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Near-Infrared Fluorescent Turn-On Probe with a Remarkable Large Stokes Shift for Imaging Selenocysteine in Living Cells and Animals

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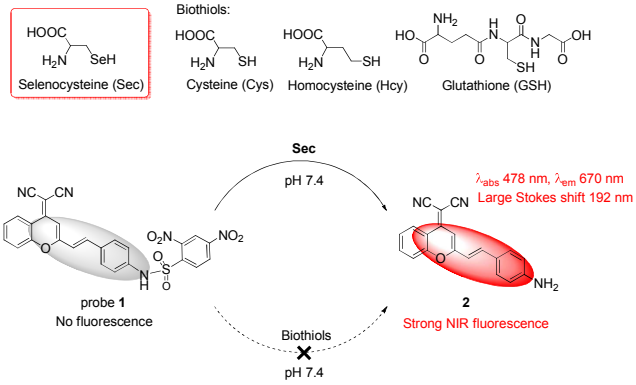
ABSTRACT: Selenocysteine (Sec) is the 21st naturally occurring amino acid and has emerged as an important sensing target in recent years. However, fluorescent detection of Sec in living systems is challenging. To date, very few fluorescent Sec probes have been reported and most of them respond fluorescence to Sec in the visible region. In this paper, a very promising near-infrared fluorescent probe for Sec was developed. This probe works in aqueous solution over a wide pH range under mild conditions and can be used for rapid, highly selective and sensitive detection of Sec with significant near-infrared fluorescent turn-on signal changes. In addition, it features a remarkable large Stokes shift (192 nm) and a low detection limit (60 nM) for Sec with a wide linear range (0-70 μM). Moreover, this probe can be conveniently used to detect Sec in serum samples, living cells, and animals, indicating it holds great promise for biological applications.

Selenium is an essential trace element in human body and is known very important to human health.[1-2] Although different forms of selenium exist, selenocysteine (Sec), a cysteine (Cys) analogue with a selenol group in place of the thiol group, appears to be the major form of biological seleniums.[3-4] Sec is the 21st naturally occurring amino acid and an essential building block for selenoproteins,[5-6] which are involved in various cellular functions and linked to several human diseases, such as inflammations, cardiovascular diseases, neurodegenerative diseases, and cancers.[7-9] Considering the important roles of Sec in biological systems, development of effective and biocompatible methods that can be used to visualize cellular Sec in real-time is of great significance.[10-13]

It is well-known that fluorescent probes with high selectivity and sensitivity and short response times are powerful tools for real-time detection of biomolecules.[14] However, designing specific fluorescent probes for selective detection of Sec in living systems is challenging mostly due to the interference of biothiols (such as Cys, Hcy, and GSH, Scheme 1) that share similar chemical properties to Sec but are much more abundant in cells. Although the first Sec-selective fluorescent probe has been reported 10 years ago,[15] the selectivity of this probe over biothiols is strictly restricted at low pH conditions (pH 5.8), which prevents its practical bioimaging applications. In fact, it was until very recently (the year of 2015) that selective detection of Sec under physiological pH and in living cells was realized with fluorescent probes,[16] and to date, fluorescent Sec probes are still very limited.[17-21] In addition, most of these reported Sec probes suffer from UV light excitation, short emission wavelength and relatively small Stokes shift (Table S1, Supporting Information), which limited their biological applications. A good solution is to develop near-infrared (NIR) fluorescent probes with large Stokes shift.[22] NIR fluorescent probes have been proved more favorable for bioimaging applications due to their minimum photodamage to biological samples, deep tissue penetration and negligible interference from background,[23-25] while fluorescent probes with large Stokes shift are more suitable for fluorescence microscopy studies because such probes have the clearly separated excitation and emission bands, which can effectively minimize the interferences caused by self-absorption or auto-fluorescence.[26-27] Therefore, development of NIR fluorescent probes with large Stokes shift for Sec detection are highly desirable.

Scheme 1. Probe **1** for NIR fluorescent Detection of Sec



Herein, we report a NIR fluorescent probe with a large Stokes shift for Sec detection (probe **1** in Scheme 1). This probe not only can be used to detect Sec over biothiols with high selectivity and sensitivity in aqueous solution under physiological pH and mild conditions, but also shows a rapid and distinct NIR fluorescent turn-on sensing process for Sec. In addition, the Stokes shift of this probe is remarkable large (192 nm), which is highly favorable to achieve reliable fluorescent detection. Moreover, this probe can be conveniently used for fluorescent detection of Sec in serum samples, living cells, and living animals, indicating this probe is very promising.

EXPERIMENTAL SECTION

Materials and Chemicals. All chemicals and solvents were used as received unless otherwise stated. Ultrapure water was used from a Millipore water ultrapurification system. The pH was measured using a PB-10 digital pH-meter. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 600 spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as an internal standard. The low-resolution mass spectra were collected on a LC-MS spectrometer. The high-resolution mass (HR-MS) data were obtained with a Bruker microTOF-Q instrument. UV-vis and fluorescence spectra were recorded on an Agilent Cary-100 UV-vis spectrophotometer and an Agilent Cary Eclipse fluorescence spectrophotometer, respectively. Cell imaging was performed with an inverted fluorescence microscopy with a 24× objective lens.

