

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

Alexander R. Lippert,[†] Elizabeth J. New,[†] and Christopher J. Chang^{*,†,‡}

[†]Department of Chemistry and [‡]Howard Hughes Medical Institute, University of California, Berkeley, California 94720, United States

S Supporting Information

ABSTRACT: Hydrogen sulfide (H₂S) 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 H₂S in living cells that exploit the H₂S-mediated reduction of azides to fluorescent amines. Sulfidefluor-1 (SF1) and Sulfidefluor-2 (SF2) respond to H₂S by a turn-on fluorescence signal enhancement and display high selectivity for H₂S over other biologically relevant reactive sulfur, oxygen, and nitrogen species. In addition, SF1 and SF2 can be used to detect H₂S in both water and live cells, providing a potentially powerful approach for probing H₂S chemistry in biological systems.

Hydrogen sulfide (H₂S) 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 status⁶ and other fundamental signaling processes⁷ involved in human health and disease. Like other gaseous signaling molecules such as nitric oxide (NO) and carbon monoxide (CO), H₂S can interact directly with downstream protein targets, most commonly through post-translational cysteine sulphydration⁸ as well as via binding to heme iron centers.⁹ H₂S 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, H₂S levels are altered in diseases ranging from Alzheimer's disease¹⁷ and Down's syndrome¹⁸ to diabetes¹⁹ and liver cirrhosis.²⁰ As such, a major challenge to elucidating the complex contributions of H₂S 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 H₂S 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 [H₂S] ranging from nano- to millimolar levels.^{25,27,28} We now present a new approach for the detection of H₂S within intact, live biological systems through the development of chemoselective H₂S-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 H₂S 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 H₂S 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 (H₂O₂) through the H₂O₂-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 H₂S, 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 ligations³³ and Huisgen 1,3-dipolar cycloaddition reactions,^{34–37} and can operate in living cells³⁸ and animals.^{39,40} In a conceptual departure from these ligation methods, we envisioned that azides could be utilized for H₂S detection via the H₂S-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 H₂S 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 azide⁴² (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 μ M SF1 or SF2 with 100 μ M NaSH (a commonly employed H₂S donor), a robust increase in fluorescence intensity was observed, accompanied by the appearance of absorption and emission bands in the visible region (SF1 product: $\lambda_{\text{max}} = 490$ nm, $\epsilon = 61\,000$ M^{−1} cm^{−1}, $\lambda_{\text{em}} = 525$ nm, $\Phi = 0.51$; SF2 product: $\lambda_{\text{max}} = 492$ nm, $\epsilon = 75\,000$ M^{−1} cm^{−1}, $\lambda_{\text{em}} = 525$ nm, $\Phi = 0.60$; 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 H₂S using SF1 and SF2 was found to be 5–10 μ M (Figure S2). Finally, ¹H NMR and LC–MS analyses of reactions of the SF probes with H₂S confirmed the production of the corresponding rhodamine dyes (Figure S3).

Received: April 20, 2011

Published: June 15, 2011

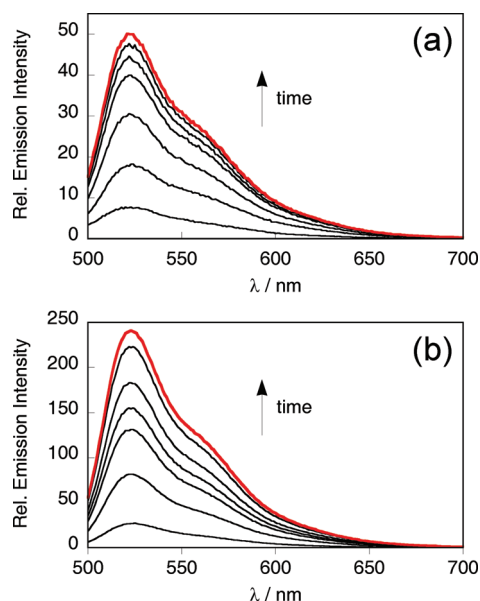
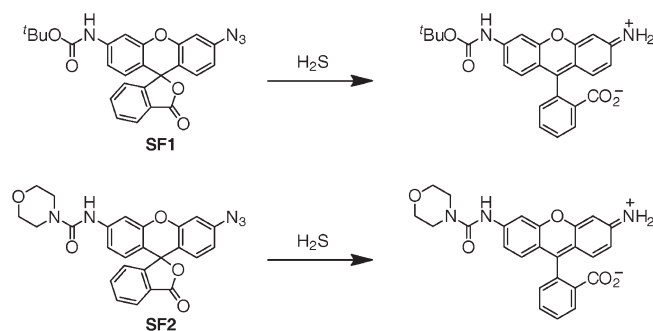
Scheme 1. Sulfidefluor-1 (SF1) and Sulfidefluor-2 (SF2) Fluorescent Probes for H₂S Detection


