H_2S donors with optical responses

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Abstract

Reactive sulfur species, including hydrogen sulfide (H_2S), are important biological mediators and play key roles in different pathophysiological conditions. Small molecules that release H_2S on demand, often referred to as "H_2S donors," constitute a key investigative tool for H_2S-related research. A significant challenge, however, is correlating the rate of H_2S release from such donors in complex systems with biological outcomes, because release rates are commonly perturbed by different biological environments. In this chapter, we outline an approach to use H_2S donors that provide a fluorescent response upon H_2S release to address this problem. These compounds leverage the intermediate release of carbonyl sulfide (COS), which is quickly converted to H_2S by the endogenous enzyme carbonic anhydrase (CA), to provide activatable donors with an optical response. The described donors are activated by biological thiols and provide a fluorescence response that correlates directly with H_2S delivery, which allows for delivered H_2S levels to be measured in real time by fluorescence techniques.

Methods in Enzymology
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2020.04.039

1. Introduction

Reactive sulfur species (RSS) have gained significant interest in the last decade due to their roles in different biological processes and systems. Of such species, hydrogen sulfide (H_2S) has garnered significant interest because it is produced endogenously from both enzymatic and non-enzymatic pathways and functions as an important signaling molecule akin to nitric oxide (NO) and carbon monoxide (CO) (Wang, 2002, 2012). As brief examples of this activity, misregulation of endogenous H_2S has been implicated in different (patho)physiological processes, including angiogenesis, wound healing, carcinogenesis, protection against ischemia/reperfusion injury, as well as other activities (Cortese-Krott, Fernandez, Kelm, Butler, & Feelisch, 2015; Filipovic, Zivanovic, Alvarez, & Banerjee, 2018; Kolluru, Shen, Bir, & Kevil, 2013; Szabo et al., 2014; Yuan, Shen, & Kevil, 2017). Complementing the expansion of biological activities associated with H_2S and related RSS, small molecule chemical tools for H_2S detection and delivery have emerged as key tools for investigate complex RSS in diverse environments (Levinn, Cerda, & Pluth, 2020; Li, Yin, & Huo, 2015; Lin, Chen, Xian, & Chang, 2015; Powell, Dillon, & Matson, 2018; Szabo & Papapetropoulos, 2017).

Of such tools, small molecules that release H_2S on demand form a cornerstone of our ability to manipulate biological levels of H_2S (Levinn et al., 2020; Powell et al., 2018; Szabo & Papapetropoulos, 2017). Simple sources of H_2S, such as Na_2S or NaSH, are commonly used in biological investigations, yet lack physiological relevance due to the rapid release and subsequent oxidation of H_2S from these salts. Building from the need for slow-releasing H_2S donors, researchers have made significant advances in the development of passive releasing donors, which are often activated by hydrolysis or reaction with common biological nucleophiles, as well as activatable donors, which are often activated by specific biological or bioorthogonal stimuli. One approach that has enabled different donors to be tuned for activation by different stimuli is the development of systems that release carbonyl sulfide (COS) as a precursor for H_2S delivery. Under biological conditions, COS is quickly converted to H_2S by the endogenous enzyme carbonic anhydrase (CA).

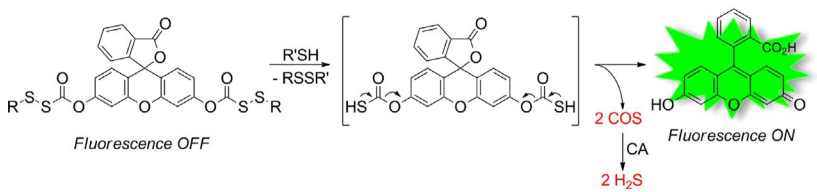
One significant challenge in using small molecule donors for H_2S or other biologically-relevant species, is relating measured release rates in buffer with H_2S-related activities in more complex systems. Release in buffer, however, typically does not match release rates in more complex systems,