Synthesis of probe 1. Probe **1** was prepared via the reaction of compound **2** with 2,4-dinitrobenzenesulfonyl chloride according to our previous report.[28]

Compound **2**, as the fluorophore of probe **1**, was first synthesized according to the reported method[29] as a deep purple-red solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (d, J = 8.3 Hz, 1H), 7.88 (t, J = 7.8 Hz, 1H), 7.76 (d, J = 8.4 Hz, 1H), 7.63 (d, J = 15.8 Hz, 1H), 7.58 (t, J = 7.7 Hz, 1H), 7.49 (d, J = 8.1 Hz, 2H), 7.09 (d, J = 15.6 Hz, 1H), 6.86 (s, 1H), 6.62 (d, J = 8.2 Hz, 2H), 6.11 (br s, 2H, NH_2). ^{13}C NMR (150 MHz, DMSO-d_6) 159.6, 152.4, 152.0, 151.9, 140.5, 134.9, 130.6, 125.8, 124.4, 122.2, 118.8, 117.7, 117.1, 116.3, 113.7, 112.3, 104.6, 57.3. ESI-MS: m/z found 312.10 (M + H$^+$). These data are consitant with our previous report.[28]

Then a mixture of compound **2** (100 mg, 0.32 mmol) in dry pyridine (10 mL) was prepared and cooled to 0˚C, and a solution of 2, 4-dinitrobenzenesulfonyl chloride (260 mg) in dry CH_2Cl_2 (5 mL) was slowly added. After stirring at 0 °C for 30 minutes, the reaction mixture was further stirred at room temperature overnight. After the completion of the reaction (monitored by TLC), the mixture was washed in turn by 50 mL of water and saturated brine and dried over anhydrous Na_2SO_4. After remove the solvent, the residue was purified by flash column chromatography to give pure probe **1** as a red solid (86 mg, yield 50%). Mp >300 °C. TLC (silica plate): R_f ~0.47 (hexane/ethyl acetate 1:1, v/v). ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.44 (br s, 1H, NH), 8.92 (s, 1H), 8.72 (d, J = 8.4 Hz, 1H), 8.61 (d, J = 8.7 Hz, 1H), 8.29 (d, J = 8.7 Hz, 1H), 7.91 (t, J = 7.3 Hz, 1H), 7.77 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 8.1 Hz, 2H), 7.67 (d, J = 16.3 Hz, 1H), 7.61 (t, J = 7.7 Hz, 1H), 7.40 (d, J = 16.3 Hz, 1H), 7.22 (d, J = 8.1 Hz, 2H), 6.99 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) 158.0, 152.8, 151.9, 149.9, 147.7, 137.7, 135.3, 131.4, 131.0, 129.4, 127.1, 126.1, 124.5, 120.3, 120.2, 118.9, 118.7, 117.1, 117.0, 115.8, 106.5, 60.0. HR-MS calcd for $C_{26}H_{16}N_5O_7S^+$ (M + H$^+$) 542.0765; Found: 542.0785.

Optical Studies. Stock solutions of probe **1** (1 mM) were prepared in HPLC grade DMSO. The Sec was prepared by the incubation of equimolar selenocystine $(Sec)_2$ with dithiothreitol (DTT) in phosphate buffer at 37 °C for 30 min and freshly used.[15-16] Stock solutions (1-10 mM) of the other analytes including thiols such as Cys, Hcy, and GSH, amino acids such as Ala, Glu, Arg, Ser, Lys, Asp, Gly, Leu, Ile, Gln, Tyr, His, Trp, Thr, Phe, Asn, Met, and Val, and common salts for anions and metal cations such as NaF, NaCl, KBr, KI, NaAcO, $NaHCO_3$, NaN_3, $NaNO_3$, NaHS, Na_2SO_4, NaSCN, $Na_2C_2O_4$, $Na_2S_2O_7$, Na_2HPO_4, $NaHSO_3$, NaHS, NaCN, NaClO, $NaNO_2$, Na_2SeO_3, LiCl, $MgCl_2$, $Al_2(SO_4)_3$, $CaCl_2$, $Zn(NO_3)_2$, $MnSO_4$, $Co(NO_3)_2$, $Cd(NO_3)_2$, $Ni(NO_3)_2$, $Cr(NO_3)_3$, $HgCl_2$, $Cu(NO_3)_2$, $FeCl_2$, $FeCl_3$, and $AgNO_3$ were prepared in ultrapure water.

For a typical optical study, a solution of probe **1** (10 μM) was incubated with a certain amount of analyte (Sec or others) in DMSO-PBS buffer solution (10 mM, 1:1, v/v, total volume 3 mL) under an indicated pH in a quartz cuvette (10 mm lightpath), and the temperature was set at 37 °C. The optical spectrum of the above solution was measured at an indicated time. Excitation wavelength for fluorescence measurements was set at 490 nm with slit width d_{ex} = d_{em} = 10 nm. For competition studies, probe **1** was firstly incubated with an analyte for 10 min, and the fluorescent intensity of the solution at 670 nm was recorded. Then, Sec (100 μM) was added to the mixture. After further incubation for 10 min, the fluorescent intensity at 670 nm of the solution was measured again.