Figure 1. Fluorescence responses of (a) 10 μ M SF1 and (b) 10 μ M SF2 to 100 μ M H₂S. Data were acquired at 25 $^{\circ}$ 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 μ M H₂S.

Moreover, the turn-on responses of SF1 and SF2 were found to be selective for H₂S over other biologically relevant RSS, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Figure 2). Both probes displayed good selectivity for H₂S over abundant biologically relevant thiols, including 5 mM glutathione and 5 mM cysteine (50 times the amount of H₂S 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 H₂O₂, *tert*-butyl peroxide (*t*BuOOH), hypochlorite (OCl[−]), nitrite (NO₂[−]), nitric oxide (NO), and superoxide (O₂[−]), did not trigger fluorescence turn-on responses to the same extent as exposure to H₂S. SF1 displayed \sim 3-fold greater response for H₂S than for most of the other species tested and \sim 2-fold selectivity versus O₂[−]. For comparison, SF2 possesses more favorable fluorescence properties than SF1 *in vitro* in terms of a greater fluorescence response to H₂S, much lower background

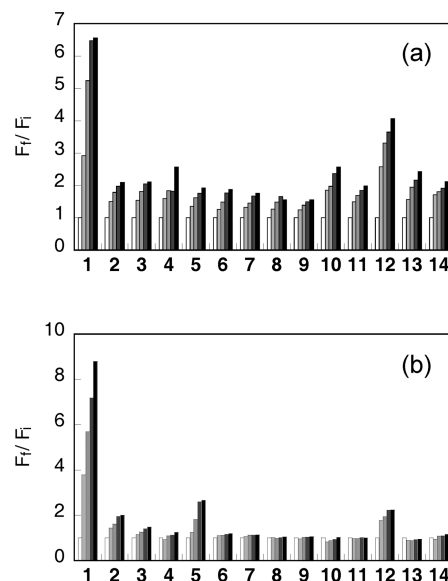


Figure 2. Fluorescence responses of (a) 10 μ M SF1 and (b) 10 μ M SF2 to biologically relevant RSS, RNS, and ROS. Bars represent relative responses at 525 nm at 0, 15, 30, 45, and 60 min after addition of RSS, RNS, or ROS. Data shown are for 5 mM glutathione, 5 mM cysteine, and 100 μ M for other RSS, RNS, and ROS. Data were acquired in 20 mM HEPES buffer (pH 7.4) with excitation at λ_{ex} = 488 nm. Legend: (1) H₂S; (2) glutathione; (3) cysteine; (4) lipoic acid; (5) Na₂SO₃; (6) Na₂O₃; (7) KSCN; (8) S-nitrosoglutathione; (9) NaNO₂; (10) NO; (11) H₂O₂; (12) O₂[−]; (13) *t*BuOOH; (14) HOCl.

