JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Reaction-Based Fluorescent Probes for Selective Imaging of Hydrogen Sulfide in Living Cells

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Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) is emerging as an important mediator of human physiology and pathology but remains difficult to study, in large part because of the lack of methods for selective monitoring of this small signaling molecule in live biological specimens. We now report a pair of new reaction-based fluorescent probes for selective imaging of H_2S in living cells that exploit the H_2S-mediated reduction of azides to fluorescent amines. Sulfidefluor-1 (SF1) and Sulfidefluor-2 (SF2) respond to H_2S by a turn-on fluorescence signal enhancement and display high selectivity for H_2S over other biologically relevant reactive sulfur, oxygen, and nitrogen species. In addition, SF1 and SF2 can be used to detect H_2S in both water and live cells, providing a potentially powerful approach for probing H_2S chemistry in biological systems.

Hydrogen sulfide (H_2S) is a member of the reactive sulfur species (RSS) family, which includes thiols,[1,2] *S*-nitrosothiols,[3] sulfenic acids,[4] and sulfite,[5] and it plays vital roles in regulating intracellular redox status[6] and other fundamental signaling processes[7] involved in human health and disease. Like other gaseous signaling molecules such as nitric oxide (NO) and carbon monoxide (CO), H_2S can interact directly with downstream protein targets, most commonly through post-translational cysteine sulfhydration[8] as well as via binding to heme iron centers.[9] H_2S contributes to a diverse array of physiological processes, including vasodilation,[10] angiogenesis,[11] oxygen sensing,[12] apoptosis,[13] inflammation,[14] and neuromodulation,[15] and it can also protect against ischemia/reperfusion injury.[16] On the other hand, H_2S levels are altered in diseases ranging from Alzheimer's disease[17] and Down's syndrome[18] to diabetes[19] and liver cirrhosis.[20] As such, a major challenge to elucidating the complex contributions of H_2S to both healthy and disease states is the dearth of methods for selective tracking of this small molecule within living biological specimens. Indeed, current techniques for H_2S detection, such as colorimetric[21−23] and electrochemical assays,[24] gas chromatography,[25] and metal-induced sulfide precipitation,[26] often require post-mortem processing and/or destruction of tissues or cell lysates and hence afford variable estimates of [H_2S] ranging from nano- to millimolar levels.[25,27,28] We now present a new approach for the detection of H_2S within intact, live biological systems through the development of chemoselective H_2S-responsive fluorescent probes. Sulfidefluor-1 (SF1) and Sulfidefluor-2 (SF2) represent a pair of first-generation reagents featuring a robust fluorescence turn-on response that is selective for H_2S over other RSS, and they can be utilized to image changes in the levels of this small signaling molecule in living cells.

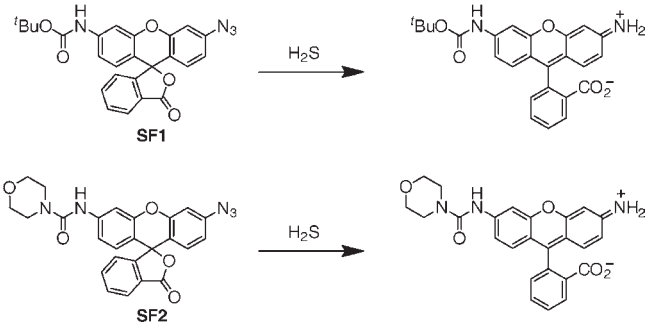
Our general strategy for H_2S detection in living biological specimens relies on monitoring this small molecule through chemoselective reactions that are bioorthogonal to native cellular processes, a strategy that our laboratory has successfully employed for sensing hydrogen peroxide (H_2O_2) through the H_2O_2-mediated oxidation of arylboronates[29,30] or α-ketoacids.[31] In this context, desirable features for reaction-based probes of this type include selectivity for H_2S, compatibility with aqueous environments, reactivity at physiological pH and ionic strength, and liberation of nontoxic byproducts. Accordingly, we became attracted to reactions involving the azide functional group, as this moiety has been extensively and elegantly employed as a bioorthogonal functional group,[32] particularly in Staudinger−Bertozzi ligations[33] and Hüisgen 1,3-dipolar cycloaddition reactions,[34−37] and can operate in living cells[38] and animals.[39,40] In a conceptual departure from these ligation methods, we envisioned that azides could be utilized for H_2S detection via the H_2S-mediated reduction of azides to amines, a reaction that has been employed in the synthesis of complex organic molecules.[41] On the basis of these design considerations, we prepared the azide-caged rhodamine analogues SF1 and SF2, reasoning that reductive reactions with H_2S would generate highly fluorescent rhodamine products (Scheme 1). To this end, we synthesized these probes in two steps starting from commercially available rhodamine 110, utilizing a Sandmeyer reaction to install the pendant azide[42] (Scheme S1 in the Supporting Information).