Cell Culture and Sec Bioimaging with Probe 1. A549 human lung carcinoma cells were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 °C, and then were seeded in a 24-well culture plate for one night before cell imaging experiments. In bioimaging experiments, as control, living cells were incubated with 20 μM of probe **1** (with 0.1% DMSO, v/v) at 37 °C for 1 h, 6 h, and 12 h and washed with PBS for three times, and then imaged, respectively. For imaging exogenous Sec in living cells, cells were cultured with 5 μM $(Sec)_2$ for 1 h (or 6 h, 12 h as indicated) at 37 °C, washed three times with PBS, and then incubated with 20 μM of probe **1** for 30 min at 37 °C and imaged immediately. In the imaging of endogenous Sec, cells were pre-treated with Na_2SeO_3 (5 μM) for 1 h (or 6 h, 12 h as indicated) at 37 °C. After washed three times with PBS buffer, the cells were then treated with probe **1** (20 μM) for 30 min at 37 °C. Cell imaging was then carried out after washing with PBS buffer.

For *in vivo* living mice imaging, Balb/c mice (weight 18-22 g) were used. The mice were purchased from School of Medcine of Wuhan University (Wuhan, China). All of the animal experiments in this study were approved by the Animal Ethical Experimentation Committee of Wuhan University and were carried out strictly in accordance with the requirements of the National Act on the use of experimental animals (China). During the experiments, the mice were anesthetized by intraperitoneal (i.p.) injection of 1% pelltobarbitalum natricum solution (0.01 mL/g), and their abdominal fur was removed with a shaver. The Balb/c mice were divided into two groups.[17] One group was only given an i.p. injection of probe **1** (10 μM, in 20 μL DMSO) in the peritoneal cavity as the control and after 30 min, the mouse was imaged; The second group was given an i.p. injection of probe **1** (10 μM, in 20 μL DMSO) and followed by i.p. injection with the $(Sec)_2$ (10 μM, in 100 μL saline) and the mouse was imaged after 5 min, 15 min, and 30 min, respectively. Imaging was carried out in a Bruker In Vivo-Xtreme imaging system with optical and X-ray dual mode. An excitation filter of 490 nm and an emission filter of 700 nm were used for fluorescence imaging.

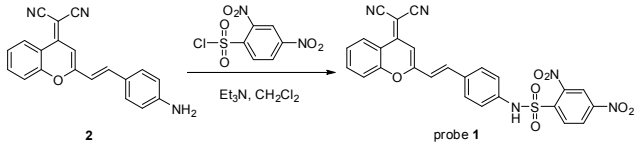
RESULTS AND DISCUSSION

Probe Structure and Synthesis. To obtain a NIR fluorescent probe for Sec, probe **1** used a dicyanomethylene-benzopyran (DCMB)-based compound **2** as the fluorophore and a 2,4-dinitrobenzenesulfonyl (DNBS) moiety as the Sec-

reaction site. Compound **2** is used because it is a known fluorophore with emissions in the NIR region, large Stokes shift, high photostability, and high quantum yield ($\Phi = 0.66$).[29-33] On the other hand, the 2,4-dinitrobenzenesulfonamide group has been proved to be a good fluorescence quenching group and a good reaction site for Sec but not for biothiols under physiological pH due to the higher nucleophilicity of Sec (pK_a of Sec: ~5.8; pK_a of biothiols: ~8.5).[16,17,19,21] Although this probe has been recently reported by us for detection of thiophenols,[28] thiolphenols are not naturally present in living systems. Moreover, recent studies revealed that a thiophenol probe can be used as a Sec probe due to the same mechanism of nucleophilic attack (Sec and thiophenol have very similar pK_a values).[16,19,22] Therefore, we can envision that probe **1** should have great potential as a NIR fluorescent probe for Sec detection in biological systems.

To prove our thoughts, probe **1** was prepared from the reaction of the compound **2** with 2,4-dinitrobenzenesulfonyl chloride (Scheme 2). The structure of this probe was confirmed by NMR and MS analyses. Detailed synthetic procedures and structure characterizations are given in the Experimental Section and in the Supporting Information.

