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RESEARCH ARTICLE

N-Positive ion activated rapid addition and mitochondrial targeting ratiometric fluorescent probes for *in vivo* cell H_2S imaging

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Abstract Heterocyclic compound quinoline and its derivatives exist in natural compounds and have a broad spectrum of biological activity. They play an important role in the design of new structural entities for medical applications. Similarly, indoles and their derivatives are found widely in nature. Amino acids, alkaloids and auxin are all derivatives of indoles, as are dyes, and their condensation with aldehydes makes it easy to construct reaction sites for nucleophilic addition agents. In this work, we combine these two groups organically to construct a rapid response site (within 30 s) for H_2S, and at the same time, a ratiometric fluorescence response is presented throughout the process of H_2S detection. As such, the lower detection limit can reach 55.7 nmol/L for H_2S. In addition, cell imaging shows that this probe can be used for the mitochondrial targeted detection of endogenous and exogenous H_2S. Finally, this probe application was verified by imaging H_2S in nude mice.

Keywords heterocyclic compound, hydrogen sulfide, ratiometric, mitochondrial targeted

1 Introduction

Mitochondria, the power factories in living cells, have been shown to be involved in signaling, cell differentiation, apoptosis and other life activities [1,2]. Therefore, mitochondrial metabolism is also associated with various diseases, such as atherosclerosis, Alzheimer's disease and Parkinson's disease [3,4]. Likewise, H_2S in mitochondria has been shown to play a protective role in oxidative stress–induced dysfunction and cell death [5,6]. Thus, the selective tracing of H_2S in mitochondria is crucial in elucidating the complex role of H_2S in physiological and pathological processes [7,8].

At present, fluorescent probe technology has been widely used in the detection of substances in organisms due to its advantageous high selectivity and sensitivity, real-time imaging, high spatial resolution and high time resolution [9–19]. Compared to normal "Turn-On" fluorescent probes, by self-correcting at different wavelengths, ratiometric fluorescent probes can eliminate most of these disturbances with probe concentration, environmental factors and light source efficiency, providing reliable quantitative information [20–30]. Therefore, ratiometric fluorescent probes can be used to more accurately explore changes to the content of molecules in cells and tissues [31–42].

In this work, we explore real-time H_2S changes in mitochondria by ratiometric fluorescent probes. To achieve this goal, the fluorescent probes must meet four standards: (a) the probe must have good biocompatibility, (b) the probe must exhibit significant rate-type variation to ensure the accuracy of the test, (c) the probe must have good mitochondrial localization and (d) the probe must have a fast response to H_2S to achieve real-time monitoring. Based on this idea, we used quinoline due to its good biocompatibility as a fluorophore; additionally, indoles iodized salt was designed to improve the emissions wavelength of the probe and act as a mitochondrial target group. Finally, we designed and synthesized the new fluorescent Probe 1 to achieve targeted mitochondrial detection of H_2S. Surprisingly, after a series of spectral testing, the response time of Probe 1 to H_2S was less than 30 s, providing an opportunity to monitor changes to H_2S

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in real time. Most importantly, Probe 1 was used to detect H_2S in living cells and *in vivo*.