such as cell culture or animal models. For example, even the presence of simple proteins, such as bovine serum albumin (BSA), has been demonstrated to significantly change the rate of H_2S release from certain donors (Zhao, Steiger, & Pluth, 2018a). H_2S release rates from donors can be readily measured in buffer or in vitro using H_2S-responsive electrodes, or analytical methods including the methylene blue (MB) or monobromobimane (mBB) methods (Hartle & Pluth, 2016). All of these methods, however, are destructive and consume H_2S and also often require significant sample preparation. One approach toward addressing this challenge is to use H_2S donors that couple an optical signal with H_2S releasing, such that H_2S delivery can be monitored in real time by common spectroscopic methods. In this chapter, we describe the procedure for preparing and using the fluorescent H_2S donors FLD-1 and FLD-3, which enable direct real-time monitoring of H_2S release using fluorescence spectroscopy and microscopy (Zhao, Cerda, & Pluth, 2019).

2. Preparation and properties of fluorescent H_2S donors

2.1 General design principles

To access H_2S donors that release H_2S slowly under physiological conditions with a concomitant fluorescence readout, we developed a system that could be readily activated by biological thiols to release both H_2S and fluorescein. By using the thiol-mediated reduction of sulfenyl thiocarbonates, reaction with biological thiols including reduced glutathione (GSH) or cysteine (Cys) results in disulfide exchange and the intermediate release COS (Scheme 1). The released COS is quickly converted to H_2S by CA. This strategy for leveraging intermediate COS release to enable H_2S delivery is now a broadly-used approach to develop H_2S donor compounds (Cerda, Mancuso, Mullen, Hendon, & Pluth, 2020; Levinn, Cerda, & Pluth, 2019), and has been used to develop donors activated by cellular nucleophiles



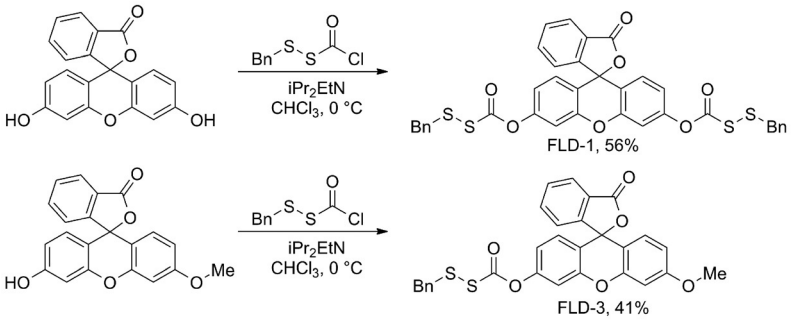
Scheme 1 Activation and response mechanism for the sulfenyl thiocarbonate donors. Reaction with cellular thiols results in disulfide reduction and COS release, which is quickly converted to H_2S by carbonic anhydrase (CA).

(Powell, Foster, Okyere, Theus, & Matson, 2016; Zhao, Steiger, & Pluth, 2018b, 2019), reactive oxygen species (Chauhan, Jos, & Chakrapani, 2018; Hu et al., 2019; Zhao, Henthorn, & Pluth, 2017; Zhao & Pluth, 2016), enzymes (Chauhan, Bora, Ravikumar, Jos, & Chakrapani, 2017; Levinn, Steiger, & Pluth, 2019; Steiger, Marcatti, Szabo, Szczesny, & Pluth, 2017), light (Sharma et al., 2017; Stacko, Muchova, Vitek, & Klan, 2018; Zhao, Bolton, & Pluth, 2017), and other stimuli (Gilbert, Zhao, Otteson, & Pluth, 2019; Steiger, Yang, Royzen, & Pluth, 2017).