Scheme 2. The Synthesis of Probe **1**



Optical Responses of Probe 1 for Sec. With probe **1** in hand, its optical sensing property for Sec was investigated in PBS buffer (10 mM, pH 7.4, with 50% of DMSO, v/v) at 37 °C. As Sec is not stable, we generated it in situ by mixing equal molar of $(Sec)_2$ with DTT according to the reported method.[15-17] As expected, probe **1** is almost non-fluorescent under this condition but shows significant NIR fluorescence enhancement upon addition of Sec and as a result, the solution started to emit red fluorescence, which can be clearly observed under a portable 365 nm light (Figure 1a). The fluorescence enhancement at 670 nm was observed to reach a plateau within 10 minutes, indicating probe **1** responds to Sec very fast. In contrast, in the absence of Sec, the probe **1** solution did not show any noticeable fluorescence changes within 30 minutes (Figure 1b). Since Sec was generated in situ by mixing equal molar of $(Sec)_2$ with DTT, we also tested the response of probe **1** for DTT and $(Sec)_2$, respectively. The results show that probe **1** only displays very small fluorescent enhancement to DTT and is not responsive to $(Sec)_2$ at all (Figure S1), which indicates that the observed response in Figure 1a is indeed from Sec. All these initial results suggested that probe **1** can be used as a good NIR fluorescent turn-on probe to rapidly detect Sec in aqueous solution under mild conditions.

We also tested the UV-vis spectra response of probe **1** for Sec. The result showed that upon addition of Sec to the probe **1** solution, the maximum absorption at 474 nm slightly red-shifted to 478 nm with the increase of intensity (Figure S2). However, this change is not that noticeable due to the small absorption spectra difference between probe **1** and compound **2** (the expected product). Therefore, the following studies were focused on the fluorescent method.

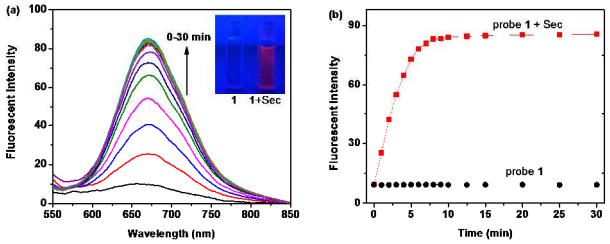


Figure 1. (a) Fluorescence spectral changes of probe **1** (10 μM) upon addition of Sec (100 μM) in PBS buffer (10 mM, pH 7.4, with 50% DMSO, v/v) at 37 °C. Insert: an emission color change under a 365 nm light. (b) The fluorescent intensity changes of probe **1** (10 μM) at 670 nm in the absence and presence of Sec (100 μM).

Selectivity. Since high selectivity is crucial to a probe, we then investigated the selectivity of probe **1** for Sec by addition of various other analytes. Delightedly, as shown in Figure 2, upon addition of the most potential interferences, biothiols such as Cys, Hcy, and GSH and amino acids such as Ala, Glu, Arg, Ser, Lys, Asp, Gly, Leu, Ile, Gln, Tyr, His, Trp, Thr, Phe, Asn, Met, and Val, the probe **1** solution did not show any noticeable changes. This is in sharp contrast to the response of probe **1** for Sec, indicating probe **1** has high selectivity for Sec over biothiols and other amino acids. Other potential interfering anions such as F^-, Cl^-, Br^-, I^-, AcO^-, HCO_3^-, N_3^-, NO_3^-, SO_4^{2-}, SCN^-, $C_2O_4^{2-}$, $S_2O_7^{2-}$, HSO_3^-, HS^-, CN^-, ClO^-, NO_2^-, HPO_4^{2-} and SeO_3^{2-}, and metal ions such as Li^+, Na^+, K^+, Mg^{2+}, Al^{3+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Ag^+ were also tested (Figure S3-4). The results showed that these ionic species also did not show any noticeable changes to the probe **1** solution (Figure S3a and S4a). Moreover, competition experiments showed that the fluorescent turn-on response of probe **1** to Sec was not interfered by all of these analytes including 5 mM level of GSH (Figure 2b, Figure S3b and S4b), indicating biothiols, amino acids, common anions and metal cations have no interference for NIR fluorescent detection of Sec with probe **1**. Overall, these results clearly showed that probe **1** has excellent selectivity and anti-interference ability for Sec, which is highly desirable for Sec detection in living systems.

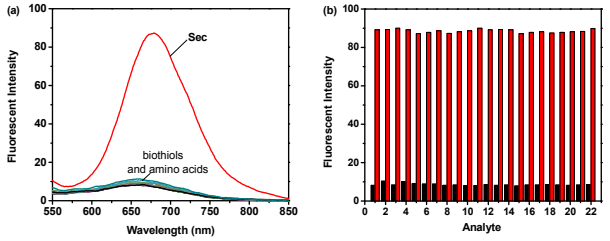


Figure 2. (a) Fluorescence responses of probe **1** (10 μM) upon addition of Sec (100 μM), biothiols (Cys, 200 μM; Hcy, 100 μM; GSH, 5 mM), and amino acids (Ala, Glu, Arg, Ser, Lys, Asp, Gly, Leu, Ile, Gln, Tyr, His, Trp, Thr, Phe, Asn, Met, and Val, 100 μM each) in PBS buffer (10 mM, pH 7.4, with 50% DMSO, v/v) at 37 °C. (b) Fluorescence intensity responses of probe **1** (10 μM) at 670 nm toward Sec in the presence of various analytes. Black bars represent the addition of a single analyte (100 μM of each unless specified). (1) none, (2) Cys (200 μM), (3) Hcy, (4) GSH (5 mM), (5) Ala, (6) Glu, (7) Arg, (8) Ser, (9) Lys, (10) Asp, (11) Gly, (12) Leu, (13) Ile, (14) Gln, (15) Tyr, (16) His, (17) Trp, (18) Thr, (19) Phe, (20) Asn, (21) Met, (22) Val. Red bars represent the subse-

quent addition of Sec (100 μM) to the mixture. Each data was obtained 10 min after mixing.

