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Imaging Agents

Construction of a Near-Infrared Fluorescent Turn-On Probe for Selenol and Its Bioimaging Application in Living Animals

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Abstract: As selenocysteine (Sec) carries out the majority of the functions of the various Se-containing species in vivo, it is of high importance to develop reliable and rapid assays with biocompatibility to detect Sec. Herein, an NIR fluorescent turn-on probe for highly selective detection of selenol was designed and synthesized. The probe exhibits large turn-on signal upon treatment with selenocysteine (R-SeH), and it was further demonstrated that the new NIR fluorescent probe can be employed to image selenol in living animals.

Selenium (Se) is a biologically essential element for cellular redox balance, immune responses, cancer prevention, and inflammation protection.[1] Many different metabolites of Se, such as hydrogen selenide, selenocysteine (Sec), selenite, selenophosphate, selenodiglutathione, and charged Sec-tRNA, are synthesized in animals in the course of converting inorganic Se to organic forms and vice versa.[2] Insufficient or excessive intake of Se has been associated with a number of diseases.[3] Sec is a cysteine (Cys) analogue with a selenium-containing selenol group in place of the sulfur containing thiol group in Cys.[4] In mammalian tissues, selenocysteine (Sec), genetically encoded as the 21st amino acid, appears to be the predominant chemical form of selenium, and is specifically incorporated into selenoproteins (SePs).[4] As Sec carries out the majority of the function of the various Se-containing species in vivo, it is of high demand to develop reliable and rapid assays with biocompatibility to determine Sec.

Since the fluorescent indicators for calcium ion were reported by Tsien in the early 1980s,[5a] fluorescent probes have been recognized as the efficient molecular tools that can help monitor and visualize trace amounts of samples in live cells or tissues because of their high sensitivity and spatiotemporal resolution.[5] However, designing highly selective probes for Sec without suffering from interference of biological thiols is challenging since thiols are usually present in high concentration (millimolar levels) in cells and have similar chemical properties as Sec. Some fluorescent probes for Sec have been achieved by taking advantage of the mechanism of nucleophilic aromatic substitution.[6,7] However, these reported fluorescent probes for Sec are located in the visible region and are only suitable for imaging studies in living cells. It is essential to develop fluorescent Sec probes with near-infrared emission for living animal imaging applications. Near-infrared (NIR) light (650–900 nm) is advantageous in biological imaging due to minimum photodamage to biological samples, deep tissue penetration, and minimum interference from background autofluorescence by biomolecules in living systems.[8,9] To the best of our knowledge, no NIR fluorescent Sec probes have been reported in the literature to date, although they are highly desirable for biological imaging of selenocysteine in living animals.

Recently, we introduced a unique family of NIR dyes (HD NIR dyes), which are superior to the traditional 7-hydroxycoumarin and fluorescein with absorption and emission in the NIR region while retaining an optically tunable hydroxyl group.[10] Based on this finding, we reasoned that their novel amino analogue HD-NH_2 could also exhibit an optically tunable function. It is known that the strongly electron-withdrawing 2,4-dinitrobenzenesulfonyl group has been used for the protection of an amino group.[11] The resulting sulfonamide can be readily cleaved by a nucleophilicity group, such as thiolate anions (Figure 1). Several probes with sulfonamide linkage have been reported to selectively detect thiolphenols at neutral pH.[12] This mechanism underlines the importance of the anion form of a nucleophilic group, which is the essentially reactive form for the reaction. The pK_a value of selenocysteine is around 5.8, whereas that of aliphatic thiols is about 8.5. In a neutral reaction medium (for example, pH 7.4), the high degree of dissociation of selenocysteine results in the predominant generation of the corresponding selenolate, which can effectively react with 2,4-dinitrobenzenesulfonamide.[7] However, under the same neutral conditions, the less reactive neutral form of aliphatic thiols predominates, thus, the cleavage of the sulfonamide is very slow.

In this work, we developed HD-Sec as the first NIR fluorescent turn-on selenocysteine probe using HD-NH_2 as the near-infrared platform and 2,4-dinitrobenzenesulfonyl group as the reactive site for selenocysteine (Figure 1). We thus anticipated that the probe HD-Sec may be essentially non-fluorescent. However, upon reaction with selenocysteine to release the 2,4-

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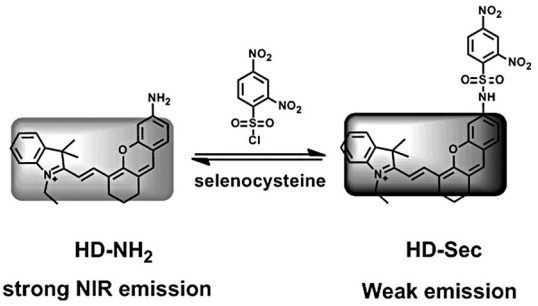

Figure 1. The design of the near-infrared fluorescent turn-on probe HD-Sec for selenocysteine.

dinitrobenzenesulfonate unit, the near-infrared fluorescence should be recovered.