On the basis of these design principles, two different fluorescein-based H_2S donors (FLD-1 and FLD-3) can be readily prepared. Treatment with either fluorescein or 3-*O*-methylfluorescein with ((benzyl)dithio)carbonyl chloride yields the fluorescent donors FLD-1 and FLD-3, respectively, in moderate yield (Scheme 2). Both compounds are readily purified by SiO_2 column chromatography and are stable at room temperature, but should be stored in a freezer over extended periods of time. These donors provide systems with a turn on fluorescence response that corresponds to H_2S donation. The chromophores used for FLD-1 ($\lambda_{ex} = 490$ nm; $\lambda_{em} =$ 500–650 nm) and FLD-3 ($\lambda_{ex} = 454$ nm; $\lambda_{em} = 500$–650 nm) correspond to commonly used FITC/GFP green channels and filter cubes used for fluorescence microscopy. Fluorescein is commercially available from many sources, and ((benzyl)dithio)carbonyl chloride and 3-*O*-methylfluorescein are readily available through simple synthetic procedures (Mugherli, Burchak, Chatelain, & Balakirev, 2006).

2.2 Materials, equipment, and reagents

- Fluorescein (CAS# 2321-07-5) or 3-*O*-methylfluorescein (CAS# 70672-05-8)
- (Benzyl(dithio))carbonyl chloride (CAS# 31331-36-9)



Scheme 2 Synthesis of FLD-1 and FLD-2.

- Chloroform
- Ethyl acetate
- Hexanes
- Silica gel
- Diisopropyl ethylamine (DIPEA)
- Magnesium sulfate ($MgSO_4$)
- Brine
- Round bottom flask
- Separatory funnel
- Column for chromatography
- NMR instrument to verify compound identity and purity
- HPLC to verify compound purity

2.3 Preparation procedures

2.3.1 General procedure for preparation of FLD-1 and FLD-3

1. Add 1.0 equiv. of fluorescein and 3.0 equiv. of ((benzyl)dithio)carbonyl chloride to $CHCl_3$ (~5–10 mL) under nitrogen.
2. Cool the reaction mixture to 0 °C for 5 min in an ice bath, and then slowly add 3.0 equiv. of diisopropyl ethylamine (DIPEA).
3. Remove the ice bath and stir the reaction mixture for 2 h, or until the reaction is complete as indicated by thin layer chromatography.
4. Quench the reaction mixture by adding 25 mL of brine.
5. Extract the aqueous solution with ethyl acetate (3×15 mL).
6. Combine the organic layers and dry over $MgSO_4$.
7. Filter off the $MgSO_4$ and remove the solvent under vacuum.
8. Purify the crude product using SiO_2 column chromatography using ethyl acetate/hexanes as the eluent.
9. The product can be isolated as a yellow solid in approximately 40–55% yield.

2.3.2 Reaction scales and product identification for FLD-1 and FLD-3

Reaction scale for FLD-1: fluorescein (332 mg, 1.00 mmol), ((benzyl)dithio) carbonyl chloride (654 mg, 3.00 mmol), DIPEA (390 mg, 3.00 mmol); SiO_2 purification with 1:3 ethyl acetate: hexanes (v/v), $R_f = 0.31$. Characterization data for FLD-1: ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.09 (d, $J = 5.0$ Hz, 1H), 7.85 (t, $J = 5.0$ Hz, 1H), 7.79 (t, $J = 5.0$ Hz, 1H), 7.38 (m, 13H), 7.01 (d, $J = 10.0$ Hz, 2H), 6.95 (d, $J = 10.0$ Hz, 2H), 4.18 (s, 4H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.8, 167.8, 152.6, 151.2, 136.5, 131.1, 130.1, 130.0, 129.0, 128.2, 125.7, 125.6, 124.6, 118.4, 117.5, 110.6, 81.1, 42.4. IR (cm^{-1}):

2981, 1744, 1608, 1408, 1420, 1237, 1143, 1107, 1060, 988, 881, 751. HRMS m/z $[M+H]^+$ calcd. For $[C_{36}H_{25}O_7S_4]^+$ 697.0483; found 697.0474.