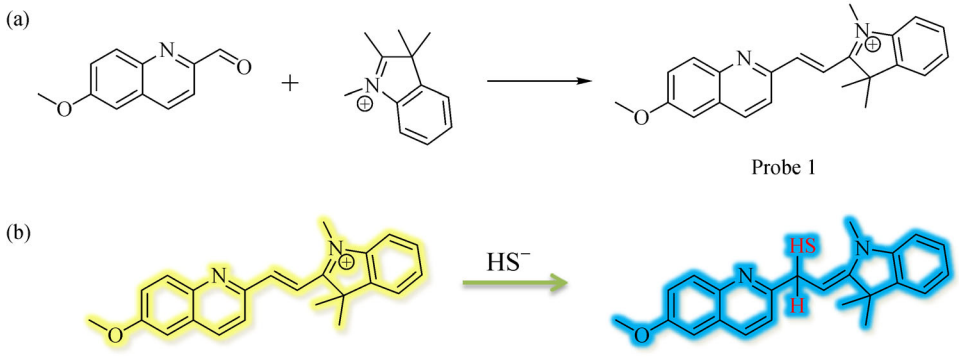
2 Experimental

2.1 The preparation and characterization of Probe 1

We prepared Probe 1 using the synthetic route in Scheme 1 (a). The syntheses of 6-methoxyquinoline-2-carbaldehyde and 1,2,3,3-tetramethyl-3H-indol-1-ium were based on the reported literature [43]. The characterization of Probe 1 was analyzed by nuclear magnetic resonance (NMR) (Figs. S1 and S2, cf. Electronic Supplementary Material (ESM)) and high-resolution mass spectrography (HRMS) (Fig. S3, cf. ESM).

2.2 Imaging experiments and general measurements

The materials and reagents used are commercially



Scheme 1 The synthetic route of Probe 1 (a) and the proposed mechanism (b).

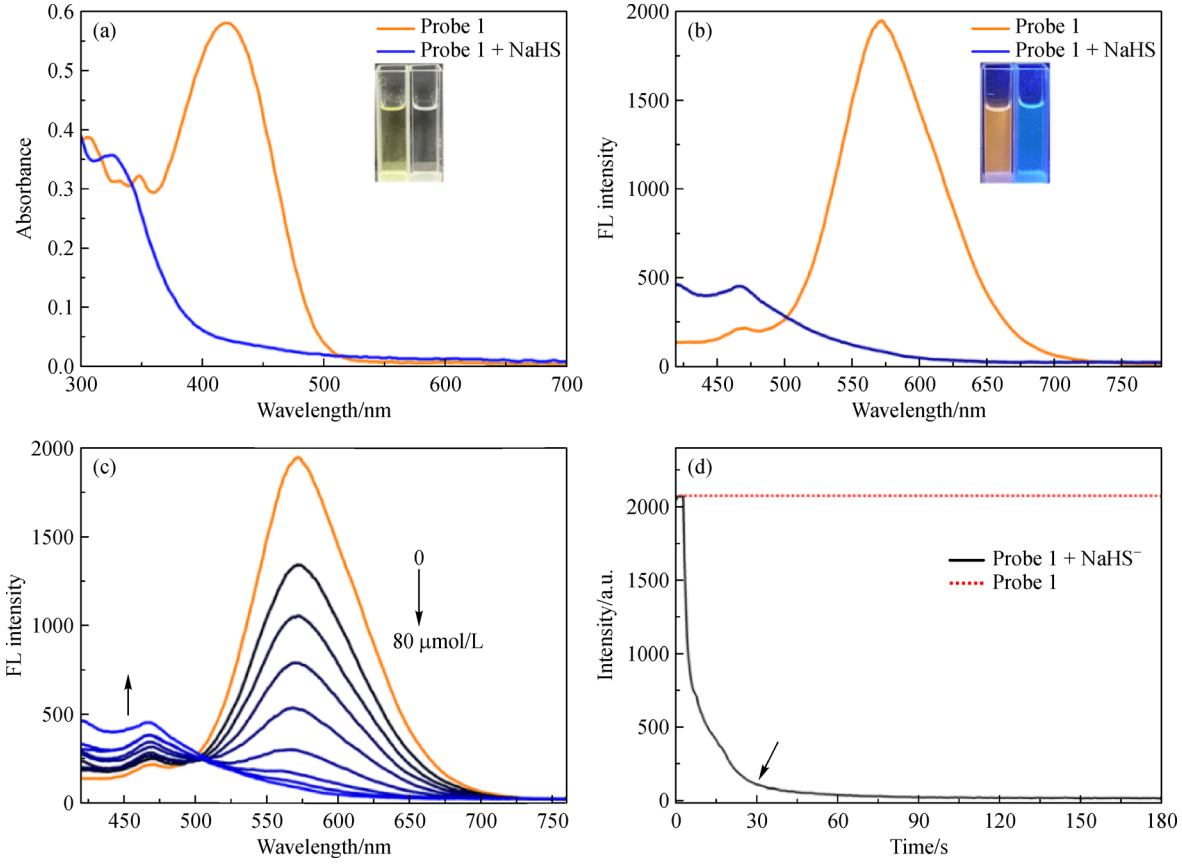


Fig. 1 UV-vis (a) and fluorescent spectra (b) change of the 10 μmol/L Probe 1 and an 80 μmol/L HS^- in a PBS buffer (20% DMSO, V/V, pH = 7.4); (c) fluorescent titration change of the 10 μmol/L Probe 1 upon addition of H_2S (0–80 μmol/L); (d) linear relationship between the fluorescence intensity ratios (F_{470}/F_{572}) and HS^- (10–80 μmol/L, where λ_{ex} = 400 nm). The inset image shows the color changes under visible light (red: probe, black: probe + NaHS).

