonstrates that there is a linear dependence of the fluorescence intensity on the peroxynitrite concentration (0.0−10.0 μM); the regression equation was F_{775nm} = 16799$[ONOO^-]$ − 10435, with r = 0.9901.

To study the specificity of Cy-PSe toward peroxynitrite, an important procedure was carried out to determine whether reactive oxygen species other than $ONOO^-$ could potentially introduce signal noise. As shown in Figure 2a, no significant fluorescence intensity changes were observed in the emission spectra except in the case of $ONOO^-$. To check whether the chemoselective selenide switch might turn on upon incubation with other ROS over time, the probe's time courses with various ROS for 1 h were measured. As Figure 2b demonstrates, Cy-PSe selectively responded to $ONOO^-$ by a turn-on fluorescence switch and avoided a host of other ROS oxidants.

Because various types of antioxidants are contained in cells, an additional important test of the probe was performed to determine whether other reducing species would act as interferents. As

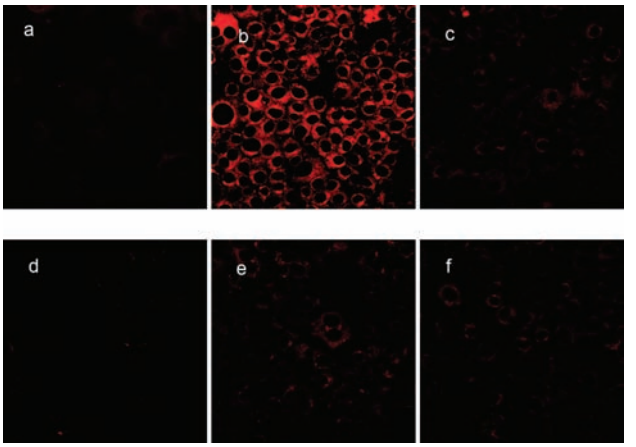

Figure 5. Confocal fluorescence images of oxidative stress in RAW264.7 cells. Macrophage cells were treated with various stimulants and then incubated with Cy-PSe (10.0 μM) for 5 min. (a) Control. (b) LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (c) AG (1 mM), LPS (1 μg/mL), and IFN-γ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (d) TEMPO (100 μM), LPS (1 μg/mL), and IFN-γ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (e) Cells treated with 50 μM H_2O_2 for 9 min. (f) Image after the administration of 50 μM NOC-5 for 3 min.

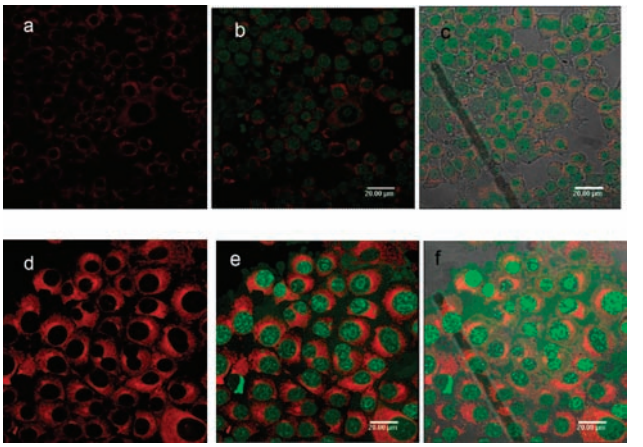

Figure 6. CFM images of RAW264.7 cells incubated with Cy-PSe (10.0 μM) for 5 min and rhodamine 6G (1.0 μM) for 10 min. (a–c) Controls. (d–f) Cells treated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h and then with PMA (10 nM) for 0.5 h. (a, d) Cells loaded with Cy-PSe; (b, e) overlay of images showing fluorescence from Cy-PSe and rhodamine 6G; (c, f) overlay of bright-field, Cy-PSe, and rhodamine 6G images.

shown in Figure 3, the probe Cy-PSeO displayed excellent selectivity response to thiols, which are the main antioxidants in vivo at an intracellular concentration of ~5 mM.[18] The average fluorescence recovery rate was up to ~97% for reduction by L-cysteine and GSH.

Kinetic assays of the probe for 2 h were also done. They showed that the probe solution was stable toward the medium, light, and air. Figure 4a indicates that the probe responded to the change in $ONOO^-$ within 8 min and was reduced by GSH within 5 min. Figure 4b shows that the reversible oxidation–reduction cycle could be repeated at least five times with no loss of fluorescence intensity. These data indicate that Cy-PSe is suitable for reversible peroxynitrite monitoring.

Since the Cy-PSe probe showed high redox sensitivity, selectivity, and reversibility with respect to the redox environment, we next asked whether it could monitor reversible $ONOO^-$ redox cycles in living cells under physiological conditions. We chose the mouse macrophage cell line RAW264.7 as a bioassay model, since the macrophage cells activate the generation of reactive oxygen and nitrogen species after exposure to lipopolysaccharide (LPS)/interferon-γ (IFN-γ) and phorbol 12-myristate 13-acetate (PMA).[19] RAW264.7 cells were stimulated by various reagents, incubated with Cy-PSe (10.0 μM) for 5 min, and then washed three times with PBS buffer. There was almost no fluorescence in the absence of stimulant (Figure 5a). However, strong fluorescence appeared after treatment with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h followed by additional stimulation with PMA (10 nM) for 0.5 h (Figure 5b). When the cells were pretreated with aminoguanidine (AG), an inhibitor of nitric oxide synthase,[20] much weaker fluorescence was detected in the stimulated cells (Figure 5c). The amount of hydroxyl radical (·OH) produced in the stimulated cells should not be decreased by the presence of AG. Therefore, the weaker fluorescence in Figure 5c illustrated that the strong fluorescence in Figure 5b was induced by peroxynitrite rather than ·OH. Additionally, after pretreatment of cells with the superoxide