Reaction scale for FLD-3: 3-*O*-methylfluorescein (69.0 mg, 0.207 mmol), ((benzyl)dithio)carbonyl chloride (136 mg, 0.623 mmol), DIPEA (81.0 mg, 0.623 mmol); SiO_2 purification with 1:1 ethyl acetate: hexanes (v/v) $R_f = 0.64$. Characterization data for FLD-3: ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.06 (d, $J = 10.0$ Hz, 1H), 7.83 (t, $J = 5.0$ Hz, 1H), 7.77 (t, $J = 5.0$ Hz, 1H), 7.36 (m, 7H), 6.98 (d, $J = 10.0$ Hz, 2H), 6.91 (d, $J = 10.0$ Hz, 2H), 6.77 (d, $J = 10.0$ Hz, 1H), 6.73 (d, $J = 10.0$ Hz, 1H), 4.18 (s, 2H), 3.84 (s, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.9, 167.8, 161.7, 152.7, 152.4, 151.9, 151.5, 136.5, 136.4, 130.9, 130.1, 130.0, 129.5, 129.0, 128.2, 126.1, 125.4, 124.5, 118.0, 117.8, 113.0, 110.9, 110.4, 101.3, 81.9, 56.2, 42.4. IR (cm^{-1}): 2981, 1747, 1607, 1491, 1420, 1241, 1220, 1144, 1103, 1060, 986, 874. HRMS m/z $[M+H]^+$ calcd. For $[C_{29}H_{21}O_6S_2]^+$ 529.0780; found 529.0779.

2.4 Donor usage in vitro

Both FLD donors react with thiols to release COS, which is subsequently converted to H_2S by CA. For simplicity, Cys can be used to verify donor activity due to the higher solubility of Cys in buffer than GSH. As an example of the reactivity of FLD-1, incubation of 10 μM of FLD-1 with 100 μM Cys in PBS buffer (pH 7.4, 10 mM) containing physiologically-relevant concentrations of CA (25 μg/mL) results in a significant fluorescent response as shown in Fig. 1A. Over the course of 2 h under these reaction conditions, a ~500-fold increase in fluorescence intensity is observed for FLD-1. The release rate from the FLD compounds is dependent on both the donor and thiol concentration. As an example of this dependence, Fig. 1B shows the Cys concentration dependence of the fluorescence response from FLD-1. Using conditions of 200 μM Cys and 10 μM FLD-1, the activation of the donor is complete within 20 min.

Both FLD-1 and FLD-3 become fluorescent when activated by thiols to release COS/H_2S, but the magnitude of this fluorescence response is different. FLD-1, which releases 2 equivalents of COS/H_2S, shows a more gradual fluorescence response because the intermediate in which 1 equivalent of COS/H_2S has been released is moderately fluorescent. By contrast, FLD-3 shows a faster fluorescence response, although a smaller dynamic range (Fig. 2).

