A semi-naphthorhodafluor-based red-emitting fluorescent probe for tracking of hydrogen polysulfide in living cells and zebrafish

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Highlights

- A semi-naphthorhodafluor-based red-emitting fluorescent probe SNARF-H2Sn for selective detection of H2Sn was designed.
- The addition of H2Sn would result in a > 1000-fold fluorescence enhancement within 10 min.
- SNARF-H2Sn was successfully employed to image exogenous/endogenous H2Sn in living cells and zebrafish.

Graphical Abstract

Abstract

Hydrogen polysulfides (H2Sn, n ≥ 2) is recently regarded as a potential signaling molecule which shows a higher efficiency than hydrogen sulfides (H2S) in regulating enzymes and ion channels. However, the development of specific fluorescent probes for H2Sn with long-wavelength emission (>600 nm) are still rare. In this work, a semi-naphthorhodafluor-based red-emitting fluorescent probe SNARF-H2Sn containing a phenyl 2-(benzoylthio) benzoate responsive unit was constructed. SNARF-H2Sn was capable of selectively detecting H2Sn over other reactive sulfur species. Treatment with H2Sn would result in a > 1000-fold fluorescence enhancement within 10 min. SNARF-H2Sn showed a low limit of detection down to 6.7 nM, and further enabled to visualize exogenous/endogenous H2Sn in living A549 cells and zebrafish.

1. Introduction

Reactive sulfur species (RSS), consisting of thiols, hydrogen sulfides (H2S), S-modified protein cysteine adducts, hydrogen polysulfides (H2Sn, n ≥ 2) and so on, are playing an indispensable role in biomedical research [1–4]. Among them, there has been a great deal of interest in the study of H2S in the past decades. Extensive studies have been revealed H2S can contribute to a large body of...
physiological and pathological processes, such as protection against oxidative stresses [5,6], regulation of blood vessel tone [7]. The H2S level variations will result in a series of diseases ranging from hypertension to diabetes, Down’s syndrome and Alzheimer’s diseases [8–10]. Recently, H2Sn have successfully aroused widespread attention chiefly because evidences show that H2Sn plays a more important role than H2S in mediating certain biological mechanisms [11,12]. Much of what we know about H2S as a signaling molecule may actually be attributed to H2Sn [13]. H2Sn can be endogenously produced from the reaction of H2S and reactive oxygen species like hypochlorite and can form redox couples with H2S coexisting in biological systems [14]. H2Sn exerts a enormous function on redox biology and is associated with a large body of physiological processes. Thus, the development of accurate and highly selective methods to monitor H2Sn levels in living organisms is necessary for an in-depth understanding of its production, degradation pathway and regulatory mechanisms.

Mass spectrometry and UV–vis spectroscopy are commonly employed for the determination of H2Sn, but these tradition methods cannot achieve in-situ measurement of H2Sn in cells or in vivo [15,16]. In this case, fluorescent probes are selected as potentially powerful tools for tracking H2Sn in living organisms due to its high spatial and temporal resolution [17–23]. Inspired by pioneering work of Xian’s group, several fluorescent probes for H2Sn have been reported on basis of different response units, which mainly include 2-fluoro-5-nitrobenzoic ester [24–31], aziridine [32], nitro [33–36], phenyl 2-(benzoylthio) benzoate [37–40], and cinnamate ester [41]. However, 2-fluoro-5-nitrobenzoic ester, aziridine and cinnamate ester may be attacked by nucleophiles to cause probe consumption, and nitro may be reduced by other reducing species such as H2S, carbon monoxide [42,43]. Moreover, there are still some other issues to sort out, such as poor selectivity, low sensitivity or limitations in vivo applications. Hence, the development of ideal fluorescent probes for H2Sn detection are still desirable.

Encouraged by the above considerations, we herein designed and synthesized a simple semi-naphthorhodafluor-based red-emitting fluorescent probe, namely SNARF-H2Sn for specific tracking of H2Sn. The probe SNARF-H2Sn bearing phenyl 2-(benzoylthio) benzoate as a responsive unit showed a remarkable fluorescence off-on response to H2Sn. We reasoned that SNARF-H2Sn enabled the release SNARF fluorophore due to the unique dual-reactivity of H2Sn, thus triggering the dramatic fluorescence changes. Furthermore, to illustrate the potential application of SNARF-H2Sn, we have successfully applied this probe for imaging exogenous/endogenous H2Sn in living A549 cells and zebrafish.