available and have not been further purified. The solution of compounds was prepared of deionized water. All the animal experiments were performed by following the protocols approved by Radiation Protection Institute of Drug Safety Evaluation Center in China (Production license: SYXK (Jin) 2018-0005). Balb/c type mouse (4–6 weeks, male) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Experiments were conducted according to the relevant laws and guidelines issued and approved by the Committee of Scientific Research in Shanxi University. Instrument parameters and related biological experiments were prepared according to previous work standards and could be found in ESM.

3 Results and discussion

3.1 Fluorescence and ultraviolet-visible (UV-vis) spectra

The ESM contained detailed characterizations of Probe 1. To explore the detection properties of Probe 1 for H_2S *in vitro*, Probe 1 was obtained after purification and first studied in solution [44]. As shown in Figs. 1(a) and 1(b), the UV-vis and fluorescent spectra of Probe 1 with H_2S were recorded. Probe 1 showed a maximum absorption peak at 420 nm and a maximum fluorescence emissions peak at 572 nm. Upon gradually increasing the amount of NaHS (the H_2S donor), the maximum absorption peak underwent an overt blue shift from 420 to 325 nm, which indicated that Probe 1 had reacted with H_2S and had produced a new substance.

Figure 1(c) shows the changes in the fluorescent emissions spectra after adding HS^-. Upon adding HS^-, Probe 1 showed significant fluorescence attenuation at 572 nm; by contrast, Probe 1 showed gradual fluorescence enhancement at 470 nm. These results showed that Probe 1 had a good response to HS^-. In addition, the color of the solution changed from yellow to colorless, indicating that Probe 1 can be used to detect H_2S by naked eye. Furthermore, Fig. 1(d) shows that these reactions can be completed within 30 s. Considering the high reactivity and volatility of H_2S, the half-life of H_2S in a biological system has been reported to be within several minutes [45]. Hence, Probe 1 would be useful in real-time H_2S imaging.

3.2 The selectivity and competitive response of Probe 1 to H_2S

Various species were examined in parallel under the same conditions for verifying the selectivity and competitiveness of Probe 1, including reactive inorganic sulfur species, reactive oxygen species, proteins, reactive nitrogen species, amino acids and other anions. As shown in Fig. 2, the fluorescence intensity (F_{470}/F_{572}) of Probe 1 to other analytes showed negligible response. Moreover, in the competitive experiment (Fig. 2(b)), the fluorescence intensity (F_{470}/F_{572}) of Probe 1 responded to H_2S effectively in the presence of other molecules.

3.3 Kinetic study in the detection process of H_2S

To investigate the sensitivity of Probe 1 to H_2S, the fluorescent intensity ratios (F_{470}/F_{572}) were obtained according to the titration profile. The correlation coefficient was $R^2 = 0.9917$ with a determined limit of 55.7 nmol/L, and the linear range was 0–80 μmol/L (Fig. 3). On the basis of the results of the pH-dependent experiment, the fluorescence intensity (F_{470}/F_{572}) of Probe 1 was stable in the 6.0–8.0 range (Fig. S4, cf. ESM). These results fully indicate that Probe 1 should be suitable for the ratiometric imaging of biological H_2S.