Sensitivity. After confirm the high selectivity of probe **1** for Sec, its sensitivity was investigated. Thus, different concentrations of Sec were added to the probe **1** solution and the fluorescence changes were measured. As shown in Figure 3, upon progressive addition of Sec, the fluorescence of the probe **1** solution gradually increases until it reaches a plateau after the addition of more than 10 equiv of Sec. One can see that, a good linear relationship between the fluorescent intensity at 670 nm and the Sec concentrations from 0 to 70 μM can be observed (Figure 3b), which indicates that probe **1** can be employed to detect Sec quantitatively in a relatively wide linear range. In addition, the detection limit of probe **1** for Sec was calculated to be about 60 nM from the calibration curve using the method of signal-to-noise ratio (S/N) of 3, indicating probe **1** has high sensitivity for Sec.

A comparison of probe **1** with previously reported fluorescent Sec probes is shown in Table S1. One can see that, the sensitivity of probe **1** for Sec is comparable or better than some of previously reported Sec probes. However, it should be noted that probe **1** has the merits of NIR emission with visible excitation wavelength, whereas most of the reported probes emit visible fluorescence and use UV light for excitation. Besides, probe **1** has a remarkable large Stokes shift (192 nm), which can effectively diminish self-quenching and measurement error caused by excitation light and scattered light[25-27] and therefore is more favorable for fluorescent detection.

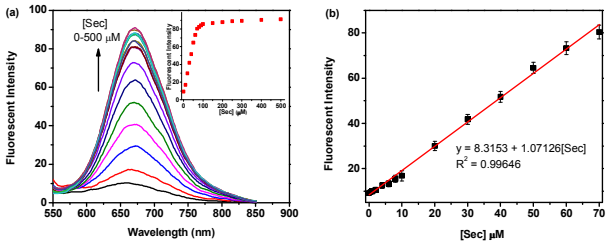


Figure 3. (a) Fluorescent spectra changes of probe **1** (10 μM) upon addition of different concentrations of Sec (0-500 μM) in DMSO-PBS buffer (10 mM, pH 7.4, 1:1, v/v) at 37 °C. Each spectrum was obtained 10 min after Sec addition. Insert: the plot of fluorescence intensity at 670 nm as a function of Sec concentrations. (b) Linear relationship of the fluorescence intensity at 670 nm against the concentration of Sec from 0 to 70 μM. The data were reported as the mean ± standard deviation of triplicate experiments and were fitted by the equation inserted.

The effect of pH. The effect of pH for fluorescent sensing of Sec with probe **1** was also investigated. As shown in Figure S5 (Supporting Information), the fluorescence of probe **1** itself is almost not responsive to the change of pH; however, in the presence of Sec, the probe **1** solution showed distinct fluorescence enhancement over a wide pH range from 4 to 10. Notably, the fluorescence response of probe **1** to Sec reached a maximum at pH about 6 and after that, it became almost constant, which should be highly favorable for Sec detection in living systems, because variation of pH around the physiological pH cannot affect the analysis.

The sensing mechanism. The above optical responses of probe **1** for Sec suggest that probe **1** reacted with Sec and produced the NIR fluorescent compound **2**. To prove this, TLC, HPLC, and mass analyses were used to analyze the reaction mixture of probe **1** and Sec. The TLC analysis showed that the reaction mixture produced a red fluorescent product, which has the same retention factor (R_f) value to the reference compound **2** (Figure S6), indicating compound **2** was produced. The HPLC and mass analyses further proved the production of compound **2** in the reaction mixture of probe **1** and Sec (Figure S7-8). Overall, all these results indicate that probe **1** actually reacted with Sec and gave out the fluorescent **2** as a product, which can be used to explain the observed optical changes.

Application Potential of Probe 1. All the above results showed that we have established a promising NIR fluorescent probe for Sec. Considering that the available fluorescent probes for Sec detection in biological samples are very rare to date, we further investigated the potential of probe **1** for detection of Sec in serum samples, living cells and animals.

*Sec Detection in Serum Samples with Probe **1***. To demonstrate the potential of probe **1** for Sec detection in serum samples, commercially available fetal bovine serum (FBS) was used. The received FBS sample was directly diluted ten times with the testing PBS buffer (10 mM, pH 7.4, with 50% DMSO, v/v) and subjected to analysis. Initial test showed that probe **1** is not responsive this diluted FBS sample, indicating the level of Sec in the diluted serum is too low to be directly detected by probe **1**. However, when this diluted FBS sample was spiked with different levels of Sec (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, and 500 μM, respectively) and then incubated with probe **1** for 10 min, we obtained concentration-dependent fluorescence responses (Figure S9), which are almost the same to the results shown in Figure 3. This result clearly indicates that probe **1** has great potential for detection of Sec in serum samples.