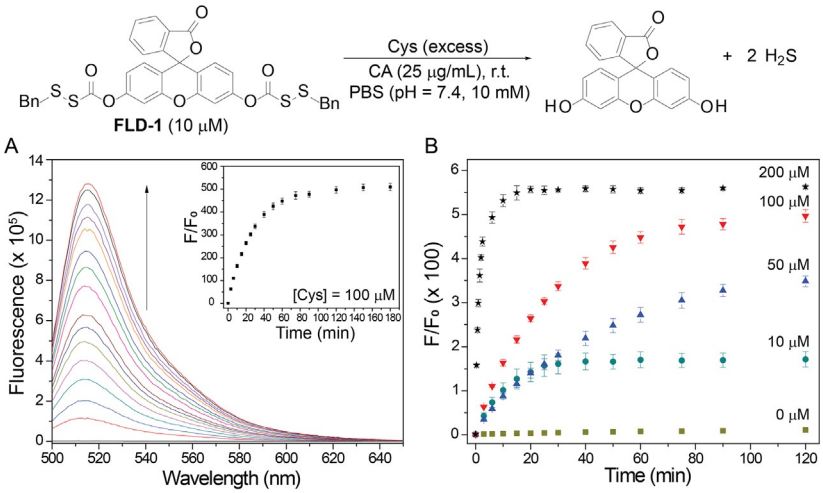


Fig. 1 (A) Fluorescence response of FLD-1 (10 μM) in PBS (pH 7.4, 10 mM) containing Cys (100 μM) and CA (25 μg/mL). (B) Cys-dependent (0–200 μM) fluorescence turn on of FLD-1 (10 μM) in PBS. General fluorescence acquisition parameters: $\lambda_{ex} = 490$ nm, $\lambda_{em} = 500$–650 nm. The data shown is the average of three replicates and errors are shown as mean ± SD ($n = 3$). *Modified from Zhao, Y., Cerda, M. M., & Pluth, M. D. (2019). Fluorogenic hydrogen sulfide (H_2S) donors based on sulfenyl thiocarbonates enable H_2S tracking and quantification.* Chemical Science, 10*(6), 1873–1878 with permission from the R.S.C.*

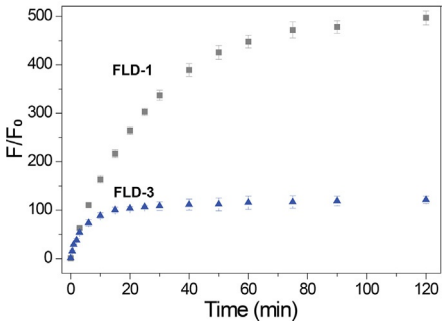


Fig. 2 Fluorescence turn on of FLD-1 (gray) and FLD-3 (blue). General conditions: 10 μM FLD in PBS (pH 7.4, 10 mM) with Cys (100 μM). $\lambda_{ex} = 490$ nm for FLD-1, $\lambda_{ex} = 454$ nm for FLD-3, $\lambda_{em} = 500$–650 nm. The data shown is the average of three replicates and errors are shown as mean ± SD (n = 3). *Modified from Zhao, Y., Cerda, M. M., & Pluth, M. D. (2019). Fluorogenic hydrogen sulfide (H_2S) donors based on sulfenyl thiocarbonates enable H_2S tracking and quantification.* Chemical Science, 10*(6), 1873–1878 with permission from the R.S.C.*

2.5 Correlating fluorescence signal to H_2S release

The H_2S release from the fluorescent FLD compounds can be readily measured and quantified using the methylene blue (MB) assay. This method allows for trapping of released H_2S to form the methylene blue dye, which can then be quantified by UV–vis spectroscopy. Incubation of FLD-3 (10 μM) with Cys (100 μM) resulted in a rapid fluorescence response with 96% of the expected H_2S release measured by the MB assay. In the absence of CA, negligible H_2S was detected, which is consistent with the intermediate formation of COS, which requires CA to be converted to H_2S (Fig. 3A). Using FLD-3, the fluorescence response can be monitored and plotted against H_2S measurements from the MB assay under the same conditions (Fig. 3B). These experiments show a linear correlation between the fluorescence response and quantified H_2S, which allows for the fluorescence response to be correlated directly with H_2S concentrations. Note that because all fluorimeters will have different sensitivities, this calibration curve should be repeated for each instrument system used to ensure accurate correlations from fluorescence to H_2S concentrations.