2. Experimental section

2.1. General method

Unless otherwise noted, the reagents and solvent in this work were commercially available and were used without further purification. Ultrapure water (18.2 MΩ·cm) was used for all spectral analysis. 1H NMR and 13C NMR spectra were measured on a Varian 600 MHz spectrometer as solutions in CDCl3. High-resolution mass spectra (HRMS) were recorded on a Bruker SolariX in positive mode. UV–vis absorption and fluorescence spectra were performed on commercial spectrophotometers (Shimadzu UV-2700 and Agilent Cary eclipse spectrophotometer). Cell images were obtained on an inverted fluorescence microscope (Olympus IX71, Japan). Zebrafish images were acquired on a stereomicroscope (Olympus SZX16, Japan). A stock solution of SNARF-H2Sn (1 mM) in dimethyl sulfoxide was prepared. The stock solution (10 mM) of various potential biological analytes, such as reactive sulfur species (Cys, GSH, Hcy, CH2SSCH3, GSG, Na2S, Na2S2O3, Na2S2O4, Na2S2, common amino acids (Ile, Ala, Arg, Gly, Ser, Pro) and l-ascorbic acid were prepared in ultrapure water. The stock solution (10 mM) of CH2SSCH3 was prepared in acetonitrile. The stock solution (5 mM) of S2 was prepared in ethanol. The solution of reactive oxygen species (H2O2, O2•−, OH•, 1O2, ClO−) were prepared according to the previous literatures [40]. All the spectra were measured in PBS buffer (50 mM, pH 7.4, containing 100 µM CTAB). The fluorescence was obtained upon the excitation of 580 nm. The excitation and emission slits were set at 5 nm/5 nm. PMT detector voltage = 600 V.

2.2. Fluorescence imaging in living cells

A549 human lung carcinoma cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin with an atmosphere containing 5% carbon dioxide at 37 °C. A549 cells were incubated with DMEM culture medium containing 10 µM SNARF-H2Sn and 50 µM CTAB for 30 min. A549 cells stained with 50 µM Na2S and 50 µM CTAB for 30 min were treated with 10 µM SNARF-H2Sn for another 30 min. A549 cells stimulated with LPS for 12 h, and then incubated with 10 µM SNARF-H2Sn for 30 min. The cells were washed with DMEM for three times, and then applied for fluorescence imaging measurements on an inverted fluorescence microscope (Olympus IX71, Japan).

2.3. Fluorescence imaging in larval zebrafish

The zebrafish larvae post-fertilization obtained from Eze-Rinka Company (Nanjing, China) were kept in 10 ml of embryonic medium supplemented with 1-phenyl-2-thiourea in a beaker at 30 °C for 96 h. The fluorescence imaging in larval zebrafish were divided into four groups. First group: the 5-day-old zebrafish alone were the control group. Second group: the zebrafish were stained with 10 µM SNARF-H2Sn for 30 min. Third group: the zebrafish were stained with 50 µM Na2S for 1 h, and further incubated with 10 µM SNARF-H2Sn for 30 min. Fourth group: the zebrafish stimulated with LPS for 12 h, and then incubated with 10 µM SNARF-H2Sn for 30 min. The zebrafish were washed with PBS for three times, and then applied for fluorescence imaging measurements on a stereomicroscope (Olympus SZX16, Japan).

2.4. Synthesis of SNARF-H2Sn

SNARF-H2Sn was synthesized by the reaction of SNARF with 2-(benzoylthio)benzoic acid according to the reported literatures [44]. To a stirred solution of SNARF (43.8 mg, 0.1 mmol), 2-(benzoylthio)benzoic acid according to the reported literatures [44]. To a stirred solution of SNARF (43.8 mg, 0.1 mmol), 2-(benzoylthio)benzoic acid (30.9 mg, 0.12 mmol), 1-[3-dimethylamino propyl]-3-ethylcarbodiimide hydrochloride (28.7 mg, 0.15 mmol) in methylene chloride (5 ml) was added 4-dimethylaminopyridine (2.4 mg, 0.02 mol). The mixture was stirred under Ar at room temperature for 6 h. The solution was evaporated in vacuum and the residue was purified by column chromatography on silica gel (methylene chloride/methanol = 30:1) to afford the desired product SNARF-H2Sn as a pale yellowish green solid (52.3 mg, yield 77%). SNARF-H2Sn 1H NMR (600 MHz, CDCl3): δ 8.62 (d, J = 9.1 Hz, 1H), 8.29 (d, J = 7.6 Hz, 1H), 8.04 (d, J = 7.9 Hz, 3H), 7.73 (d, J = 7.6 Hz, 1H), 7.67–7.57 (m, 6H), 7.49–7.45 (m, 3H), 7.34 (d, J = 8.7 Hz, 1H), 7.14 (d, J = 7.5 Hz, 1H), 6.76 (d, J = 8.7 Hz, 1H), 6.65 (d, J = 9.0 Hz, 1H), 6.63 (d, J = 2.0 Hz, 1H), 6.42 (d, J = 9.0, 2.1 Hz, 1H), 3.40 (q, J = 7.1 Hz, 4H), 1.21 (t, J = 7.1 Hz, 6H), 13C NMR (150 MHz, CDCl3): δ 189.28, 169.70, 164.65, 153.65, 152.37, 127.54, 126.95, 125.06, 124.84, 124.13, 124.02, 122.56, 122.00, 121.36, 118.64, 112.74, 108.89, 104.95, 97.66, 84.26, 44.45.
3. Results and discussion

