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A near-infrared reversible fluorescent probe for peroxynitrite and imaging of redox cycles in living cells†

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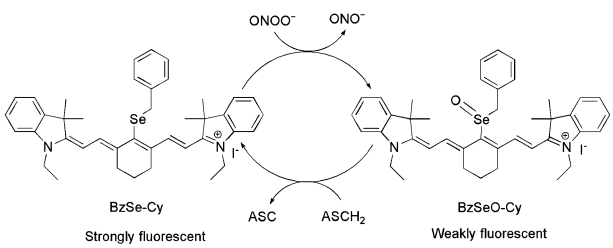
BzSe-Cy is a small-molecule fluorescent probe containing Se, which can respond reversibly to changes in $ONOO^-$ or reduced ascorbate and exhibit high sensitivity and selectivity for $ONOO^-$.

Redox homeostasis is critical for proper cellular function.[1] The reduction–oxidation state of the cell is primarily a consequence of the precise balance between the levels of reactive oxygen species (ROS) and reducing equivalents.[2] Unregulated production of ROS results in potentially cytotoxic "oxidative stress".[3] Many diseases (*e.g.* Alzheimer's disease) are linked with ROS damage as a result of an imbalance between radical-generating and radical-scavenging systems.[4] Thus, the study of intracellular redox cycles has an important physiological and pathological significance. However, only a few reversible fluorescent probes based on the modified fluorescent proteins for redox activity[5] and one small-molecule fluorescent probe designed for monitoring redox cycles in living cells have been described in the past several years.[6] Nowadays, new fluorescent probes which can not only respond reversibly to redox events but also exhibit high selectivity for one kind of ROS should be more useful for studying dynamic redox chemistry in biosystems.

Peroxynitrite ($ONOO^-$), a powerful oxidizing agent formed from a diffusion-controlled reaction between NO and $O_2^{-\bullet}$ in cells, plays an important role in biological processes.[7] Depending on the concentration, $ONOO^-$ can be either "friend" or "foe". A well-controlled level of $ONOO^-$ drives important cellular functions such as keeping cellular integrity and organ homeostasis, whereas a high level of $ONOO^-$ can induce DNA damage and initiate lipid peroxidation in biomembranes.[8] Among the reported methods for detecting $ONOO^-$, the use of fluorescent probes had its apparent advantages over other methods. For example, a few fluorescent probes such as HKGreen[9] and $[Eu^{3+}/Tb^{3+}(DTTA)]$[10] have been developed for the selective detection of $ONOO^-$. Furthermore, fluorescent probes that can respond reversibly to changes in concentration of $ONOO^-$ would be much more valuable for studying the generation, metabolism of $ONOO^-$ and its dynamic damaging of living cells. Selenium, long known to be an important dietary "antioxidant", is now recognized as an essential component of the active sites of many enzymes.[11] Moreover, depending on the redox cycling of selenium, many organoselenium compounds are capable of simulating catalytic functions demonstrated by natural enzymes.[12] One of the important biochemical functions of these compounds is the protection against $ONOO^-$.[13] Although there is an increasing amount of evidence showing that selenium redox cycling can enhance the protective effects of organoselenium compounds against $ONOO^-$, integrating selenium into a fluorescent probe to detect $ONOO^-$ and monitor the redox cycles in living cells has not been reported to date.

In this report, we chose tricarbocyanine, a near-infrared (NIR) fluorescent dye, as a fluorophore and divalent selenium as a redox-responsive group to synthesize a new reversible organoselenium probe benzylselenide-tricarbocyanine (BzSe-Cy, Scheme S1, ESI†) for the selective detection of $ONOO^-$ and monitoring redox cycles in living cells. The structure of BzSe-Cy was characterized with ^1H NMR, ^{13}C-NMR, IR and ESI-MS (see ESI†). The redox cycling of the probe—whereby the oxidized probe produced from BzSe-Cy by $ONOO^-$ (Scheme 1) is then recycled back to the BzSe-Cy by reduced ascorbate ($ASCH_2$)—can be repeated at least 8 times, and the real-time imaging of cellular redox cycles was achieved successfully in living RAW 264.7 cells. In addition, another benefit of this probe is its NIR emission wavelength, which can lead to minimize photodamage and cell autofluorescence.[14]



Scheme 1 The redox cycle of BzSe-Cy.