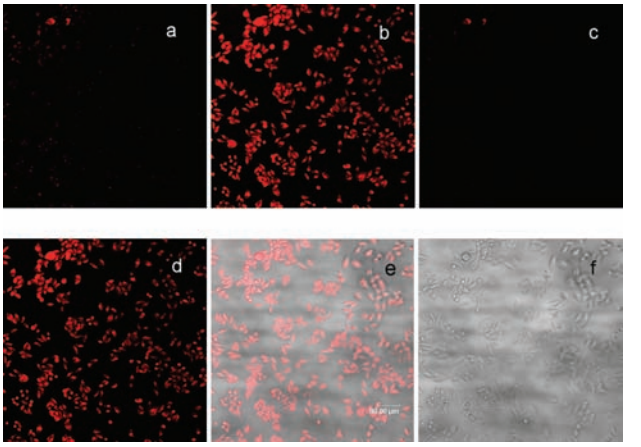

Figure 7. Confocal fluorescence images of reversible redox cycles in living RAW264.7 cells. (a) RAW264.7 cells loaded with 10.0 μM Cy-PSe for 5 min. (b) Dye-loaded cells treated with 10.0 μM SIN-1 for 10 min. (c) Dye-loaded, SIN-1-treated cells incubated with GST (125 units/mL) for 10 min. (d) Cells exposed to a second dose of SIN-1 for an additional 10 min. (e) Merged images of red (b) and bright-field (f) channels. (f) Bright-field image of (a).

scavenger 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO),[20] no fluorescence from the stimulated cells was observed (Figure 5d). Live RAW264.7 cells loaded with 50 μM H_2O_2 for 9 min and 50 μM 3-aminopropyl-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) for 3 min also showed faint fluorescence (Figure 5e,f). These results indicate that Cy-PSe can be used to visualize $ONOO^-$ selectively in cells.

We applied Cy-PSe to probe the subcellular locations of endogenous peroxynitrite in RAW264.7 cells using confocal fluorescence microscopy (CFM). Weak fluorescence in the cytoplasm of RAW264.7 cells was detected when the cells were loaded with Cy-PSe (10.0 μM, incubated for 5 min; Figure 6a). Co-staining with rhodamine 6G (1.0 μM, incubated for 10 min)

revealed the location of the probe in the cytoplasm of these living RAW264.7 cells (Figure 6b,c).[21] After treating the cells with the same stimulants used in Figure 5b, we observed greater fluorescence enhancement (Figure 6d) than with the control cells, indicating an increase in the peroxynitrite level in the cytoplasm. By co-staining with rhodamine 6G and overlaying with bright-field images, we confirmed that the probe was retained in the cytoplasm (Figure 6e,f).

We next asked whether the probe could be used to monitor $ONOO^-$ reversible redox cycles in living cells. Living RAW264.7 cells loaded with 10.0 μM Cy-PSe for 5 min showed only faint fluorescence (Figure 7a). However, the same Cy-PSe-loaded cells incubated with 10.0 μM 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor,[4] resulted in a substantial increase in intracellular fluorescence after 10 min as the probe detected oxidative stress (Figure 7b). The cells were then treated with the ROS scavenger glutathione S-transferase (GST, EC 2.5.1.18; 125 units/mL). After 10 min, the intracellular fluorescence decreased to the baseline level as GST reduced the cell environment (Figure 7c). Addition of a second aliquot of SIN-1 oxidant resulted in another burst of oxidative stress and an increase in intracellular fluorescence (Figure 7d). These experiments demonstrated that the low levels of intracellular fluorescence of Figure 7c were not due to photobleaching or dye leakage. To evaluate the cytotoxicity of the probe, we performed an MTT assay on RAW264.7 cells with probe concentrations from 0.01 μM to 10.0 mM. The results showed an IC_{50} value of 500 μM, clearly demonstrating that the probe was of low toxicity toward cultured cell lines under the experimental conditions at the concentration of 10.0 μM. This result suggests that Cy-PSe is capable of sensing redox cycles through reversible fluorescence responses in living cells.

In summary, we have developed an NIR reversible fluorescent probe that exhibits high sensitivity and selectivity in monitoring peroxynitrite oxidation and reduction events under physiological conditions in aqueous solution and living cells. The probe effectively avoids the influence of autofluorescence in biological systems and poses minimal toxicity to cells. It can also be used for real-time imaging of living cells. Our results show that the Cy-PSe probe can be used to visualize intracellular peroxynitrite levels with negligible background fluorescence and cellular toxicity.

■ ASSOCIATED CONTENT

Supporting Information. Additional CFM images; more experimental materials for the paper; theoretical and computational methods; synthesis; ^1H NMR, ^{13}C NMR, ^{77}Se NMR, and MS spectra of adducts; and complete ref 16c. This material is available free of charge via the Internet at http://pubs.acs.org.

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