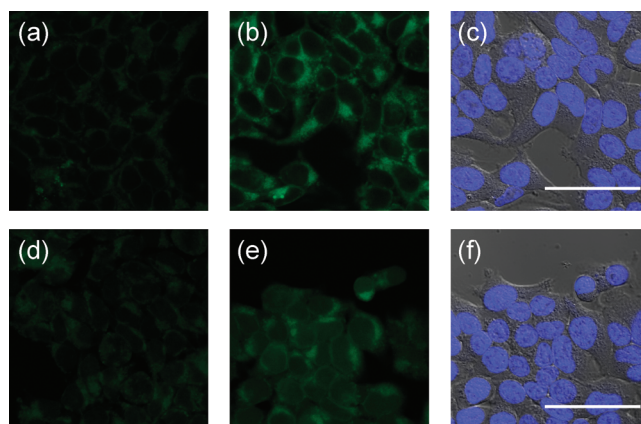


Figure 3. Confocal microscopy images of H₂S detection in live HEK293T cells using SF1 and SF2. (a) HEK293T cells incubated with SF1 for 60 min at 37 $^{\circ}$ C. (b) HEK293T cells incubated with SF1 for 60 min at 37 $^{\circ}$ C with 250 μ M NaSH added for the final 30 min. (c) Bright-field images of the field of cells in (b) overlaid with images of 1 μ M Hoechst stain at 37 $^{\circ}$ C. (d) HEK293T cells incubated with SF2 for 60 min at 37 $^{\circ}$ C. (e) HEK293T cells incubated with SF2 for 60 min at 37 $^{\circ}$ C with 250 μ M NaSH added for the final 30 min. (f) Bright-field images of the field of cells in (e) overlaid with images of 1 μ M Hoechst stain at 37 $^{\circ}$ C. Scale bars represent 50 μ M.

reactivity to other analytes, and better selectivity versus glutathione (\sim 5-fold), sulfite (\sim 4-fold), and O₂[−] (\sim 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 H₂S in aqueous media at physiological pH.

Next, we tested the abilities of SF1 and SF2 to visualize changes in H₂S levels in live-cell imaging mode using confocal microscopy (Figure 3). HEK293T cells were incubated with 5 μ M SF1 or SF2 for 30 min and then treated with a blank control or 250 μ M NaSH for an additional 30 min; this concentration is well within the range that has been used to elicit physiological responses (10–600 μ M).^{10,11,15} A patent increase in intracellular fluorescence intensity was observed in the H₂S-treated cells (Figure 3b,e) relative to the 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 H₂S 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 H₂S detection through the synthesis and evaluation of the azide-based fluorescent probes SF1 and SF2. These reagents are highly selective for detection of H₂S in aqueous media and can be used for imaging H₂S in living cells. We are actively pursuing more sensitive and responsive analogues for fluorescence imaging of H₂S in living cells, tissues, and animals as well as the utilization of these probes to study the endogenous production of H₂S in living cells and its contributions to physiological and pathological processes.

■ ASSOCIATED CONTENT

S 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>.

■ AUTHOR INFORMATION

Corresponding Author
chrischang@berkeley.edu

■ 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.

