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

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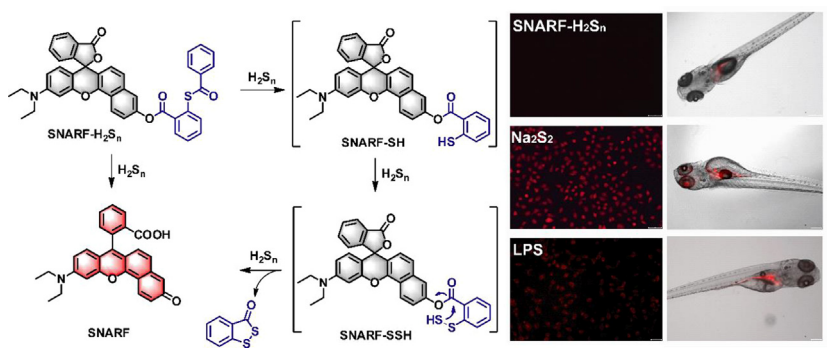
HIGHLIGHTS

- A semi-naphthorhodafluor-based red-emitting fluorescent probe SNARF-H_2S_n for selective detection of H_2S_n was designed.
- The addition of H_2S_n would result in a > 1000-fold fluorescence enhancement within 10 min.
- SNARF-H_2S_n was successfully employed to image exogenous/endogenous H_2S_n in living cells and zebrafish.

GRAPHICAL ABSTRACT



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ABSTRACT

Hydrogen polysulfides (H_2S_n, $n \geq 2$) is recently regarded as a potential signaling molecule which shows a higher efficiency than hydrogen sulfides (H_2S) in regulating enzymes and ion channels. However, the development of specific fluorescent probes for H_2S_n with long-wavelength emission (>600 nm) are still rare. In this work, a semi-naphthorhodafluor-based red-emitting fluorescent probe SNARF-H_2S_n containing a phenyl 2-(benzoylthio) benzoate responsive unit was constructed. SNARF-H_2S_n was capable of selectively detecting H_2S_n over other reactive sulfur species. Treatment with H_2S_n would result in a > 1000-fold fluorescence enhancement within 10 min. SNARF-H_2S_n showed a low limit of detection down to 6.7 nM, and further enabled to visualize exogenous/endogenous H_2S_n in living A549 cells and zebrafish.

1. Introduction

Reactive sulfur species (RSS), consisting of thiols, hydrogen sulfides (H_2S), S-modified protein cysteine adducts, hydrogen polysulfides (H_2S_n, $n \geq 2$) 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 H_2S in the past decades. Extensive studies have been revealed H_2S can contribute to a large body of

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physiological and pathological processes, such as protection against oxidative stresses [5,6], regulation of blood vessel tone [7]. The H_2S level variations will result in a series of diseases ranging from hypertension to diabetes, Down's syndrome and Alzheimer's diseases [8–10]. Recently, H_2S_n have successfully aroused widespread attention chiefly because evidences show that H_2S_n plays a more important role than H_2S in mediating certain biological mechanisms [11,12]. Much of what we know about H_2S as a signaling molecule may actually be attributed to H_2S_n [13]. H_2S_n can be endogenously produced from the reaction of H_2S and reactive oxygen species like hypochlorite and can form redox couples with H_2S coexisting in biological systems [14]. H_2S_n 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 H_2S_n 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 H_2S_n, but these tradition methods cannot achieve in-situ measurement of H_2S_n in cells or in vivo [15,16]. In this case, fluorescent probes are selected as potentially powerful tools for tracking H_2S_n 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 H_2S_n 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 H_2S, 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 H_2S_n 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-H_2S_n, for specific tracking of H_2S_n. The probe SNARF-H_2S_n bearing phenyl 2-(benzoylthio) benzoate as a responsive unit showed a remarkable fluorescence off-on response to H_2S_n. We reasoned that SNARF-H_2S_n enabled the release SNARF fluorophore due to the unique dual-reactivity of H_2S_n, thus triggering the dramatic fluorescence changes. Furthermore, to illustrate the potential application of SNARF-H_2S_n, we have successfully applied this probe for imaging exogenous/endogenous H_2S_n 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. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian 600 MHz spectrometer as solutions in $CDCl_3$. 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-H_2S_n (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, CH_3SSSCH_3, GSSG, Na_2S, $Na_2S_2O_3$, Na_2SO_3, Na_2SO_4, Na_2S_2), common amino acids (Ile, Ala, Arg, Gly, Ser, Pro) and L-ascorbic acid were prepared in ultrapure water. The stock solution (10 mM) of CH_3SSSCH_3 was prepared in acetonitrile. The stock solution (5 mM) of S_8 was prepared in enthanol. The solution of reactive oxygen species (H_2O_2, $O_2^{\bar{}}$, ·OH, $^1O_2$, 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-H_2S_n and 50 μM CTAB for 30 min. A549 cells stained with 50 μM Na_2S_2 and 50 μM CTAB for 30 min were treated with 10 μM SNARF-H_2S_n for another 30 min. A549 cells stimulated with LPS for 12 h, and then incubated with 10 μM SNARF-H_2S_n 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-H_2S_n for 30 min. Third group: the zebrafish were stained with 50 μM Na_2S_2 for 1 h, and further incubated with 10 μM SNARF-H_2S_n for 30 min. Fourth group: the zebrafish stimulated with LPS for 12 h, and then incubated with 10 μM SNARF-H_2S_n 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-H_2S_n