3.4 Reaction mechanism of Probe 1 for detecting H_2S

The mechanism of the Probe 1 response to H_2S was

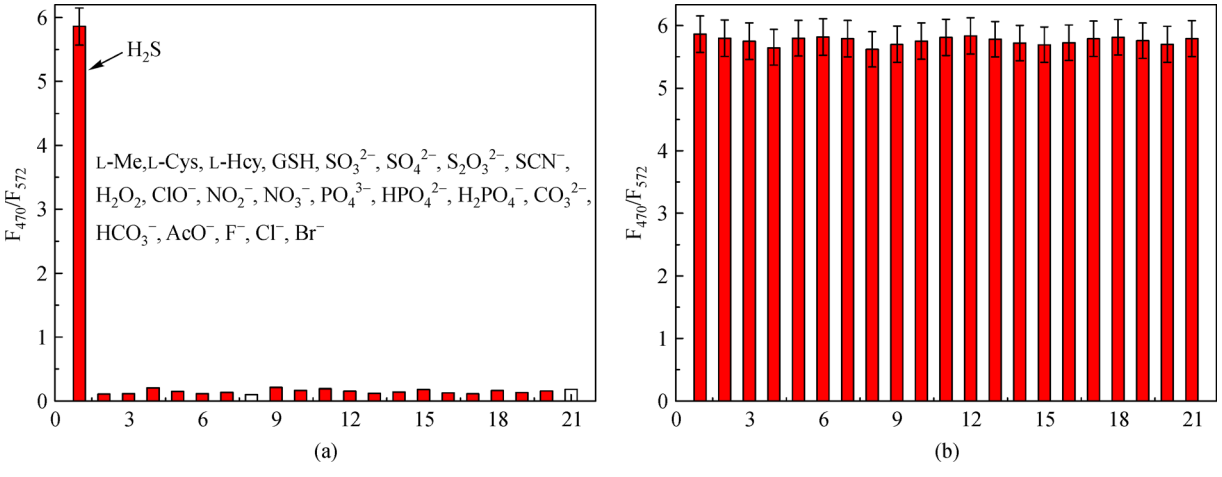


Fig. 2 (a) Fluorescent intensity (F_{470}/F_{572}) responses of Probe 1 (10 μmol/L) to analytes (λ_{ex} = 400 nm); (b) The competitive test response of Probe 1 under the presence of various analytes.

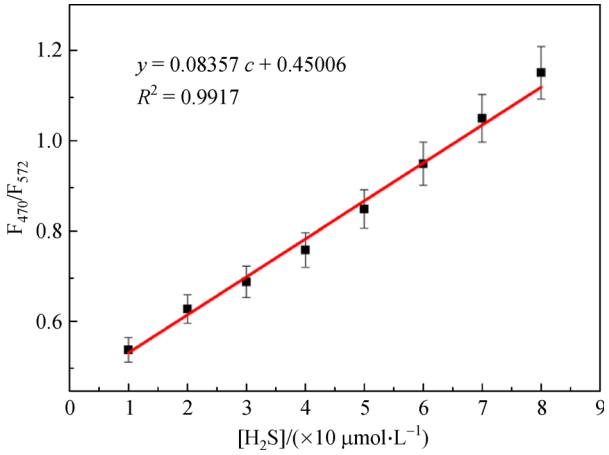


Fig. 3 Plot of the fluorescence intensity of Probe 1 (10 μmol/L) *vs*. the H_2S concentration (0–80 μmol/L).

evaluated. As shown in Scheme 1(b), the nucleophilic addition of HS^- toward the indolium group of the probe shortened the conjugation system occurring in the fluorescent and UV-vis spectral change, as well as the color change. To further confirm our hypothesis, Probe 1 toward HS^- was carried out by an ^1H NMR titration experiment. Its nicely stacking graph powerfully proves this mechanism (Fig. 4). After the addition of excess HS^-, the number of aromatic protons remained 11, except for some that split and moved up field. H^1 ($1CH_3$) shifted from d 3.96 to 2.91 ($1'CH_3$) ppm, and H^2 ($2CH_3$) shifted from 1.84 to 1.22 ($2'CH_3$) ppm. The nuclear magnetic resonance signal of H^3 (CH_3–O–) farther from indolium iodide barely changed. H^4 and H^5 markedly shifted up-field and appeared at 5.61 and 6.61 ppm after the addition of HS^-. A clear H signal appeared, however, at 1.51 ppm, which was identified as –HS. The mixture of Probe 1 and HS^- was detected by HRMS. This result exhibited an *m*/*z* peak at 377.1679 in accordance with the adduct (Fig. S5, cf. ESM).