■ REFERENCES

- (1) Sies, H. *Free Radical Biol. Med.* **1999**, *27*, 916–921.
- (2) Estrela, J. M.; Ortega, A.; Obrador, E. *Crit. Rev. Clin. Lab. Sci.* **2006**, *43*, 143–181.
- (3) Seth, D.; Stamler, J. S. *Curr. Opin. Chem. Biol.* **2007**, *15*, 1–8.
- (4) Paulsen, C. E.; Carroll, K. S. *ACS Chem. Biol.* **2010**, *5*, 47–62.
- (5) Garrett, R. M.; Johnson, J. L.; Graf, T. N.; Feigenbaum, A.; Rajagopalan, K. V. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6394–6398.
- (6) Kabil, O.; Banerjee, R. J. *Biol. Chem.* **2010**, *285*, 21903–21907.
- (7) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169–187.
- (8) Mustafa, A. K.; Gadalla, M. M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S. K.; Barrow, R. K.; Yang, G.; Wang, R.; Snyder, S. H. *Sci. Signaling* **2009**, *2*, No. ra72.
- (9) Blackstone, E.; Morrison, M.; Roth, M. B. *Science* **2005**, *308*, 518.
- (10) Yang, G.; Wu, L.; Jiang, B.; Yang, B.; Qi, J.; Cao, K.; Meng, Q.; Mustafa, A. K.; Mu, W.; Zhang, S.; Snyder, S. H.; Wang, R. *Science* **2008**, *322*, 587–590.
- (11) Papapetropoulos, A.; Pyriochou, A.; Altaany, Z.; Yang, G.; Marazioti, A.; Zhou, Z.; Jeschke, M. G.; Branski, L. K.; Herndon, D. N.; Wang, R.; Szabó, C. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 21972–21977.
- (12) Peng, Y.-J.; Nanduri, J.; Raghuraman, G.; Souvannakitti, D.; Gadalla, M. M.; Kumar, G. K. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10719–10724.
- (13) Yang, G.; Wu, L.; Wang, R. *FASEB J.* **2006**, *20*, 553–555.
- (14) Li, L.; Bhatia, M.; Zhu, Y. Z.; Zhu, Y. C.; Ramnath, R. D.; Wang, Z. J.; Anuar, F. B.; Whiteman, M.; Salto-Tellez, M.; Moore, P. K. *FASEB J.* **2005**, *19*, 1196–1198.
- (15) Abe, K.; Kimura, H. *J. Neurosci.* **1996**, *16*, 1066–1071.
- (16) Nicholson, C. K.; Calvert, J. W. *Pharmacol. Res.* **2010**, *62*, 289–297.
- (17) Eto, K.; Asada, T.; Arima, K.; Makifuchi, T.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1485–1488.
- (18) Kamoun, P.; Belardinelli, M.-C.; Chabli, A.; Lallouchi, K.; Chadeaux-Vekemans, B. *Am. J. Med. Genet.* **2003**, *116A*, 310–311.
- (19) Yang, W.; Yang, G.; Jia, X.; Wu, L.; Wang, R. *J. Physiol.* **2005**, *569*, 519–531.
- (20) Fiorucci, S.; Antonelli, E.; Mencarelli, A.; Orlandi, S.; Renga, B.; Rizzo, G.; Distrutti, E.; Shah, V.; Morelli, A. *Hepatology* **2005**, *42*, 539–548.
- (21) Fisher, E. *Chem. Ber.* **1883**, *16*, 2234–2236.
- (22) Choi, M. M. F. *Analyst* **1998**, *123*, 1631–1634.
- (23) Choi, M. G.; Cha, S.; Lee, H.; Jeon, H. L.; Chang, S. K. *Chem. Commun.* **2009**, 7390–7392.
- (24) Lawrence, N. S.; Davis, J.; Jiang, L.; Jones, T. G. J.; Davies, S. N.; Compton, R. G. *Electroanalysis* **2000**, *18*, 1453–1460.
- (25) Furne, J.; Saeed, A.; Levitt, M. D. *Am. J. Physiol.* **2008**, *295*, R1479–R1485.
- (26) Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signaling* **2009**, *11*, 205–214.
- (27) Warenycia, M. W.; Goodwin, L. R.; Benishin, C. G.; Reiffenstein, R. J.; Francom, D. M.; Taylor, J. D.; Dieken, F. P. *Biochem. Pharmacol.* **1989**, *38*, 973–981.
- (28) Han, Y.; Qin, J.; Chang, X.; Yang, Z.; Du, J. *Cell. Mol. Neurobiol.* **2006**, *26*, 101–107.
- (29) Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 15392–15393.
- (30) Miller, E. W.; Tulyathan, O.; Isacoff, E. Y.; Chang, C. J. *Nat. Chem. Biol.* **2007**, *3*, 263–267.
- (31) Lippert, A. R.; Keshari, K. R.; Kurhanewicz, J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 3776–3779.
- (32) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974–6998.
- (33) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007–2010.
- (34) Huisgen, R. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley: New York, 1984; pp 1–176.
- (35) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.
- (36) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
- (37) Dieterich, D. C.; Lee, J. J.; Link, A. J.; Graumann, J.; Tirrell, D. A.; Schuman, E. M. *Nat. Protoc.* **2007**, *2*, 532–540.
- (38) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253–2255.
- (39) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873–877.
- (40) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664–667.
- (41) Pang, L.-J.; Wang, D.; Zhou, J.; Zhang, L.-H.; Ye, X.-S. *Org. Biomol. Chem.* **2009**, *7*, 4252–4266.
- (42) Abe, H.; Wang, J.; Furukawa, K.; Oki, K.; Uda, M.; Tsuneda, S.; Ito, Y. *Bioconjugate Chem.* **2008**, *19*, 1219–1226.