SNARF-H_2S_n 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 (30.9 mg, 0.12 mmol), 1-(3-dimethylamino propyl)-3ethylcarbodiimide 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-H_2S_n as a pink solid (52.3 mg, yield 77%). SNARF-H_2S_n ^{1}H NMR (600 MHz, $CDCl_3$): 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 (dd, J = 9.0, 2.1 Hz, 1H), 3.40 (q, J = 7.1 Hz, 4H), 1.21 (t, J = 7.1 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 189.28, 169.70, 164.65, 153.65, 152.37, 149.93, 149.55, 147.42, 137.22, 136.45, 134.93, 134.85, 133.88, 133.77, 132.57, 131.57, 129.63, 129.48, 128.87, 128.83, 128.76, 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,

12.53; HRMS *m/z*: $C_{42}H_{31}NO_6S$ $[M+H]^+$ calcd for 678.1950 found 678.1947.

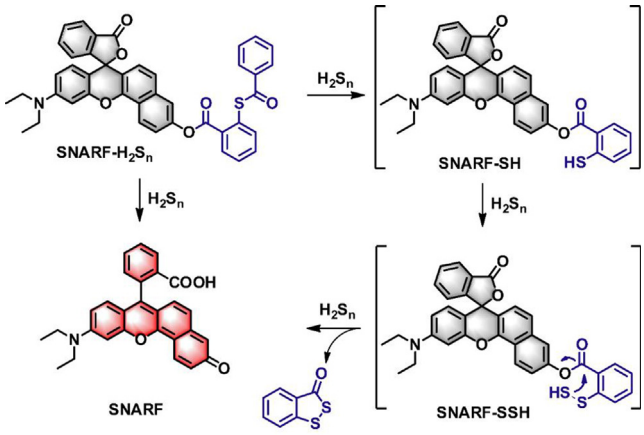
3. Results and discussion

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, H_2S_n possessed a unique dual reactivity including electrophilicity and nucleophilicity. Based on the dual reactivity of H_2S_n, we designed and prepared a SNARF-based off-on fluorescent probe for sensing H_2S_n by tagging a H_2S_n-specific response unit phenyl 2-(benzoylthio) benzoate to SNARF scaffold. SNARF-H_2S_n was synthesized via a two-step reaction, and its structure was determined by ^{1}H NMR, ^{13}C NMR and HRMS. As depicted in Scheme 1, the reaction of SNARF-H_2S_n and H_2S_n took place in three steps: (i) H_2S_n acted as a nucleophile to attack phenyl 2-(benzoylthio) benzoate to form intermediate SNARF-SH; (ii) SNARF-SH was trapped by H_2S_n 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-H_2S_n with H_2S_n 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-H_2S_n

