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Selective turn-on fluorescent probes for imaging hydrogen sulfide in living cells†

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Abstract

Hydrogen sulfide (H₂S) is an important biological messenger but few biologically-compatible methods are available for its detection. Here we report two bright fluorescent probes that are selective for H₂S over cysteine, glutathione and other reactive sulfur, nitrogen, and oxygen species. Both probes are demonstrated to detect H₂S in live cells.

Hydrogen sulfide (H₂S)¹ has emerged as the most recent biosynthetic gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO).^{2–7} Although historically known for its toxicity and characteristic rotten egg smell, H₂S has gained substantial interest as an established signaling molecule. Despite this interest, few methods for its detection offer the spatial or temporal resolution required for typical cell-based or live tissue experiments. Only recently have fluorescence-based methods emerged for the study of H₂S.^{8–17} Based on our interest in developing chemical tools to study the biological roles of H₂S, we have developed two new turn-on fluorescent probes for H₂S that can be used to image H₂S in live cells.

Hydrogen sulfide is biosynthesized endogenously by three enzymes: cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3MST).^{18–21} Much like how NO production in different tissues results from different isoforms of nitric oxide synthase (NOS),^{22–24} H₂S genesis in different tissues is thought to differ depending on which enzymes are present. Unlike NO and CO, H₂S is highly water soluble, with an aqueous solubility of approximately 90 mM at 37 °C.²⁵ Despite this heightened solubility, biological concentrations of H₂S vary by approximately five orders of magnitude although exact H₂S concentrations remain controversial.⁶

Hydrogen sulfide has potent cardioprotective and neuroprotective effects and is an active neuromodulator.^{18,26–29} High endogenous levels of neuronal H₂S suggest that H₂S has an important physiological function in the central nervous system (CNS). Moreover, H₂S is thought to be involved in long-term potentiation (LTP), calcium homeostasis, suppression of oxidative stress, and modulation of neurotransmission.^{2–7} In humans, H₂S is involved in blood pressure potentiation and abnormal concentrations have been correlated with CNS diseases such as Down syndrome, Alzheimer's disease, and other diseases of mental deficiency.^{21,30–34} Notably, much higher concentrations of exogenous H₂S are typically required to elicit the same physiological response as endogenous H₂S, suggesting that local

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concentrations of H₂S may be higher than those measured in mean bulk tissue experiment,³⁵ thus underscoring the need for selective probes for intracellular H₂S detection.

When coupled with fluorescence microscopy techniques, small-molecule fluorescent probes offer high spatiotemporal resolution and are important tools used to study biological signaling pathways.^{36–38} In our design of fluorescent probes able to detect H₂S, the amino-naphthalimide fluorophore platform was particularly attractive due to its ease of synthetic modification at both the amide and 4-position of the dye, ease of fluorescence modulation by functionalization of the amine moiety, and large Stokes shift. Furthermore, naphthalimide dyes, along with other members of the Lucifer Yellow family, have been used for a variety of fluorescence and colorimetric sensing applications.^{39,40} Based on previous reports of using H₂S to reduce either RNO₂ or RN₃ to the parent amine moieties,^{41–44} we sought to exploit this chemistry for H₂S sensing. By masking a fluorogenic amine as a nitro or azido group, mild reduction with H₂S regenerates the parent amine and results in fluorescence turn-on.

Both developed probes were prepared as outlined in Scheme 1. Treatment of naphthalic anhydride **1** with 2-methoxyethylamine in ethanol afforded Hydrosulfide Naphthalimide-1 (HSN1) in good yield (Scheme 1). Prior to reaction with H₂S, HSN1 is weakly fluorescent ($\lambda_{\text{max}} = 356 \text{ nm}$, $\epsilon = 13100 \pm 300 \text{ M}^{-1} \text{ cm}^{-1}$, $\Phi = 0.0020 \pm 0.0005$). Reaction of naphthalic anhydride **2** with 2-methoxyethylamine in ethanol afforded substituted naphthalimide bromide **3**. Subsequent azide incorporation using NaN₃ in NMP at elevated temperature resulted in formation of Hydrosulfide Naphthalimide-2 (HSN2) (Scheme 1). HSN2 is also weakly fluorescent in its unreacted state ($\lambda_{\text{max}} = 376 \text{ nm}$, $\epsilon = 13500 \pm 200 \text{ M}^{-1} \text{ cm}^{-1}$, $\Phi = 0.0020 \pm 0.0005$). Upon treatment with H₂S, both probes are converted efficiently to fluorescent amine **4** ($\lambda_{\text{max}} = 432 \text{ nm}$, $\epsilon = 11700 \pm 300 \text{ M}^{-1} \text{ cm}^{-1}$, $\lambda_{\text{em}} = 542 \text{ nm}$, $\Phi = 0.096 \pm 0.001$). Product **4** was also prepared independently from ± 4 -amino naphthalic anhydride and does not show any photobleaching during the typical timecourse of an experiment (see SI).