*Sec Detection in Living Cells with Probe **1***. Before bioimaging experiments, MTT assays were used to investigate the cytotoxicity of probe **1**. The results indicate that probe **1** (used up to 50 μM) has very low cytotoxicity to living cells (Figure S10). Fluorescent imaging of Sec with probe **1** in living cells was then investigated. As shown in Figure 4, when living A549 human lung cells were incubated with probe **1** (20 μM), almost no fluorescence can be observed even after 12 h of incubation (Figure 4a), indicating probe **1** is not responsive to the species inside the cells including the abundant biothiols. However, when cells were preincubated with $(Sec)_2$ (5 μM), and then incubated with probe **1** (20 μM), the cells showed time-dependent red fluorescence (Figure 4b). This can be ascribed to the formation of Sec by the reaction of $(Sec)_2$ with intracellular biothiols,[16-17] which can be proved by the responses of probe **1** to the mixture of $(Sec)_2$ with 2 equiv of Cys or GSH in the *in vitro* test (Figure S11). Therefore, these results clearly indicate that probe **1** can be used to detect exogenous Sec in living cells.

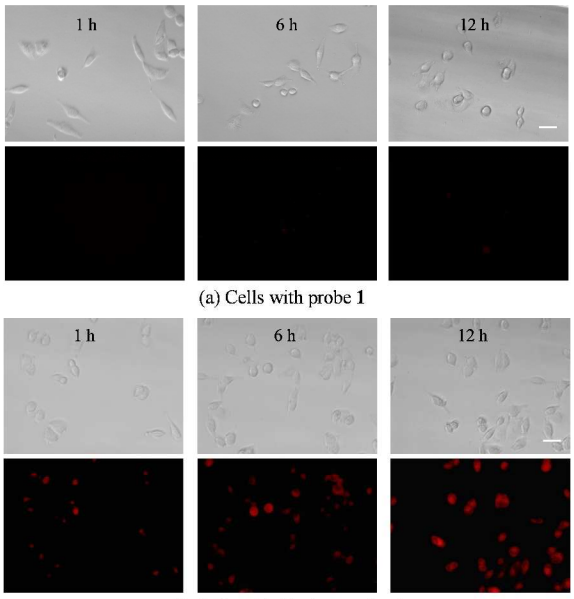


Figure 4. Fluorescent imaging of Sec in A549 cells. (a) Cells were treated with probe **1** (20 μM) at different incubation times (1 h, 6 h and 12 h). (b) Cells were preincubated with 5 μM $(Sec)_2$ for 1 h, 6 h and 12 h, and then treated with probe **1** (20 μM) for 30 min, respectively. Top row of a and b: bright field images. Bottom row of a and b: the fluorescence images with excitation wavelength at 460-550 nm. Scale bar = 30 μm.

It has been reported that Na_2SeO_3 can be metabolized to selenol via a series of physiological processes.[34] Although our *in vitro* experiment showed that probe **1** is not responsive to Na_2SeO_3, supplement of cells with a small amount of Na_2SeO_3 has been reported to significantly increase the Sec level in cells and this process can be tracked by fluorescent Sec probes.[16-21] Thus, we also decided to investigate the potential of probe **1** to track endogenously produced Sec in living cells through Na_2SeO_3 stimulation. As shown in Figure 5, after the cells were stimulated with Na_2SeO_3 (5 μM) and then incubated with probe **1**, a marked time-dependent fluorescence enhancement was also clearly observed. This indicates that probe **1** can be used to track the endogenously produced Sec in living cells.

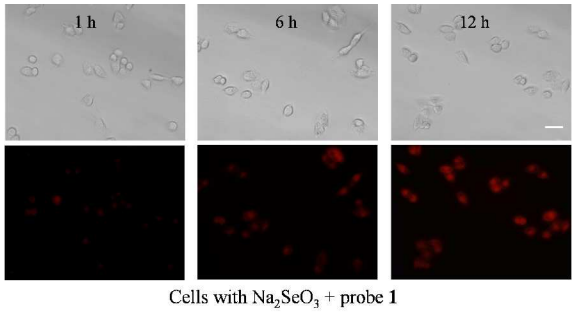


Figure 5. Fluorescent imaging of endogenously produced Sec through Na_2SeO_3 stimulation in A549 cells. Cells were preincubated with Na_2SeO_3 (5 μM) for 1 h, 6 h and 12 h, and then treated with probe **1** (20 μM) for 30 min, respectively. Top row: bright field images. Bottom row: the corresponding fluorescence images with excitation wavelength at 460-550 nm. Scale bar = 30 μm.

Using probe **1**, we also observed that incubation of cells with selenocompounds DBDS and SeO_2 can significantly induce the red fluorescence inside the cells (Figure S12). This is consistent with previous report, which showed that the metabolism of these selenocompounds in living cells can produce selenol.[16] Thus, all of above cell imaging experiments showed that probe **1** can be conveniently used to detect both exogenous and endogenous Sec in living cells.