With SNARF-H_2S_n in hand, we first evaluated the spectral performance in 50 mM phosphate buffer solution (pH 7.4, containing 100 μM CTAB). Surprisingly, SNARF-H_2S_n was non-emissive before addition of H_2S_n upon excitation at 580 nm. The fluorescence emission centered at 640 nm exhibited a gradual increase by adding various amounts of H_2S_n (0–50 μM) (Fig. S4). Upon addition of H_2S_n (25 μM), SNARF-H_2S_n fluorescence reached the maximum, showing a > 1000-fold enhancement compared to free SNARF-H_2S_n (Fig. 1a). Particularly, an excellent linear relationship ($F_{640\ nm}$ = 13.96 × $[Na_2S_2]$ μM + 0.1559, R^2 = 0.9983) within the H_2S_n 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 H_2S_n probes, which also demonstrated SNARF-H_2S_n was potentially used for tracking low concentration levels of H_2S_n in living organisms (Table S1). The time course of the reaction of SNARF-H_2S_n with H_2S_n was also investigated (Fig. 1c). Over 10 min, the emission intensity (λ_{em} = 640 nm) of SNARF-H_2S_n 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-H_2S_n toward H_2S_n. As can be seen in Fig. 1d, SNARF-H_2S_n fluorescence was weak and remained constant, revealing SNARF-H_2S_n was very stable over a range of pH 3.0–11.0. In the presence of H_2S_n, a drastic increase in the emission intensity (λ_{em} = 640 nm) at pH levels of 6.0–11.0. The obtained results illustrated SNARF-H_2S_n enabled to sense H_2S_n under the normal physiological range.



Scheme 1. Reaction mechanism of SNARF-H_2S_n toward H_2S_n.

Selectivity was one of the important criterions to evaluate the sensing performance of the probe. Next, treating SNARF-H_2S_n with various potential biological analytes, such as reactive sulfur species (Cys, GSH, Hcy, CH_3SSSCH_3, GSSG, Na_2S, $Na_2S_2O_3$, Na_2SO_3, Na_2SO_4, S_8), common amino acids (Ile, Ala, Arg, Gly, Ser, Pro) and L-ascorbic acid, to evaluate the selectivity of SNARF-H_2S_n toward H_2S_n. Based on the obtained results from Fig. 1e, other biological analytes could hardly trigger SNARF-H_2S_n fluorescence change except Na_2S_2. It was reported that H_2S_n might be produced from the reaction between H_2S and reactive oxygen species (ROS) in living system. To validate the scenario, we chose various ROS including H_2O_2, O_2^-, ·OH, $^1O_2$ 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-H_2S_n were seen in the presence of ROS. However, after mixing ROS and H_2S, SNARF-H_2S_n 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-H_2S_n toward H_2S_n and possible biosynthetic pathway of H_2S_n.

3.3. Cellular fluorescence imaging

The excellent spectroscopic response performance prompted us to explore whether SNARF-H_2S_n was capable of monitoring exogenous/endogenous H_2S_n by fluorescence microscope. Prior to cellular imaging experiments, a standard CCK-8 assay was carried out to explore the cytotoxicity of SNARF-H_2S_n on A549 cells. Results from Fig. S6 showed the cells maintained a high cell viability when the concentration of SNARF-H_2S_n increased to 20 μM. It was clearly seen from Fig. 2a that almost no fluorescence signal was observed after addition of SNARF-H_2S_n, demonstrating the low level of H_2S_n in A549 cells. As a control, the cells sequentially stained with Na_2S_2 and SNARF-H_2S_n presented a very strong fluorescence signal. As reported, cystathionine g-lyase (CSE) mRNA could be overexpressed by the stimulation of lipopolysaccharides (LPS), expediting the production of endogenous H_2S_n. Therefore, the effect of incubation of LPS stimulation on the production of H_2S_n in A549 cells was evaluated. When the cells stimulated with LPS for 12 h, and then incubated with SNARF-H_2S_n, the increase in fluorescence was seen in Fig. 2b, which indicated LPS could induce the upregulation of H_2S_n. These results demonstrated SNARF-H_2S_n featured excellent biocompatibility and enabled effectively to track exogenous/endogenous H_2S_n in cells.