3.5 CLSM experiments of Probe 1 in cells

To affirm Probe 1's bio-application in living HeLa cells, the Cell Counting Kit-8 (CCK-8) assay was carried out to verify its low acute toxicity [46]. The viabilities were found to be higher than 85% at 5 h or 10 h when 1–80 mmol/L of Probe 1 was incubated in the HeLa cells, showing that the probe has the potential to realize cell imaging (Fig. S6, cf. ESM).

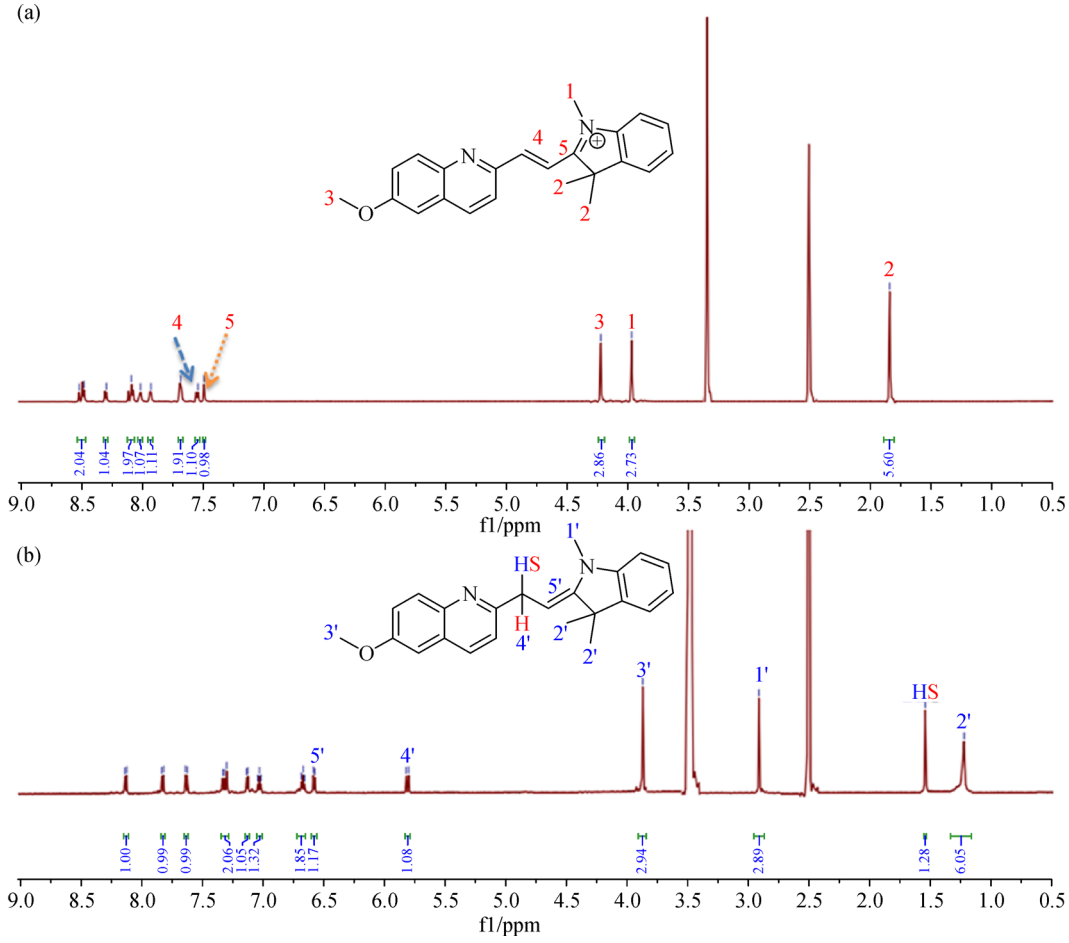


Fig. 4 ^1H NMR spectra of Probe 1 in DMSO-d_6 (a) and Probe 1 + NaHS in DMSO-d_6/D_2O (b).