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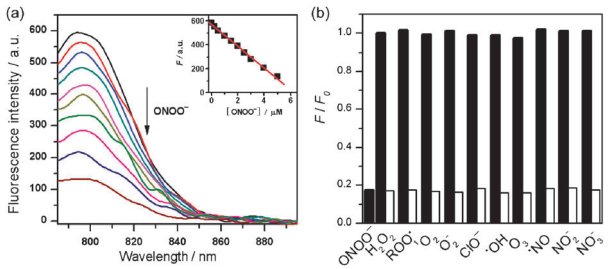

Fig. 1 (a) Fluorescence responses of 5 μM BzSe-Cy to different concentrations of $ONOO^-$. Inset: a linear correlation between emission intensity and concentrations of $ONOO^-$. (b) Fluorescence responses of BzSe-Cy to ROS and RNS (5 μM for $ONOO^-$, 50 μM for $^{\bullet}OH$, O_3 and ClO^-, 0.5 mM for others). Bars represent the final (F) over the initial (F_0) integrated emission. Black bars represent the addition of one of these bioanalytes to a 5 μM solution of BzSe-Cy. White bars represent the addition of $ONOO^-$ plus one of these interferences to the probe solution. All spectra were acquired in 30 mM phosphate buffer with pH 7.4 at 37 °C ($\lambda_{ex}/\lambda_{em}$ = 770/800 nm).

Spectroscopic evaluation of BzSe-Cy was performed under simulated physiological conditions (phosphate buffer, pH 7.4) and the optimal measurement conditions were established (Fig. S1–S2, ESI†). Initially, we tested the fluorescence reponses of the probe to $ONOO^-$. As expected, BzSe-Cy exhibits apparent fluorescence in phosphate buffer (ε = 28 778 M^{-1} cm^{-1}, Φ_F = 0.106, τ = 2.21 ns). Upon the reaction of BzSe-Cy with $ONOO^-$, the probe was converted to the oxidized BzSe-Cy (BzSeO-Cy), which is weakly fluorescent upon 770 nm excitation due to a photo-induced electron-transfer process. The structure of BzSeO-Cy was confirmed by mass spectrometry and ^{77}SeNMR (Fig. S3–S4, ESI†). As shown in Fig. 1a, the addition of $ONOO^-$ triggered 5-fold decrease of the fluorescence intensity. When BzSe-Cy reacted with different concentrations of $ONOO^-$, there was a good linearity between relative fluorescence intensity and concentrations of $ONOO^-$, the equation of the regression analysis was F = 570.36 − 91.40 [$ONOO^-$] (μM) with a correlation coefficient of 0.9975, which indicates that the probe could detect $ONOO^-$ quantitatively in the linear range.

To study the selectivity of BzSe-Cy toward $ONOO^-$, we tested its responses toward other bioanalytes. Considering the antioxidative function of selenium, the effect of interference of other ROS and reactive nitrogen species (RNS) was detected. As shown in Fig. 1b, BzSe-Cy exhibits a significant decrease in fluorescence intensity upon reaction with $ONOO^-$, whereas the response toward other bioanalytes is negligible. An error of ±5.0% in the relative fluorescence intensity was considered to be tolerable. The experiments show that BzSe-Cy is highly specific to $ONOO^-$. The change of fluorescence is due to the interconversion between selenides and selenoxides, and the oxidation potential of BzSe-Cy (0.622 V, see ESI†) is higher than that of most other reported cyanine dyes,[15] which largely hampers the unwanted oxidation of BzSe-Cy to the corresponding oxindole by ROS. These features made BzSe-Cy an $ONOO^-$-specific probe. In addition, the selectivity of this probe has been further identified *in vivo* (Fig. S5, ESI†).