3.4. In vivo fluorescence imaging

Furthermore, in order to inquiry the potential of SNARF-H_2S_n for imaging H_2S_n 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-H_2S_n-stained zebra-

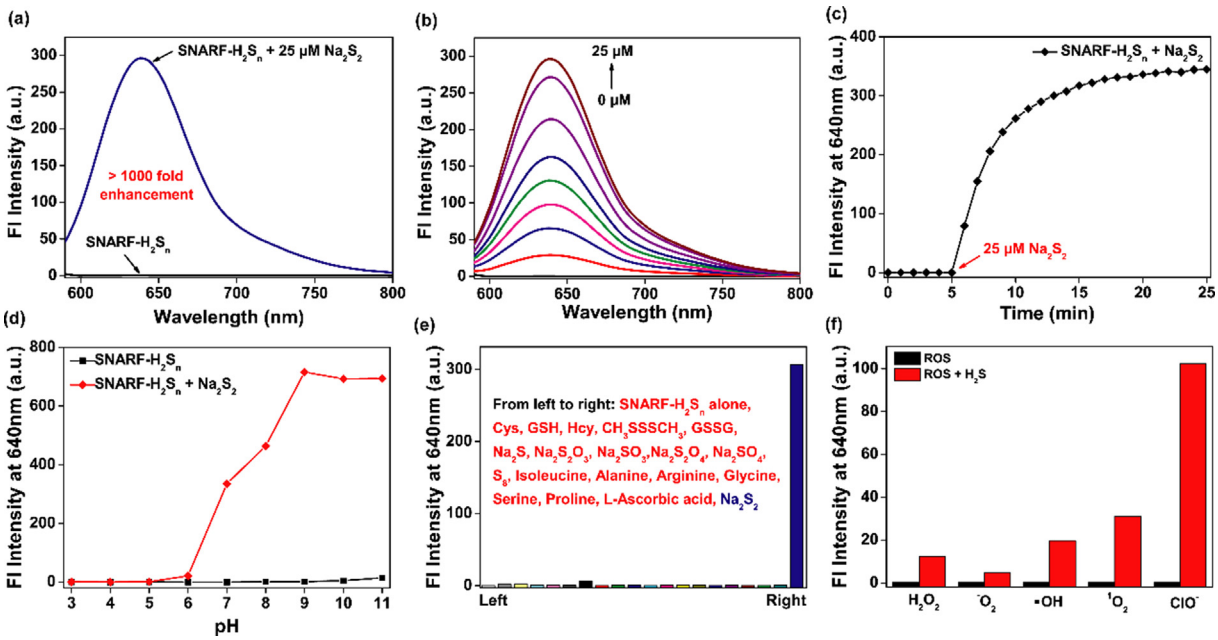


Fig. 1. (a) Fluorescence spectra of SNARF-H_2S_n (10 μM) in the presence and absence of Na_2S_2 (25 μM). (b) Fluorescence responses of SNARF-H_2S_n (10 μM) upon addition of Na_2S_2 (0–25 μM) for 10 min. (c) Time courses of fluorescence intensities at 640 nm before and after addition of Na_2S_2 (25 μM). (d) Influence of pH on the response of SNARF-H_2S_n (10 μM) in the presence and absence of Na_2S_2 (25 μM). (e) Fluorescence responses of SNARF-H_2S_n (10 μM) at 640 nm toward various potential biological analytes. From left to right: blank, Cys (200 μM), GSH (1 mM), Hcy (200 μM), CH_3SSSCH_3 (100 μM), GSSG (100 μM), Na_2S (100 μM), $Na_2S_2O_3$ (100 μM), Na_2SO_3 (100 μM), Na_2SO_4 (100 μM), S_8 (50 μM), Ile (100 μM), Ala (100 μM), Arg (100 μM), Gly (100 μM), Ser (100 μM), Pro (100 μM), L-ascorbic acid (100 μM), Na_2S_2 (25 μM). (f) Fluorescence responses of SNARF-H_2S_n (10 μM) at 640 nm in the presence of ROS (with or without 100 μM Na_2S). From left to right: H_2O_2 (200 μM), (2) O_2^- (100 μM), ·OH (100 μM), $^1O_2$ (100 μM) and ClO^- (100 μM).