*Sec Detection in Living Mouse with Probe **1***. Encouraged by the above results, the suitability of probe **1** for *in vivo* detecting of Sec was further investigated. Living Balb/c mice were used in these experiments. As shown in Figure 6, when the mouse was injected with probe **1** only (10 μM, in 20 μL DMSO) as the control, almost no fluorescence was observed (Figure 6a). However, when the mouse was given an intraperitoneal injection of probe **1** (10 μM, in 20 μL DMSO) and followed by injection with $(Sec)_2$ (10 μM, in 100 μL saline), a time dependent NIR fluorescence enhancement can be clearly observed (Figure 6b-d). The quantified relative fluorescent intensity from the abdominal area of the mice is shown in Figure 6e, and one can see that, a large fluorescence enhancement (~7.2-fold) can be observed when the mouse was incubated with $(Sec)_2$ for 30 min. These results indicate that probe **1** can be used to image Sec in living animals.

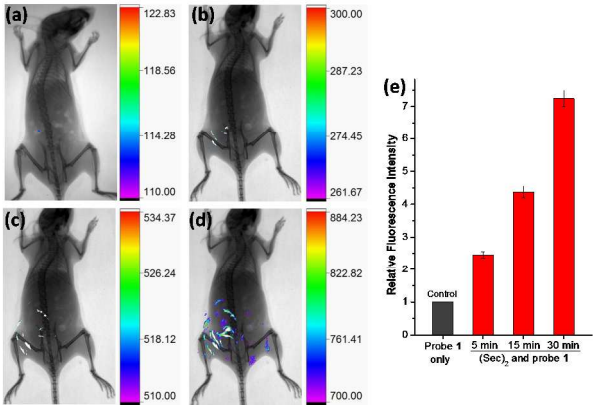


Figure 6. Fluorescent detection of Sec in living mice with probe **1**. (a) The mouse was given an intraperitoneal injection of only probe **1** and imaged after 30 min. (b-d) The mouse was given an intraperitoneal injection of probe **1** and followed by intraperitoneal injection with $(Sec)_2$ and imaged after 5 min, 15 min, and 30 min, respectively. (e) The relative fluorescence intensity from the abdominal area of the mice of a-d. All images were taken with a Bruker In Vivo-Xtreme Imaging system with optical and X-ray dual mode. Excitation was set at 490 nm and emission was collected around 700 nm. The quantified fluorescence intensity for each mouse was indicated on the right side of each figure of a-d.

CONCLUSION

In summary, we reported a new NIR fluorescent probe for detection of the biologically important Sec. This probe has the advantage of a remarkable large Stokes shift and displays a rapid, highly selective and sensitive NIR fluorescence turn-on response for Sec over biothiols, other amino acids, and various anions and metal ions. In addition, we also showed that this probe has low cytotoxicity and can be conveniently used to detect or image Sec in serum, living cells and animals. Overall, this work provided a very promising NIR fluorescent probe with desired sensing properties for Sec detection in biological samples.

ASSOCIATED CONTENT

Supporting Information

Structure characterizations of probe **1** and compound **2** and the additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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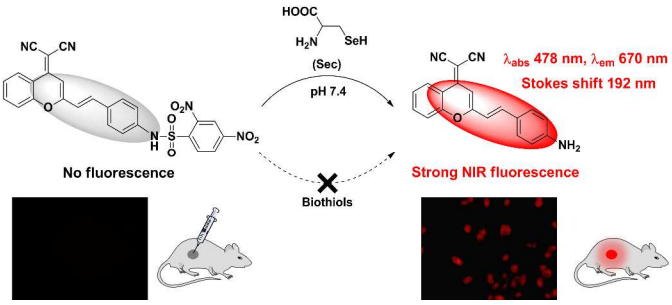
REFERENCES