Probe HD-Sec was readily synthesized in four steps (Figure 2). The staring material **1** was prepared according to a reported procedure[13] and HD-NH_2 was synthesized according to the similar procedure for HD dyes.[10] Then, the HD-NH_2 dye was treated with 2,4-dinitrobenzenesulfonyl chloride under the basic conditions to give the target compound HD-Sec. Reference compound HD-$NHCOCH_3$ was synthesized by acetylation of HD-NH_2 (Scheme S1 in the Supporting Information). All new compounds were characterized by NMR spectroscopy and mass spectrometry.

To further support our design concept of the NIR fluorescent turn-on probes HD-Sec, we proceeded to investigate their optical properties. The absorption and emission profiles of compounds HD-NH_2 and HD-$NHCOCH_3$ in CH_3CH_2OH solvent is shown in Figure S1 in the Supporting Information. The HD-NH_2 dye displays a maximum absorption peak at around 698 nm, while HD-$NHCOCH_3$ has two blue-shift absorption bands at around 612 and 663 nm (Figure S1a). The fluorescence spectrum of HD-NH_2 exhibits strong fluorescence with a maximum emission peak at around 720 nm. As expected, the reference compound HD-$NHCOCH_3$, which bears no electron-donating group, displays weak fluorescence (Figure 1 b).

The molar extinction coefficients of the new compounds HD-NH_2 and HD-$NHCOCH_3$ in CH_3CH_2OH are 99580 and 41420 $M^{-1}cm^{-1}$, respectively. Importantly, the dye HD-NH_2 has a fluorescence quantum yield of 0.53 in CH_3CH_2OH, which is relatively large for NIR dyes. Furthermore, the fluorescence quantum yield of the compound HD-$NHCOCH_3$ is only 0.02, much less than that of the dye HD-NH_2. Thus, these data suggest that the optical properties of the new dye HD-NH_2 can be regulated by structural modifications on the key amine group.

With the probe HD-Sec in hand, we first evaluated the capability of the probe to detect selenocysteine (R-SeH) in PBS buffer (pH 7.4). As Sec is not stable, we generated it in situ by mixing equal molar of dithiothreitol (DTT) and selenocysteine ($(Sec)_2$).[14] As designed, the free probe is almost non-fluorescent in PBS (Figure 3 a). However, addition of selenocysteine (R-SeH) induces a dramatic change in the fluorescence spectra. A significant fluorescence turn-on response at 712 nm (up to 65-fold) was observed (Figure 3 b). Consistently, a marked red-shift from 596 to 690 nm was noted in the absorption spectra upon treatment of the probe with equal molar of DTT and $(Sec)_2$ (Figure S2 in the Supporting Information). Notably, the emission wavelength of the probe is in the NIR region, which is favourable for fluorescent imaging studies in living animals. Furthermore, the fluorescence intensities at 712 nm have an excellent linear relationship with the concentrations of equal molar of DTT and selenocysteine ($(Sec)_2$; Figure S3 in the Supporting Information). The probe HD-Sec responded rapidly to selenocysteine (Figure S4a in the Supporting Information). The reaction was almost complete within 10 min, and the pseudo-first-order rate constant was calculated to be $k = 0.1644\ min^{-1}$ (Figure S4b). The pH effect studies suggest that the probe HD-Sec is suitable for detecting selenocysteine (R-SeH) at physiological pH (Figure S5 in the Supporting Information).

To confirm the sensing process, we decided to study the product of HD-Sec reaction with selenocysteine by both NMR spectroscopy and mass spectrometry. Both the mass spectrometry and NMR analyses confirmed that the fluorescence turn-on is indeed due to the selenocysteine-mediated removal of the 2,4-dinitrobenzenesulfonate moiety (Figure S6 and Figure S7 in the Supporting Information).