Next, we implemented a ratiometric H_2S cell imaging experiment by confocal laser scanning microscope (CLSM) (Fig. 5). The capabilities of Probe 1 were first tested on the detection of exogenous H_2S (Figs. 5(a–c)). As Fig. 5(a) shows, HeLa cells were incubated with Probe 1 (10 μmol/L) for 30 min at 37 °C, after which the cells showed a significantly yellow fluorescent signal in the channel 1 (550 ± 30 nm, λ_{ex} = 400 nm). Then, upon adding HS^- (20 and 80 μmol/L) into the HeLa cells, the yellow fluorescent signal was found to have gradually disappeared, but the blue fluorescent signal gradually appeared in channel 2 (Figs. 5(b) and 5(c)). Hence, Probe 1 can be used to image exogenous H_2S specifically in HeLa cells.

After this, the imaging of endogenous H_2S in HeLa cells was also carried out. Sodium nitroprusside dehydrate (SNP, a commercial NO donor) [47] was usually used to induce the production of endogenous H_2S. We incubated 10 μmol/L Probe 1 30 min, then incubated SNP (100 μmol/L) to stimulate the cells to produce H_2S for 30 min (Figs. 5(d) and 5(e)). The yellow fluorescent signal in channel 1 weakened, and the blue fluorescent signal in channel 2 grew, indicating the generation of endogenous H_2S in HeLa cells. These results suggest that Probe 1 can recognize endogenous H_2S in living HeLa cells.

In the previous work of our group [48], the commercially available mitochondrial dye Mito Tracker Red (MTR) was used for localization studies (Fig. 5(f)). First, the HeLa cells loaded with Probe 1 (10 mmol/L) were incubated for 30 min. Then, the cells were dyed using MTR (1 mmol/L, 20 min). The probe provided a clear fluorescent image in the yellow channel. MTR was imaged in the red channel (690 ± 30 nm, λ_{ex} = 400 nm). A composograph showed that the tinting of Probe 1 could overlap well with MTR. It also showed a good colocalization of Probe 1 and MTR with a high Pearson's coefficient of 0.83. Thus, the results revealed that Probe 1 can be located the mitochondria of the living HeLa cells.

3.6 Bioimaging applications of Probe 1 for H_2S in nude mice

The fluorescent imaging applicability of Probe 1 for H_2S imaging in mice was carried out and was inspired by pretty cell imaging results. Due to the background fluorescence of the mice, we selected a fluorescence signal of 500–580 nm to detect H_2S in the mice (Fig. 6). As Fig. 6 shows, Probe 1 was injected subcutaneously into the mice, and a strong fluorescence signal was observed. Then, HS^- (80 mmol/L) was injected into the same area, after which the fluorescence intensity decreased around the injected area with time. This indicates that Probe 1 can be used to detect exogenous H_2S in nude mice.

4 Conclusions

In summary, the applicability of a novel ratiometric fluorescent probe (Probe 1) for H_2S in HeLa cells and nude mice through bioimaging was shown. Probe 1 showed a fast response (within 30 s) toward H_2S. It exhibited high selectivity and sensitivity toward H_2S, and its limit of detection was 55.7 nmol/L. In addition, Probe 1 was successfully used to monitor exogenous/endogenous H_2S in HeLa cells, and more importantly, Probe 1 could also detect exogenous H_2S in nude mice. Moreover, Probe 1 has the ability to target mitochondria in HeLa cells. These research results provide a powerful design strategy for the development of a ratiometric fluorescence sensor