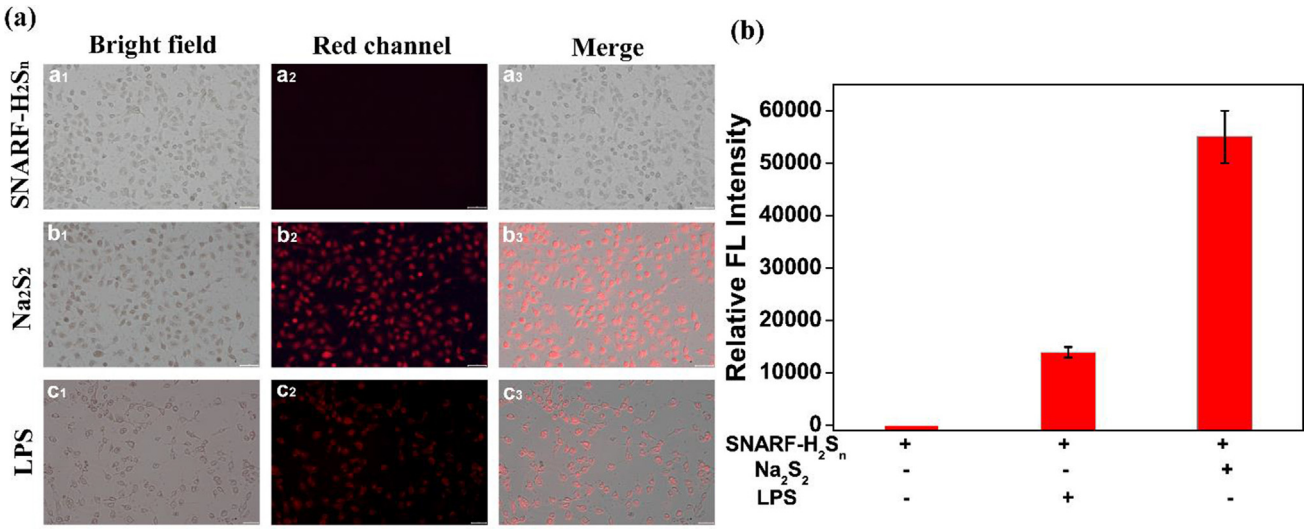


Fig. 2. (a) Fluorescence imaging of exogenous/endogenous H_2S_n in living A549 cells. (a_1–a_3) The cells were incubated with 10 μM SNARF-H_2S_n for 30 min; (b_1–b_3) the cells stained with 50 μM Na_2S_2 for 30 min were treated with 10 μM SNARF-H_2S_n for another 30 min; (c_1–c_3) the cells stimulated with LPS for 12 h, and then incubated with 10 μM SNARF-H_2S_n for 30 min. (a_1, b_1, c_1) bright field images; (a_2, b_2, c_2) red channel images; (a_3, b_3, c_3) merged images. Scale bar = 50 μM. (b) Relative fluorescence intensity of red channel in panel A. Values represent mean standard error (n = 3).

fish increased compared to control group, showing the existence of basal H_2S_n in vivo. As expected, treatment with Na_2S_2 and SNARF-H_2S_n in turn resulted in further increase in fluorescence signal. To reflect whether SNARF-H_2S_n could efficiently response to endogenous H_2S_n 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-H_2S_n, we observed a pronounced fluorescence enhancement due to the reaction of SNARF-H_2S_n with LPS-induced endogenous H_2S_n (Fig. 3b). Collectively, all these results manifested that SNARF-H_2S_n could detect and visualize exogenous/endogenous H_2S_n activity in zebrafish.