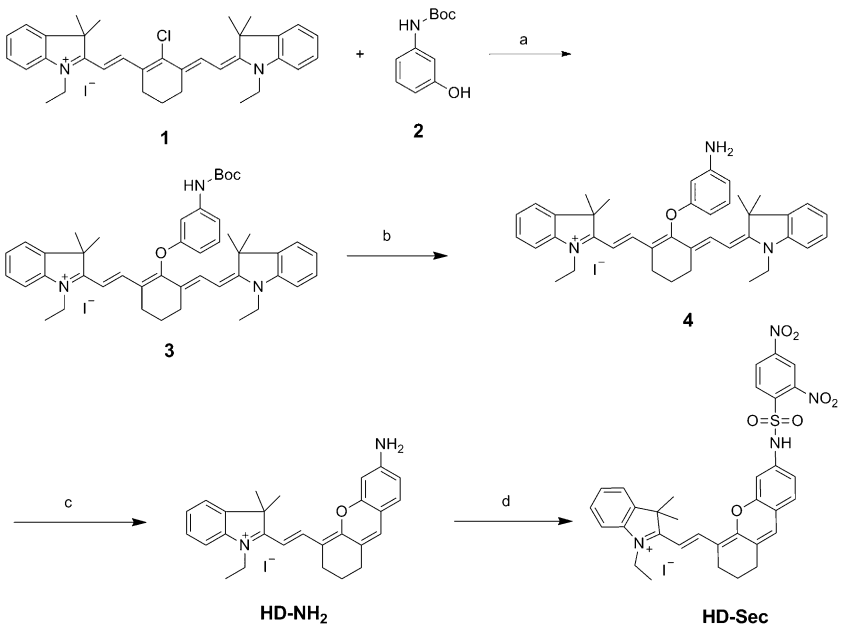

Figure 2. Synthesis of the probe HD-Sec. Conditions: a) NaH, DMF, 50 °C, 2 h; b) CF_3COOH, CH_2Cl_2, room temperature, 6 h; c) NaH, DMF, 90 °C, 5 h; d) Cs_2CO_3, CH_2Cl_2, 2,4-dinitrobenzenesulfonyl chloride, room temperature, 1 h.

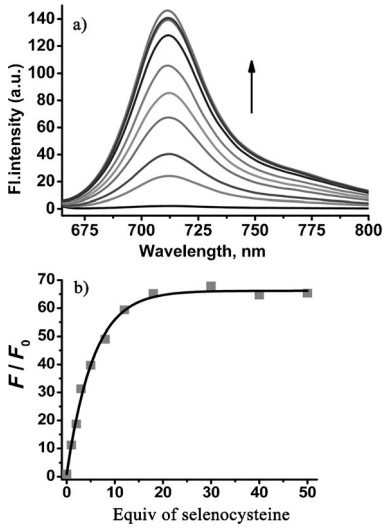

Figure 3. a) Emission spectra (excited at 650 nm) of the novel probe HD-Sec (5 μM) in the presence of increasing concentrations of selenocysteine at room temperature in PBS (pH 7.4, containing 5% DMSO as a co-solvent), generated by the treatment of equal molar $(Sec)_2$ with DTT; b) fluorescence intensity ratio (F/F_0) changes at 712 nm of the probe (5 μM) with increasing concentrations of selenocysteine at room temperature in PBS, generated by the treatment of equal molar $(Sec)_2$ with DTT.

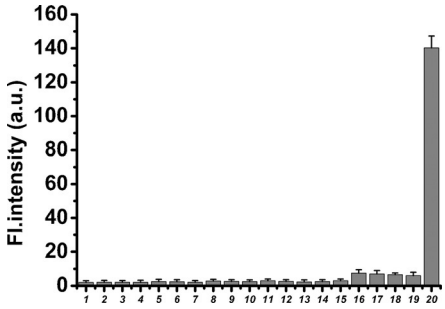

Figure 4. Fluorescence probe HD-Sec (5 μM) in the presence of various analytes in PBS buffer (pH 7.4, containing 5% DMSO as a co-solvent). 1: blank; 25 μM for: 2: Arg, 3: Glu, 4: Val, 5: Ser, 6: Leu, 7: Lys, 8: K^+, 9: Ca^{2+}, 10: Na^+, 11: Mg^{2+}, 12: Zn^{2+}, 13: DTT, 14: NADH, 15: glucose, 16: NaHS; 17: GSH (10 mM), 18: Hcy (1 mM), 19: Cys (1 mM), 20: DTT (25 μM); and selenocysteine (25 μM). Excitation at 650 nm and emission at 712 nm. Data are expressed as mean ±SD of three experiments.