The reversibility of the probe was then tested. Fig. 2 shows the time courses of fluorescence intensity of the probe for redox cycles mediated by $ONOO^-$ and $ASCH_2$ in 30 mM

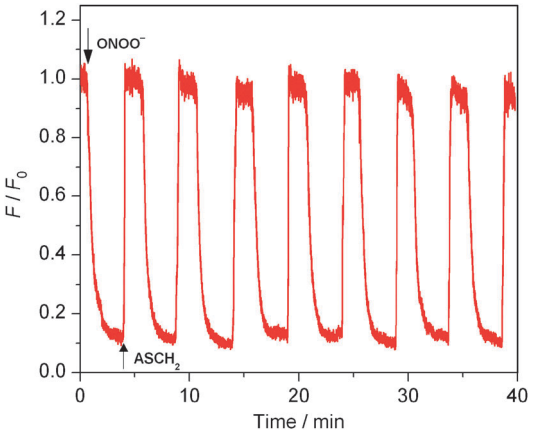

Fig. 2 Redox cycling of BzSe-Cy in 30 mM phosphate buffer with pH 7.4 at 37 °C ($\lambda_{ex}/\lambda_{em}$ 770/800 nm), showing reversible fluorescence quenching upon alternating addition of $ONOO^-$ and $ASCH_2$. A solution of 20 μM BzSe-Cy was treated with 15 μM $ONOO^-$ and then reduced with 40 μM $ASCH_2$.

buffer solution with pH 7.40 at 37 °C. Upon treatment of BzSe-Cy with $ONOO^-$, a remarkable fluorescence decrease was observed. And then $ASCH_2$ was added into the above solution, the fluorescence intensity of which increased rapidly and reached a constant value after only <15 s. This reversible redox cycle can be repeated at least 8 times under the same conditions. The experiment results show that BzSe-Cy is a sensitive redox probe, which is ideal for monitoring changes in the intracellular $ONOO^-$ levels.

The monitoring of the redox cycles in living cells by using BzSe-Cy was further undertaken by confocal laser scanning microscopy. RAW 264.7 cells incubated with 20 μM BzSe-Cy for 30 min at 37 °C showed strong fluorescence (Fig. 3a), indicating that the probe can be easily internalized by the living cells. Next, a marked fluorescence decrease in Fig. 3b appeared after stimulating the BzSe-Cy-loaded cells with 20 μM SIN-1 ($ONOO^-$ donor)[16] for 1 h (the incubation time was

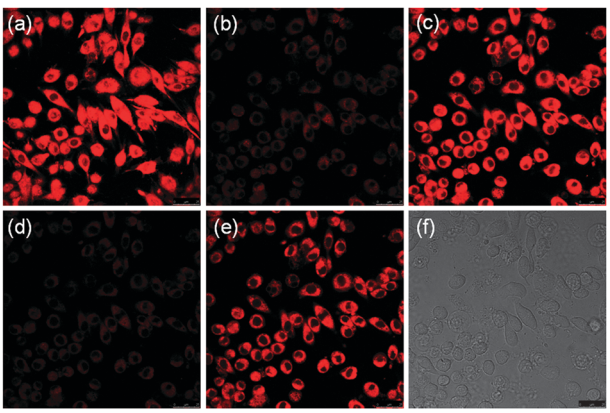

Fig. 3 Live-cell imaging of redox cycles by confocal microscopy at 37 °C. (a) RAW 264.7 cells loaded with 20 μM BzSe-Cy for 30 min. (b) Probe-loaded cells treated with 20 μM SIN-1 for 1 h. (c) Probe-loaded, SIN-1-treated cells incubated with 50 μM $ASCH_2$ for 30 min. (d) Cells exposed to a second dose of 20 μM SIN-1 for an additional 1 h. (e) Cells in panel (d) incubated with 50 μM $ASCH_2$ for another 30 min. (f) Brightfield image of live RAW 264.7 cells in panels (a)–(e). Scale bar = 25 μm.