4. Conclusion

In this work, by introducing phenyl 2-(benzoylthio) benzoate as a H_2S_n-active trigger to SNARF scaffold, we reported a new type of red-emitting fluorescent probe named SNARF-H_2S_n allowed for

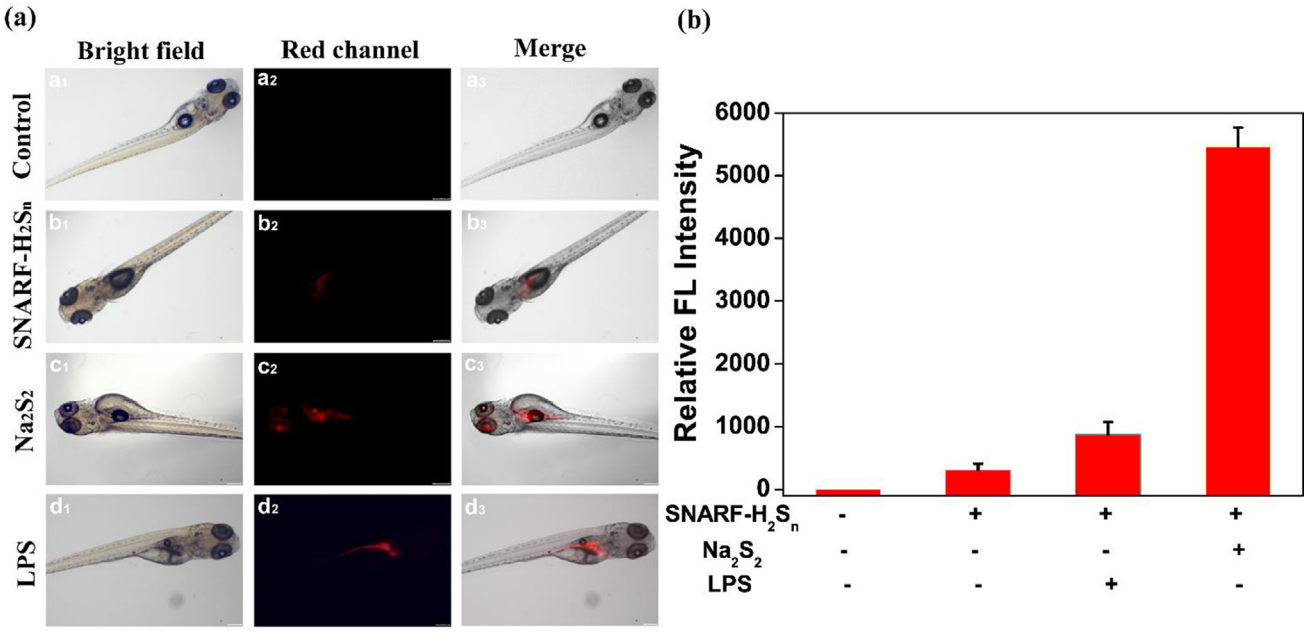


Fig. 3. Fluorescence imaging of exogenous/endogenous H_2S_n in zebrafish. (a_1–a_3) control group; (b_1–b_3) the zebrafish were stained with 10 μM SNARF-H_2S_n for 30 min; (c_1–c_3) the zebrafish were stained with 50 μM Na_2S_2 for 1 h, and further incubated with 10 μM SNARF-H_2S_n for 30 min; (d_1–d_3) the zebrafish stimulated with LPS for 12 h, and then incubated with 10 μM SNARF-H_2S_n for 30 min. (a_1, b_1, c_1, d_1) bright field images; (a_2, b_2, c_2, d_2) red channel images; (a_3, b_3, c_3, d_3) merged images. Scale bar = 200 μM. (b) Relative fluorescence intensity of red channel in panel A. Values represent mean standard error (n = 3).

detection of H_2S_n. On basis of dual reactivity of H_2S_n, SNARF-H_2S_n was able to respond H_2S_n with extreme high selectivity and nanomolar detection limit. Leveraging SNARF-H_2S_n, we enabled to track the level fluctuations of exogenous H_2S_n in living cells and zebrafish by fluorescence microscope. What's more, our results further revealed that SNARF-H_2S_n successfully realized the imaging of endogenous H_2S_n stimulated by LPS in vitro and in vivo. We anticipated that SNARF-H_2S_n might be a valuable tool for researchers to reveal more information about H_2S_n-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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2020.119105.

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