3.1. Molecular design and synthesis

SNARF, a semi-naphthorhodafluor red-emitting dye, was selected due to its desirable optical properties such as long-wavelength emission (>600 nm), good photostability, moderate Stokes shift and fluorescence quantum yield. The spirolactone ring structure changes of SNARF could trigger obvious color changes, which was easily observed by naked-eyes and potentially applied for visual detection. Thus, SNARF was commonly used in the construction of functional fluorescent probes. As reported, H₄Sn₃, possessed a unique dual reactivity including electrophilicity and nucleophilicity. Based on the dual reactivity of H₄Sn₃, we designed and prepared a SNARF-based off-on fluorescent probe for sensing H₂Sn by tagging a H₂Sn-specific response unit phenyl 2-(benzoylthio) benzoate to SNARF scaffold. SNARF-H₂Sn was synthesized via a two-step reaction, and its structure was determined by ¹H NMR, ¹³C NMR and HRMS. As depicted in Scheme 1, the reaction of SNARF-H₂Sn and H₂Sn took place in three steps: (i) H₂Sn acted as a nucleophile to attack phenyl 2-(benzoylthio) benzoate to form intermediate SNARF-SH; (ii) SNARF-SH was trapped by H₂Sn as an electrophile to generate SNARF-SSH; (iii) SNARF-SSH released the fluorophore SNARF and by-products benzodithiolone through intramolecular cyclization. To confirm the sensing mechanism, the reaction of SNARF-H₂Sn with H₂Sn was performed in the acetonitrile/PBS buffer (v/v = 1:1, 50 mM, pH 7.4, containing 100 µM CTAB). As a result, SNARF and benzodithiolone were obtained with good yield.

3.2. Response performances of SNARF-H₂Sn

With SNARF-H₂Sn in hand, we first evaluated the spectral performance in 50 mM phosphate buffer solution (pH 7.4, containing 100 µM CTAB). Surprisingly, SNARF-H₂Sn was non-emissive before addition of H₂Sn upon excitation at 580 nm. The fluorescence emission centered at 640 nm exhibited a gradual increase by adding various amounts of H₂Sn (0–50 µM) (Fig. S4). Upon addition of H₂Sn (25 µM), SNARF-H₂Sn fluorescence reached the maximum, showing a >1000-fold enhancement compared to free SNARF-H₂Sn. Particularly, an excellent linear relationship (R² = 0.9983) within the H₂Sn concentration range of 0–20 µM was obtained, and the limit of detection (LOD = 3σ/k) was found to be 6.7 nM (Fig. 1b, S5). Such a low detection limit was below most of the previous reported H₂Sn probes, which also demonstrated SNARF-H₂Sn was potentially used for tracking low concentration levels of H₂Sn in living organisms (Table S1). The time course of the reaction of SNARF-H₂Sn with H₂Sn was also investigated (Fig. 1c). Over 10 min, the emission intensity (λ_em = 640 nm) of SNARF-H₂Sn was close to saturation. Therefore, we chose 10 min as the response time for spectral analysis. Furthermore, we explored the effects of different pH values on the reactivity of SNARF-H₂Sn toward H₂Sn. As can be seen in Fig. 1d, SNARF-H₂Sn fluorescence was weak and remained constant, revealing SNARF-H₂Sn fluorescence was very stable over a range of pH 3.0–11.0. In the presence of H₂Sn, a drastic increase in the emission intensity (λ_em = 640 nm) at pH levels of 6.0–11.0. The obtained results illustrated SNARF-H₂Sn enabled to sense H₂Sn under the normal physiological range.