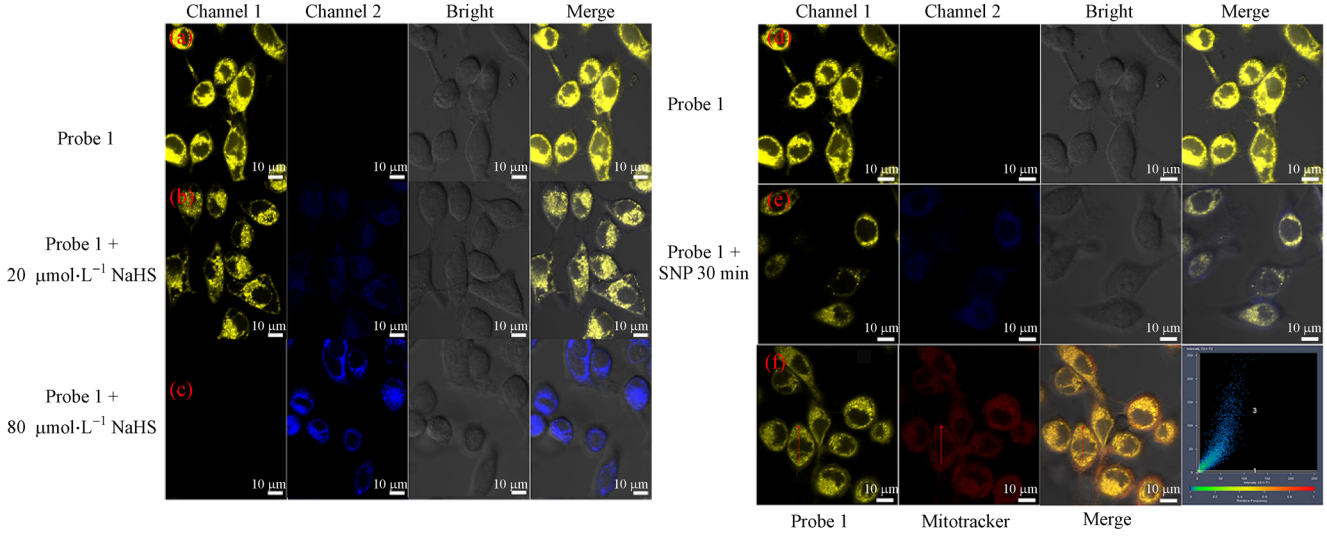


Fig. 5 (a, b, c) CLSM images of 10 μmol/L Probe 1-loaded HeLa cells incubated with HS^- (20 mmol/L and 80 μmol/L, 30 min); (d, c) CLSM images of 10 μmol/L Probe 1-loaded HeLa cells incubated with SNP (100 μmol/L, 30 min); (f) CLSM images of 10 μmol/L Probe 1-loaded HeLa cells incubated with 1 μmol/L MTR at 37 °C for 20 min, where λ_{ex} = 400 nm, λ_{em} = 580 ± 30 nm and the scale bar = 10 μm.

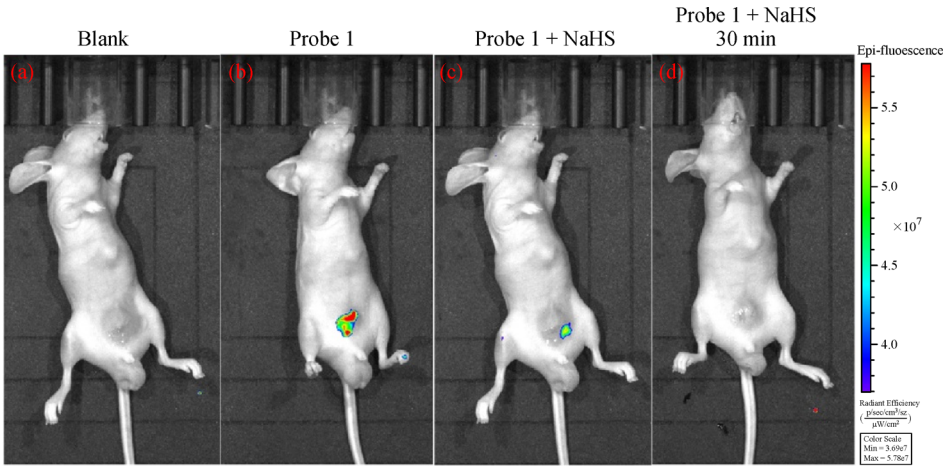


Fig. 6 *In vivo* images of nude mice: (a) fluorescence imaging of the control group, (b) after the 20.0 μmol/L Probe 1 was injected and (c–d) injection with Probe 1 (20.0 μmol/L) after being injected with 80.0 μmol/L HS^- for 30 min, where λ_{ex} = 400 nm and λ_{em} = 550–580 nm.

for H_2S imaging, which would be beneficial in the research of the various physiological and pathological functions of H_2S.

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Compliance with Ethics Guidelines Yan Shi, Fangjun Huo, Yongkang Yue and Caixia Yin declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s11705-021-2048-8 and is accessible for authorized users.

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