We then tested the fluorescence properties of these two probes in aqueous solutions buffered at physiological pH (20 mM HEPES, pH 7.4) (Figure 1). In their protected forms, SF1 and SF2 adopt a closed lactone conformation and exhibit no absorption features in the visible region (Figure S1 in the Supporting Information). However, upon treatment of 10 μM SF1 or SF2 with 100 μM NaSH (a commonly employed H_2S donor), a robust increase in fluorescence intensity was observed, accompanied by the appearance of absorption and emission bands in the visible region (SF1 product: λ_{max} = 490 nm, ε = 61 000 M^{-1} cm^{-1}, λ_{em} = 525 nm, Φ = 0.51; SF2 product: λ_{max} = 492 nm, ε = 75 000 M^{-1} cm^{-1}, λ_{em} = 525 nm, Φ = 0.60; see Figure S1). Within 1 h of reaction under these conditions, SF1 and SF2 produced 7- and 9-fold turn-on responses, respectively (Figure 1). The in vitro detection limit for H_2S using SF1 and SF2 was found to be 5−10 μM (Figure S2). Finally, ^{1}H NMR and LC−MS analyses of reactions of the SF probes with H_2S confirmed the production of the corresponding rhodamine dyes (Figure S3).

Received: April 20, 2011
Published: June 15, 2011

dx.doi.org/10.1021/ja203661j | *J. Am. Chem. Soc.* 2011, 133, 10078–10080

Scheme 1. Sulfidefluor-1 (SF1) and Sulfidefluor-2 (SF2) Fluorescent Probes for H_2S Detection



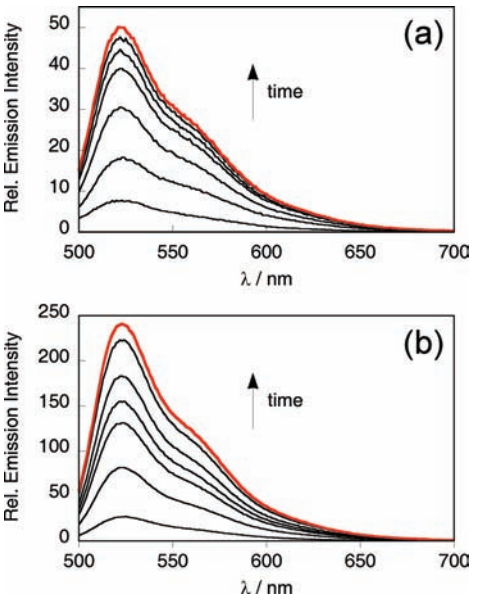


Figure 1. Fluorescence responses of (a) 10 μM SF1 and (b) 10 μM SF2 to 100 μM H_2S. Data were acquired at 25 °C in 20 mM HEPES buffer (pH 7.4) with excitation at λ_{ex} = 488 nm. Emission was collected between 498 and 700 nm. Time points represent 0, 10, 20, 30, 40, 50, and (red trace) 60 min after addition of 100 μM H_2S.

Moreover, the turn-on responses of SF1 and SF2 were found to be selective for H_2S over other biologically relevant RSS, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Figure 2). Both probes displayed good selectivity for H_2S over abundant biologically relevant thiols, including 5 mM glutathione and 5 mM cysteine (50 times the amount of H_2S tested). Additionally, other relevant RSS, including sulfite, thiosulfate, thiocyanate, *S*-nitrosoglutathione, and lipoic acid, showed limited fluorescence responses. Finally, exposing SF1 and SF2 to a panel of biologically relevant ROS and RNS, including H_2O_2, *tert*-butyl peroxide (^{t}BuOOH), hypochlorite (OCl^-), nitrite (NO_2^-), nitric oxide (NO), and superoxide (O_2^-), did not trigger fluorescence turn-on responses to the same extent as exposure to H_2S. SF1 displayed ~3-fold greater response for H_2S than for most of the other species tested and ~2-fold selectivity versus O_2^-. For comparison, SF2 possesses more favorable fluorescence properties than SF1 in vitro in terms of a greater fluorescence response to H_2S, much lower background