Selectivity was one of the important criterions to evaluate the sensing performance of the probe. Next, treating SNARF-H₂Sn with various potential biological analytes, such as reactive sulfur species (Cys, GSH, Hcy, CH₃SSCH₂CH₃, GSSG, Na₂S, Na₂S₂O₃, Na₂S₄O₆, S₈), common amino acids (Ile, Ala, Arg, Gly, Ser, Pro) and L-ascorbic acid, to evaluate the selectivity of SNARF-H₂Sn toward H₂Sn. Based on the obtained results from Fig. 1e, other biological analytes could hardly trigger SNARF-H₂Sn fluorescence change except Na₂S. It was reported that H₂Sn might be produced from the reaction between H₂S and reactive oxygen species (ROS) in living system. To validate the scenario, we chose various ROS including H₂O₂, O₂−, OH, ¹O₂ and ClO− to carry out the following tests. As shown in Fig. 1f, insignificant changes in the emission intensity (λ_em = 640 nm) of SNARF-H₂Sn were seen in the presence of ROS. However, after mixing ROS and H₂S, SNARF-H₂Sn showed varying degrees of fluorescence response, especially the addition of ClO− induced a remarkable fluorescence intensity change. These results clarified the excellent selectivity of SNARF-H₂Sn toward H₂Sn and possible biosynthetic pathway of H₂Sn.

3.3. Cellular fluorescence imaging

The excellent spectroscopic response performance prompted us to explore whether SNARF-H₂Sn was capable of monitoring exogenous/endogenous H₂Sn by fluorescence microscope. Prior to cellular imaging experiments, a standard CCK-8 assay was carried out to explore the cytotoxicity of SNARF-H₂Sn on A549 cells. Results from Fig. S6 showed the cells maintained a high cell viability when the concentration of SNARF-H₂Sn increased to 20 µM. It was clearly seen from Fig. 2a that almost no fluorescence signal was observed after addition of SNARF-H₂Sn, demonstrating the low level of H₂Sn in A549 cells. As a control, the cells sequentially stained with Na₂S₂ and SNARF-H₂Sn presented a very strong fluorescence signal. As reported, cystathionine γ-lyase (CSE) mRNA could be overexpressed by the stimulation of lipopolysaccharides (LPS), expediting the production of endogenous H₂Sn. Therefore, the effect of incubation of LPS stimulation on the production of H₂Sn in A549 cells was evaluated. When the cells stimulated with LPS for 12 h, and then incubated with SNARF-H₂Sn, the increase in fluorescence was seen in Fig. 2b, which indicated LPS could induce the upregulation of H₂Sn. These results demonstrated SNARF-H₂Sn featured excellent biocompatibility and enabled effectively to track exogenous/endogenous H₂Sn in cells.

3.4. In vivo fluorescence imaging

Furthermore, in order to inquire the potential of SNARF-H₂Sn, for imaging H₂Sn in vivo, we selected larval zebrafish as a model organism because of its similar genetic structure with human. As displayed in Fig. 3a, the fluorescence of SNARF-H₂Sn-stained zebra-
fish increased compared to control group, showing the existence of basal H₂Sn in vivo. As expected, treatment with Na₂S₂ and SNARF-H₂Sn in turn resulted in further increase in fluorescence signal. To reflect whether SNARF-H₂Sn could efficiently response to endogenous H₂Sn in zebrafish, we performed the following experiment. While the zebrafish was pretreated with LPS for 12 h followed by a 30 min incubation with SNARF-H₂Sn, we observed a pronounced fluorescence enhancement due to the reaction of SNARF-H₂Sn with LPS-induced endogenous H₂Sn (Fig. 3b). Collectively, all these results manifested that SNARF-H₂Sn could detect and visualize exogenous/endogenous H₂Sn activity in zebrafish.

4. Conclusion

In this work, by introducing phenyl 2-(benzoylthio) benzoate as a H₂Sn-active trigger to SNARF scaffold, we reported a new type of red-emitting fluorescent probe named SNARF-H₂Sn allowed for...
detection of H₂Sn. On basis of dual reactivity of H₂Sn, SNARF-H₂Sn was able to respond H₂Sn with extreme high selectivity and nanomolar detection limit. Leveraging SNARF-H₂Sn, we enabled to track the level fluctuations of exogenous H₂Sn in living cells and zebrafish by fluorescence microscope. What’s more, our results further revealed that SNARF-H₂Sn successfully realized the imaging of endogenous H₂Sn stimulated by LPS in vitro and in vivo. We anticipated that SNARF-H₂Sn might be a valuable tool for researchers to reveal more information about H₂Sn-related diseases.

CRediT authorship contribution statement

Yingying Ma: Methodology, Data curation, Writing - original draft. Zhencai Xu: Methodology, Data curation. Qi Sun: Validation, Writing - review & editing, Funding acquisition. Linlin Wang: Validation, Writing - review & editing. Heng Liu: Conceptualization, Supervision, Project administration, Funding acquisition. Fabiao Yu: Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References