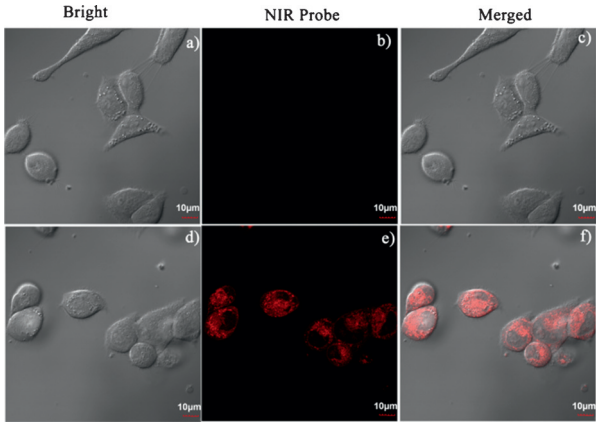

Figure 5. a–c) MCF-7 cells co-incubated with HD-Sec (5 μM) for 30 min. a) Brightfield image; b) emission from the red channel; and c) overlay of the brightfield image and red channel. d–f) MCF-7 cells pre-incubated with $(Sec)_2$ (5 μM) for 6 h, and then co-incubated with HD-Sec (5 μM) for 30 min. d) Brightfield image; e) emission from the red channel; f) overlay of the brightfield image and red channel. Scale bars: 10 μm.

To verify the specific response of probe HD-Sec toward selenocysteine (R-SeH), we then examined the selectivity of the probe HD-Sec toward other species (such as representative amino acids, glucose, metal ions, reducing agents, nucleosides, and small-molecule thiols) in buffer solution (25 mM phosphate buffer, pH 7.4) and monitored the response by emission spectroscopy. As exhibited in Figure 4, no noticeable changes were observed upon addition of amino acids (Arg, Glu, Val, Ser, Leu, and Lys), metal ions (K^+, Ca^{2+}, Na^+, Mg^{2+}, and Zn^{2+}), a reducing agent (DTT, NADH, and NaHS) or glucose. Notably, small-molecule thiols such as glutathione (GSH) at 10 mM, Cys and Hcy at 1 mM trigger only a minor fluorescence enhancement (Figure 4). By contrast, upon treatment of equal molar of DTT and selenocysteine ($(Sec)_2$) with the probe, a large fluorescence signal (65-fold fluorescence enhancement) was observed. Thus, these data demonstrate that the probe has a high selectivity for selenocysteine over other biological species tested including GSH and cysteine at the biologically relevant concentrations, suggesting that the new NIR probe is favourable for imaging studies in biological systems.

For the preliminary fluorescence imaging applications, the probe HD-Sec was incubated with living MCF-7 cells pretreated with or without $(Sec)_2$ (Figure 5 and Figure 6).

As the physiological concentration of Sec is low in cells, the probe HD-Sec cannot be directly used to image endogenous selenocysteine. As shown in Figure 5b, the cells treated with only the probe exhibit relatively weak fluorescence in the red channel. By contrast, when the cells were pretreated with $(Sec)_2$ for 6 h and then incubated with HD-Sec, much more intense fluorescence in the red channel was observed (Figure 5e). Prompted by the above desirable results of imaging exogenous selenocysteine in living cells, we decided to examine the possibility of the probe to monitor endogenously produced selenocysteine in living cells. Sodium selenite (Na_2SeO_3) is a precursor of Sec biosynthesis, and supplement of cells with sodium selenite could significantly increase the Sec level in cells.[15] Sodium selenite (Na_2SeO_3) itself did not react with the probe HD-Sec. After the cells were stimulated with sodium selenite for 6 h, a marked fluorescence appeared (Figure 6e). HD-Sec was also incubated with HeLa cells, and similar imaging results were obtained (Figure S8 and Figure S9 in the Supporting Information). Taken together, these results indicate that HD-Sec is cell membrane permeable and capable of sensing selenocysteine in living cells.

The prominent features of HD-Sec include NIR absorption and emission, rapid response, excellent selectivity, and good cell membrane permeability. These desirable attributes encouraged us to further investigate the suitability of the probe for detecting selenocysteine in the context of living animals. Kunming mice were divided into three groups (Figure 7). One group was given saline (100 μL) in the peritoneal cavity, fol-