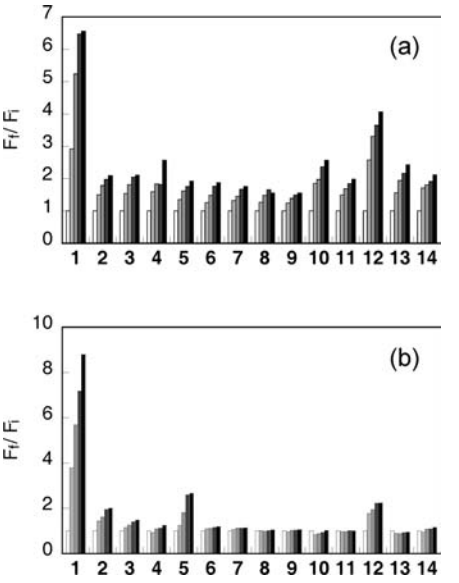


Figure 2. Fluorescence responses of (a) 10 μM SF1 and (b) 10 μM SF2 to biologically relevant RSS, RNS, and ROS. Bars represent relative responses at 525 nm at 0, 15, 30, 45, and 60 min after addition of RSS, RNS, or ROS. Data shown are for 5 mM glutathione, 5 mM cysteine, and 100 μM for other RSS, RNS, and ROS. Data were acquired in 20 mM HEPES buffer (pH 7.4) with excitation at λ_{ex} = 488 nm. Legend: (1) H_2S; (2) glutathione; (3) cysteine; (4) lipoic acid; (5) Na_2SO_3; (6) NaS_2O_3; (7) KSCN; (8) *S*-nitrosoglutathione; (9) $NaNO_2$; (10) NO; (11) H_2O_2; (12) O_2^-; (13) ^{t}BuOOH; (14) HOCl.

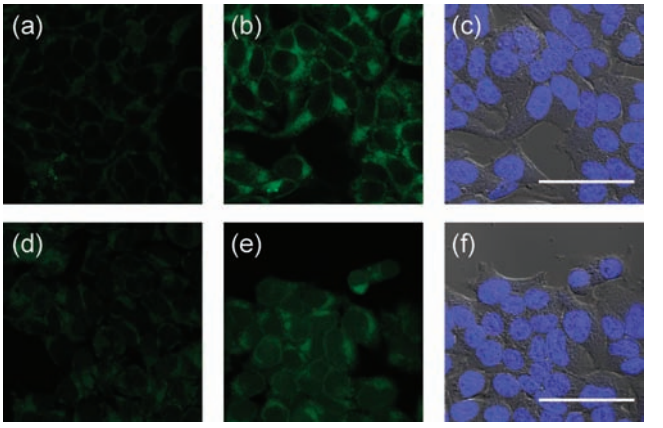


Figure 3. Confocal microscopy images of H_2S detection in live HEK293T cells using SF1 and SF2. (a) HEK293T cells incubated with SF1 for 60 min at 37 °C. (b) HEK293T cells incubated with SF1 for 60 min at 37 °C with 250 μM NaSH added for the final 30 min. (c) Bright-field images of the field of cells in (b) overlaid with images of 1 μM Hoescht stain at 37 °C. (d) HEK293T cells incubated with SF2 for 60 min at 37 °C. (e) HEK293T cells incubated with SF2 for 60 min at 37 °C with 250 μM NaSH added for the final 30 min. (f) Bright-field images of the field of cells in (e) overlaid with images of 1 μM Hoescht stain at 37 °C. Scale bars represent 50 μM.

reactivity to other analytes, and better selectivity versus glutathione (~5-fold), sulfite (~4-fold), and O_2^- (~4-fold). Taken together, these selectivity assays demonstrate that the chemoselective reduction of an azide to an amine can be used for fluorescence detection of H_2S in aqueous media at physiological pH.

dx.doi.org/10.1021/ja203661j |*J. Am. Chem. Soc.* 2011, 133, 10078–10080

Next, we tested the abilities of SF1 and SF2 to visualize changes in H_2S levels in live-cell imaging mode using confocal microscopy (Figure 3). HEK293T cells were incubated with 5 μM SF1 or SF2 for 30 min and then treated with a blank control or 250 μM NaSH for an additional 30 min; this concentration is well within the range that has been used to elicit physiological responses (10−600 μM).[10,11,15] A patent increase in intracellular fluorescence intensity was observed in the H_2S-treated cells (Figure 3b,e) relative the to control samples (Figure 3a,d). Moreover, bright-field images with Hoescht nuclear staining confirmed the viability of the cells over the course of the experiments (Figure 3c,f). Interestingly, SF1 gave a higher turn-on response than SF2 for the detection of H_2S in cells (Figure S4), which may be due to increased lipophilicity and cellular retention of SF1 ($\log P = 1.98 \pm 0.1$) relative to SF2 ($\log P = 1.21 \pm 0.15$).

To close, we have presented a new approach to biological H_2S detection through the synthesis and evaluation of the azide-based fluorescent probes SF1 and SF2. These reagents are highly selective for detection of H_2S in aqueous media and can be used for imaging H_2S in living cells. We are actively pursuing more sensitive and responsive analogues for fluorescence imaging of H_2S in living cells, tissues, and animals as well as the utilization of these probes to study the endogenous production of H_2S in living cells and its contributions to physiological and pathological processes.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis and experimental details, including procedures for the synthesis and characterization of new compounds, selectivity assays, spectroscopy, and cellular imaging experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This work was supported by Amgen, Astra Zeneca, Novartis, and the Packard Foundation. E.J.N. acknowledges the Royal Commission for the Exhibition of 1851 for additional support. C. J.C. is an Investigator of the Howard Hughes Medical Institute. We thank Ann Fischer and Michelle Yasukawa (UCB Tissue Culture Facility) for expert technical assistance.

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