Treatment of a 5 μM solution of HSN1 or HSN2 in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7.4) with 100 equiv. of H₂S resulted in a marked increase in fluorescence. For HSN1, a 15-fold turn-on was observed after 90 min,⁴⁵ and for HSN2 a 60-fold turn-on was observed after 45 min (Fig. 1).⁴⁶ The detection limits of HSN1 and HSN2 for H₂S after 60 min incubation are 5–10 μM and 1–5 μM , respectively. Although not instantaneous, the rate of fluorescence turn-on is comparable to that of commonly-used nitric oxide fluorescent probes based on *o*-diamine scaffolds, which have been used for many biological studies of NO.⁴⁷ The large change in fluorescence of HSN2 upon exposure to H₂S should allow for fluorescence detection of H₂S in biological samples even if reaction with the probe has not proceeded to completion.

Based on the large fluorescence turn-on observed with H₂S, the selectivity of the developed probes for H₂S over other reactive sulfur, oxygen, and nitrogen species (RSONs) including cysteine, glutathione, α -lipoic acid (ALA), nitric oxide (NO), hydrogen peroxide (H₂O₂), sulfite (SO₃²⁻), and thiosulfate (S₂O₃²⁻) was examined. In these experiments, 100 equiv. of each RSON was added to the probe and the integrated fluorescence response was monitored over time (Fig. 2). In addition, because cysteine and glutathione (GSH) are often found in millimolar concentrations in biological milieu, these analytes were also tested at 10 mM (2000 equiv.). HSN1 shows good selectivity for H₂S over other RSONs, but addition of 2000 equiv. of cysteine or GSH eroded much of the selectivity. By contrast, HSN2 showed both greater fluorescence turn-on and increased selectivity for H₂S over other RSONs by comparison to HSN1. Most importantly, the selectivity for H₂S over cysteine and GSH was maintained even when 2000 equiv. were added. Furthermore, we demonstrated that the

fluorescence response of **HSN2** for H_2S is maintained in the presence of cysteine or GSH (see SI).

Having demonstrated the selectivity of **HSN1** and **HSN2** for H_2S , we next tested the ability of the developed probes to be used to visualize H_2S in live cells. HeLa cells were incubated with $5\ \mu\text{M}$ **HSN1** or **HSN2** for 30 min and then treated with either buffer or buffer containing $250\ \mu\text{M}$ H_2S ; a concentration of H_2S comparable with physiological H_2S levels. After further incubation for 30 min, the cells were imaged. As shown in Fig. 3, addition of H_2S to the cells resulted in distinct change in the observed fluorescence, thus demonstrating the ability of the developed probes for use as tools to visualize H_2S in live cells. Although both probes are able to effectively image H_2S in live cells, **HSN2** provides a more robust intensity change consistent with the selectivity and turn-on studies.

In summary, we have reported two fluorescent probes for H_2S detection that exhibit a robust response both in vitro and in live cells. The high selectivity of **HSN1** and **HSN2** for H_2S over other reactive sulfur, oxygen, and nitrogen species highlight the utility of these probes. We are currently pursuing these and other strategies for developing selective fluorescent probes for H_2S and using such probes to study the physiological roles of endogenous H_2S .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

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Fig. 1. Fluorescence turn-on of (a) HSN1 and (b) HSN2 (5 μ M probe, 50 mM PIPES, 100 mM KCl, pH 7.4, 37 $^{\circ}$ C, λ_{ex} = 435 nm.) after treatment with 100 equiv. of H_2S . The insets show the time dependence of the fluorescence enhancement.

Fig. 2.

Selectivity of (a) HSN1 and (b) HSN2 with other reactive oxygen, nitrogen, and sulfur species. (5 μ M probe, 100 equiv. of RSONS, 50 mM PIPES, 100 mM KCl, pH 7.4, 37 $^{\circ}$ C, λ_{ex} = 435 nm, λ_{scan} = 450–750 nm). Data were acquired before analyte addition and 1, 5, 10, 15, 30, 45, 60, 75, and 90 min after analyte addition for HSN1. Time points at 75 and 90 min are omitted for HSN2 due to the faster reactivity. Comparisons of 2,000 equiv. (10 mM) of cysteine and GSH are shown at right. The data shown are the average of at least three independent runs.

Fig. 3.

Fluorescence imaging of H₂S in HeLa cells incubated with 5 μ M HSN1 or HSN2. Cells were incubated with HSN1 or HSN2 for 30 min, after which either buffer or 250 μ M H₂S was added. After further incubation for 30 min the cells were imaged. (a) HSN1; (b) HSN1 with H₂S; (c) HSN2; (d) HSN2 with H₂S. Top: DIC image with nuclear stain overlay. Bottom: fluorescence image. Scale bars = 25 μ m.

Scheme 1.

Synthesis of HSN1 and HSN2. Both probes are converted to fluorescent amine 4 upon treatment with H_2S .