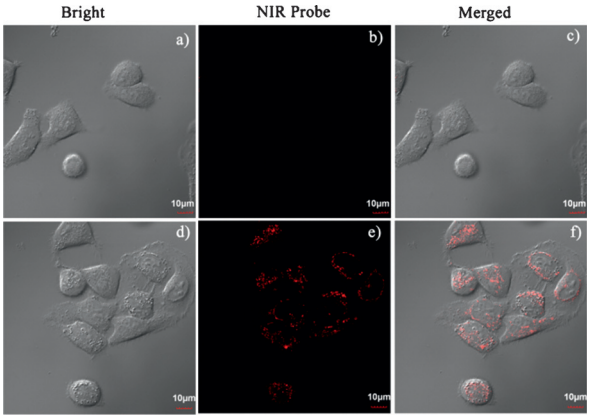

Figure 6. a–c) MCF-7 cells co-incubated with HD-Sec (5 μM) for 30 min. a) Brightfield image; b) emission from the red channel; and c) overlay of the brightfield image and red channel. d–f) MCF-7 cells pre-incubated with Na_2SeO_3 (5 μM) for 6 h, and then co-incubated with HD-Sec (5 μM) for 30 min. d) Brightfield image; e) emission from the red channel; f) overlay of the brightfield image and red channel. Scale bars: 10 μm.

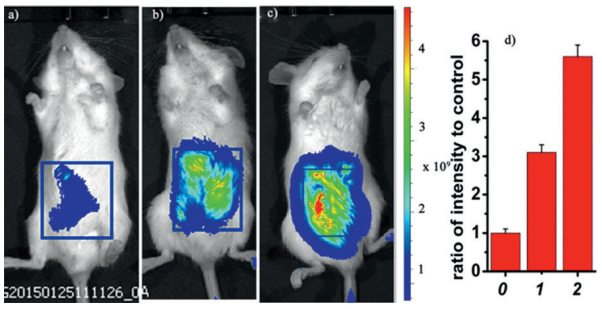

Figure 7. Representative fluorescent images (pseudo-colour) of selenocysteine in mice, 30 min after injection of HD-Sec: a) only the probe HD-Sec was injected in the peritoneal cavity of the mouse; b) the mouse was given an i.p. injection of $(Sec)_2$ and followed by i.p. injection with the sensor HD-Sec after 1 h; c) the mouse was given an i.p. injection of $(Sec)_2$ and followed by i.p. injection with the sensor HD-Sec after 6 h. The mice were imaged using an IVIS Lumina XR (IS1241N6071) in vivo imaging system with an excitation filter of 640 nm and an emission range of 695–770 nm; d) quantification of fluorescence emission intensity from the abdominal area of the mice of groups a–c: 0 (a), 1 (b), 2 (c). Data are expressed as mean ± S.D. of three experiments.

lowed by intraperitoneal (i.p.) injection with HD-Sec (20 μM, in 20 μL DMSO) as the negative control group; The second group was given an i.p. injection of $(Sec)_2$ (20 μM in 100 μL saline) and followed by i.p. injection with the probe HD-Sec (20 μM, in 20 μL DMSO) after 1 h; and the third group was given an i.p. injection of $(Sec)_2$ (20 μM in 100 μL saline) and followed by i.p. injection with the probe HD-Sec (20 μM, in 20 μL DMSO) after 6 h. The mice were anesthetized, and the abdominal fur was removed. After the probe HD-Sec was injected, the mice were imaged using an IVIS Lumina XR (IS1241N6071) in vivo imaging system. As shown in Figure 7 b, the mice pretreated with $(Sec)_2$ after 1 h and then treated with the probe HD-Sec exhibit a much higher fluorescence readout (pseudocolor) than the mice treated with only the probe (Figure 7 a), but less than the mice pretreated with $(Sec)_2$ after 6 h and then treated with the probe (Figure 7 c). The fluorescent intensity from the abdominal area of the mice was quantified, and the data indicate that the mice injected with treated $(Sec)_2$ after 6 h and then treated with the probe have approximately 5.6-fold higher fluorescence intensity than the mice injected with only the probe (Figure 7 d). Thus, these results demonstrate that the new NIR probe HD-Sec can image selenocysteine in the living animals. To the best of our knowledge, this represents the imaging of selenol in living animals for the first time.

In summary, we have designed and synthesized the first NIR fluorescent turn-on probe HD-Sec for specific detection of selenol. The probe HD-Sec exhibits high selectivity at physiological pH. In particular, the probe shows a 65-fold enhancement in the presence of selenol. Importantly, we have demonstrated that the probe HD-Sec is suitable for fluorescence imaging of selenol not only in living cells, but also in living animals. Further applications of the near-infrared probe for the investigation of the biological functions and pathological roles of selenol in living systems are in progress.

Kunming mice were purchased from Experimental Animal Center of Xiangya School of Medicine Central South Univeristy (Changsha, China). All animal procedures for this study were approved by the Animal Ethical Experimentation Committee of Central South University according to the requirements of the National Act on the use of experimental animals (China).

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Keywords: fluorescence sensing · fluorescent probes · imaging agents · NIR-fluorescent fluorophores · selenocysteine

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