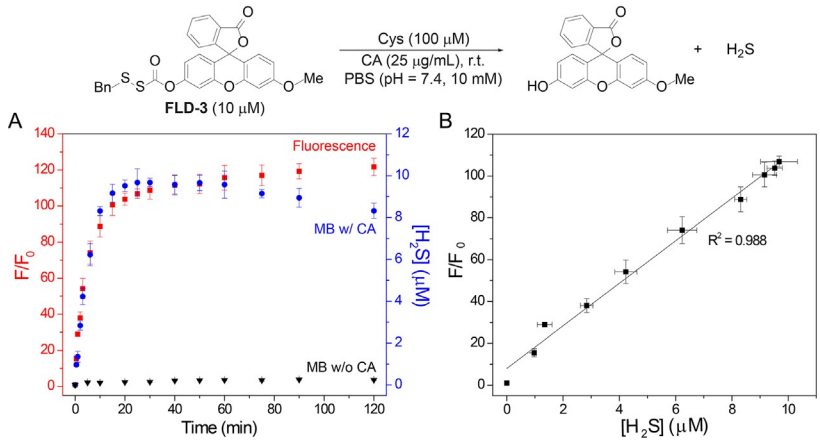


Fig. 3 Fluorescence response (red) and H_2S release (blue) after treatment of FLD-3 (10 μM) in PBS (pH 7.4, 10 mM) with Cys (100 μM) and CA (25 μg/mL). No H_2S was detected in the absence of CA (black). $\lambda_{ex} = 454$ nm, $\lambda_{em} = 500$–650 nm. (B) Correlation between quantified H_2S released and fluorescence response. The data shown is the average of three replicates and errors are shown as mean ± SD ($n = 3$). *Modified from Zhao, Y., Cerda, M. M., & Pluth, M. D. (2019). Fluorogenic hydrogen sulfide (H_2S) donors based on sulfenyl thiocarbonates enable H_2S tracking and quantification.* Chemical Science, 10*(6), 1873–1878 with permission from the R.S.C.*

2.6 Materials, equipment, and reagents

- Carbonic anhydrase (from bovine erythrocytes, CAS# 9001-03-0; EC# 4.2.1.1)
- Quartz fluorescent cuvette
- Disposable plastic cuvettes
- Fluorimeter
- UV–vis spectrophotometer
- Cysteine
- Zinc acetate ($Zn(OAc)_2$ dihydrate; CAS# 5970-45-6)
- Iron trichloride ($FeCl_3$ hexahydrate; CAS# 10025-77-1)
- Hydrochloric acid (HCl)
- *N,N*-dimethyl-*p*-phenylene diamine (HCl salt, CAS# 536-46-9)
- Sodium hydrosulfide (NaSH, CAS# 16721-80-5)
- Dimethyl sulfoxide (DMSO)
- PBS buffer (pH 7.4, 10 mM PBS)

2.7 Measurement of fluorescence intensity of FLD donors

1. Prepare a 10.0 mM stock solution of the desired FLD donor in DMSO.
2. Add 3.00 μL of the stock solution to 3.00 mL of PBS (pH 7.4, 10 mM) containing carbonic anhydrase (25.0 μg/mL) in a quartz cuvette.
3. Prepare a 10.0 mM stock solution of cysteine in PBS buffer.
4. Measure the background fluorescence spectrum of the FLD donor ($\lambda_{ex} = 454$ or 490 nm depending on the donor; $\lambda_{scan} = 500$–650 nm).
5. Add 30 μL of the cysteine stock solution to the cuvette.
6. Repeat fluorescence measurements of the sample after the addition of cysteine until the reaction is complete (~120 min).
7. Integrate the fluorescence signal from 500 to 650 nm and plot as a function of time.

2.8 Measurement of H_2S release from FLD donors

1. Prepare a 10 mM stock solution of the FLD donor in DMSO and add 20.0 μL of the stock solution to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 μg/mL) in a 20 mL scintillation vial.
2. Prepare a 100 mM cysteine stock solution in PBS buffer.
3. Prepare stock solutions for the methylene blue (MB) analysis. These stock solutions include 1.00% (w/v) $Zn(OAc)_2$, 30.0 mM $FeCl_3$ in 1.20 M HCl, and 20.0 mM *N,N*-dimethyl-*p*-phenylene diamine in 7.20 M HCl.

4. Remove a 300 μL aliquot from the FLD solution as a time = 0 point, and add this to a disposable plastic UV–vis cuvette containing 300 μL of MB cocktail (60.0 μL $Zn(OAc)_2$ (1.00% w/v), 120 μL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 120 μL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)).
5. Add 20.0 μL of the cysteine stock solution to the FLD donor solution in the scintillation vial.
6. Remove a 300 μL aliquot from the reaction mixture and add it to a disposable plastic UV–vis cuvette containing 300 μL of MB cocktail (60.0 μL $Zn(OAc)_2$ (1.00% w/v), 120 μL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 120 μL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)) at different time points.
7. Allow each aliquot to equilibrate for 1 h, and then measure the absorbance value at 670 nm.
8. Plot the absorbance values at 670 nm as a function of time. To convert this data to quantitative H_2S levels, prepare an H_2S calibration curve (described below).