(1) Rayman, M. P. *Lancet* **2012**, *379*, 1256–1268.
(2) Reich, H. J.; Hondal, R. J. *ACS Chem. Biol.* **2016**, *11*, 821–841.
(3) Weekley, C. M.; Harris, H. H. *Chem. Soc. Rev*. **2013**, *42*, 8870–8894.
(4) Fairweather-Tait, S. J.; Bao, Y.; Broadley, M. R.; Collings, R.; Ford, D.; Hesketh, J. E.; Hurst, R. *Antioxid. Redox Signaling* **2011**, *14*, 1337−1383.
(5) Böck, A.; Forchhammer, K.; Heider, J.; Leinfelder, W.; Sawers, G.; Veprek, B.; Zinoni, F. *Mol. Microbiol.* **1991**, *5*, 515-520.
(6) Roman, M.; Jitaru, P.; Barbante, C. *Antioxid. Redox Sign*. **2007**, *9*, 775–806.
(7) Labunskyy, V. M.; Hatfield, D. L.; Gladyshev, V. N. *Physiol. Rev*. **2014**, *94*, 739–777.
(8) Brown, K. M; Arthur, J. R. *Public Health Nutr*. **2001**, *4*, 593–599.
(9) Hatfield, D. L.; Tsuji, P. A.; Carlson, B. A.; Gladyshev, V. N. *Trends Biochem. Sci*. **2014**, *39*, 112–120.
(10) Hu, B.; Cheng, R.; Liu, X.; Pan, X.; Kong, F.; Gao, W.; Xu, K.; Tang, B. *Biomaterials* **2016**, *92*, 81–89.
(11) Liu, X.; Hu, B.; Cheng, R.; Kong, F.; Pan, X.; Xu, K.; Tang, B. *Chem. Commun*. **2016**, *52*, 6693–6696.
(12) Nan, Y.; Zhao, W.; Xu, X.; Au, C.-T.; Qiu, R. *RSC Adv*. **2015**, *5*, 69299–69306.
(13) Wu, D.; Chen, L.; Kwon, N.; Yoon, J. *Chem* **2017**, *1*, 674–698.
(14) Ding, S.; Zhang, Q.; Xue, S.; Feng, G. *Analyst* **2015**, *140*, 4687–4693.
(15) Maeda, H.; Katayama, K.; Matsuno, H.; Uno, T. *Angew. Chem., Int. Ed*. **2006**, *45*, 1810−1813.
(16) Zhang, B.; Ge, C.; Yao, J.; Liu, Y.; Xie, H.; Fang, J. *J. Am. Chem. Soc*. **2015**, *137*, 757−769.
(17) Chen, H.; Dong, B.; Tang, Y.; Lin, W. *Chem.-Eur. J*. **2015**, *21*, 11696−11700.
(18) Kong, F.; Hu, B.; Gao, Y.; Xu, K.; Pan, X.; Huang, F.; Zheng, Q.; Chen, H.; Tang, B. *Chem. Commun*. **2015**, *51*, 3102−3105.
(19) Sun, Q.; Yang, S.-H.; Wu, L.; Dong, Q.-J.; Yang, W.-C.; Yang, G.-F. *Anal. Chem*. **2016**, *88*, 6084–6091.
(20) Areti, S.; Verma, S. K.; Bellare, J.; Rao, C. P. *Anal. Chem*. **2016**, *88*, 7259−7267.
(21) Wang, Q.; Zhang, S.; Zhong, Y.; Yang, X.-F., Li, Z.; Li, H. *Anal. Chem*. **2017**, *89*, 1734–1741.
(22) Li, M.; Feng, W.; Zhai, Q.; Feng, G. *Biosens. Bioelectron*. **2017**, *87*, 894–900.
(23) Yuan, L.; Lin, W.; Zheng, K.; He, L.; Huang, W. *Chem. Soc. Rev*. **2013**, *42*, 622–661.
(24) Guo, Z.; Park, S.; Yoon J.; Shin, I. *Chem. Soc. Rev*., **2014**, *43*, 16–29.
(25) Yu, D.; Zhai, Q.; Yang S.; Feng, G. *Anal. Methods* **2015**, *7*, 7534–7539.
(26) Peng, X.; Song, F.; Lu, E.; Wang, Y.; Zhou, W.; Fan, J.; Gao, Y. *J. Am. Chem. Soc*. **2005**, *127*, 4170−4171.
(27) He, X.; Wang, Y.; Wang, K.; Chen, M.; Chen, S. *Anal. Chem*. **2012**, *84*, 9056**–**9064.
(28) Yu, D.; Huang, F.; Ding, S.; Feng, G. *Anal. Chem*. **2014**, *86*, 8835−8841.
(29) Sun, W.; Fan, J.; Hu, C.; Cao, J.; Zhang, H.; Xiong, X.; Wang, J.; Shuang, C.; Sun, S.; Peng, X. *Chem. Commun*. **2013**, *49*, 3890−3892.
(30) Gu, K.; Liu, Y.; Guo, Z.; Lian, C.; Yan, C.; Shi, P.; He Tian, H.; Zhu, W.-H. *ACS Appl. Mater. Interfaces* **2016**, *8*, 26622−26629.
(31) Wu, X. M.; Sun, X. R.; Guo, Z. Q.; Tang, J. B.; Shen, Y. Q.; James, T. D.; Tian, H.; Zhu, W. H. *J. Am. Chem. Soc*. **2014**, *136*, 3579−3588.
(32) Fan, J.; Sun, W.; Wang, Z.; Peng, X.; Li, Y.; Cao, J. *Chem. Commun*. **2014**, *50*, 9573−9576.
(33) Cao, S.; Pei, Z.; Xu, Y.; Pei, Y. *Chem. Mater*. **2016**, *28*, 4501−4506.
(34) Kryukov, G. V.; Castellano, S.; Novoselov, S. V.; Lobanov, A. V.; Zehtab, O.; Guigo, R.; Gladyshev, V. N.; *Science* **2003**, *300*, 1439–1443.

For TOC only


HOOC
H2N
SeH
(Sec)
pH 7.4
NC
CN
O2N
NO2
N
H
S
O
No fluorescence
Biothiols
λabs 478 nm, λem 670 nm
Stokes shift 192 nm
NH2
Strong NIR fluorescence