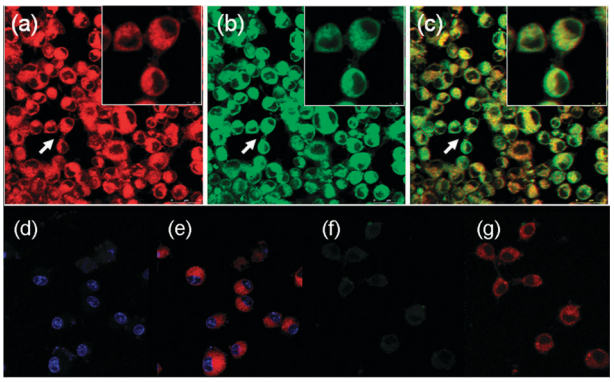

Fig. 4 Confocal fluorescence images of living RAW 264.7 cells. (a) Cells incubated with 20 μM BzSe-Cy at 37 °C for 30 min and then incubated with 50 nM Mito Tracker Green FM for 10 min, using a 633 nm laser. (b) The above cells were excited by a 488 nm laser. (c) One overlay image of (a) and (b). (d) Cells incubated with 20 μM BzSe-Cy at 37 °C for 30 min and then incubated with 10 μg mL^{-1} DAPI for 15 min, using a 405 nm laser. (e) One overlay image of (d) and an image of the (d) cells excited by a 633 nm laser. (f) Cells incubated with 20 μM BzSe-Cy at 37 °C for 30 min and then incubated with 50 μg mL^{-1} PI for 5 min, using a 543 nm laser. (g) One overlay image of (f) and an image of the (f) cells excited by a 633 nm laser.

optimized in Fig. S6 (ESI†)), while a bright fluorescence was again observed at the same field of view after addition of 50 μM $ASCH_2$ into the medium and incubation for another 30 min at 37 °C (Fig. 3c). To further prove that BzSe-Cy has a good reversibility, addition of a second aliquot of SIN-1 resulted in another decrease in intracellular fluorescence (Fig. 3d), and when the same cells were incubated with $ASCH_2$ again, the bright fluorescence appeared (Fig. 3e). The result indicates that the fluorescence changes in the RAW 264.7 cells are due to the changes in the intracellular $ONOO^-$ levels, and BzSe-Cy can also monitor the redox cycles in living cells. The bright-field image (Fig. 3f) confirmed that the cells were viable throughout the imaging experiments. In addition, the internalization assay has been carried out both at 37 °C and 4 °C, proving that BzSe-Cy exhibits excellent cell membrane permeability (Fig. S7, ESI†).

Furthermore, we assessed the ability of BzSe-Cy to target the mitochondria in living cells. RAW 264.7 cells incubated with 20 μM BzSe-Cy for 30 min at 37 °C showed bright fluorescence in discrete subcellular locations as determined by confocal microscopy (Fig. 4a). A co-staining experiment with Mito Tracker Green FM (a commercially available mitochondrial green-fluorescent probe) has been carried out to establish that BzSe-Cy is localized to the mitochondria (Fig. 4b). The overlay image confirms that the probe is retained in the mitochondria of these living cells (Fig. 4c). Finally, to further prove that BzSe-Cy has a low toxicity to the cultured cell lines, the MTT assay (Fig. S8, ESI†) and nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI, which can stain both live and dead cells) and propidium iodide (PI, which can only stain dead cells) were performed. The experiments showed that the cell morphology was scatheless as determined by comparison with the image of cells dyed with DAPI in Fig. 4d and e, and the cells were viable and not stained by PI as shown in Fig. 4f and g. These results show that the probe has low cytotoxicity under these experimental conditions.

To conclude, we have developed a NIR organoselenium fluorescent probe for detecting $ONOO^-$ and imaging of redox cycles in living cells. BzSe-Cy is a unique small-molecule indicator containing Se that responds reversibly to changes in $ONOO^-$ or $ASCH_2$ concentration, and it exhibits high sensitivity and selectivity for $ONOO^-$. Moreover, the new probe was successfully applied to the imaging of multiple cycles of oxidative stress and reductive repair in RAW 264.7 cells. This work provides a new approach to studying the generation, metabolism of $ONOO^-$ and its dynamic damage process of living cells with related chemical tools.

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