2.9 Prepare an H_2S calibration curve

1. Add 500 μL of the MB cocktail (100 μL $Zn(OAc)_2$ (1.00% w/v), 200 μL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 200 μL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)) and 500 μL of PBS (pH 7.4, 10 mM) to a 1.5 mL disposable cuvettes.
2. Prepare a 1.00 mM NaSH stock solution in PBS buffer.
3. Add aliquots of the 1.00 mM NaSH stock solution to the cuvettes containing the MB cocktail to result in final concentration of 1, 3, 5, 10, 15, and 20 μM sulfide.
4. Incubate the cuvettes for 1 h prior to measuring the absorbance at 670 nm.
5. Plot the absorbance as a function of sulfide concentration. The resultant plot should produce a straight line, which can be used as a calibration curve for quantitation experiments.

3. Probe usage and application in cell models

The FLD donor compounds can also be used to deliver H_2S in live cell environments after activation by endogenous thiols. As an example of this activity, treatment of HeLa cells with FLD-1 results in donor internalization and release of COS/H_2S by endogenous thiols and CA. As shown in

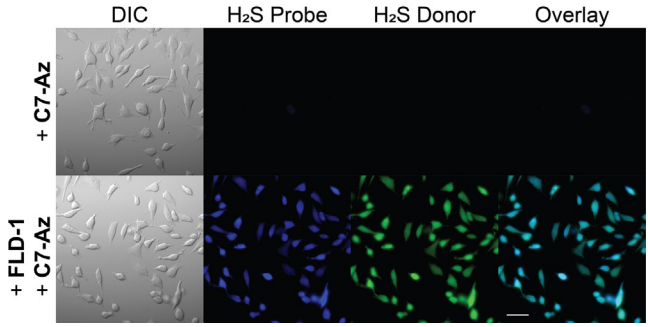


Fig. 4 H_2S delivery from FLD-1 in HeLa cells. HeLa cells were treated with the H_2S-responsive probe C7-Az (50 μM) in DMEM only (top row) or DMEM containing FLD-1 (50 μM) (bottom row) for 30 min. Cells were then washed with PBS and cell images were taken in PBS using a fluorescent microscope. Bar scale: 50 μm. *Modified from Zhao, Y., Cerda, M. M., & Pluth, M. D. (2019). Fluorogenic hydrogen sulfide (H_2S) donors based on sulfenyl thiocarbonates enable H_2S tracking and quantification.* Chemical Science, 10*(6), 1873–1878 with permission from the R.S.C.*

Fig. 4, donor activation results in an increase in fluorescence in the green channel due to liberation of fluorescein after donor activation. The released H_2S can be visualized by using a reaction-based H_2S responsive probe that does not overlap with the FLD-1 emission spectrum. Co-incubation with the commercially available, H_2S-responsive 7-azido-4-methylcoumarin (C7-Az) fluorescent probe results in C7-Az activation, confirming H_2S release from FLD-1 (Fig. 4). In the absence of FLD-1, minimal background fluorescence from C7-Az is observed.

A second example of cell-based applications of the FLD system is blocking NO formation in Raw 264.7 macrophage cells treated with lipopolysaccharide (LPS). Prior reports have demonstrated that H_2S can reduce inflammatory responses and decrease NO levels in LPS-stimulated macrophage cells, and this assay has been used previously to investigate anti-inflammatory response of H_2S-releasing compounds (Whiteman et al., 2010). To measure the impacts of FLD-1 on NO formation, cultured Raw 264.7 macrophage cells were treated with FLD-1 (0–25 μM) for 2 h followed by LPS (0.5 μg/mL) administration and further incubation 24 h. After this incubation period, accumulated nitrate (NO_2^-), which is the downstream oxidation product of NO, was measured using a commercially available Griess assay kit. From these experiments, a dose-dependent decrease in NO_2^- formation was observed, which is consistent with both H_2S release from FLD-1 under these experimental conditions and also anti-inflammatory activity of the FLD donor (Fig. 5).

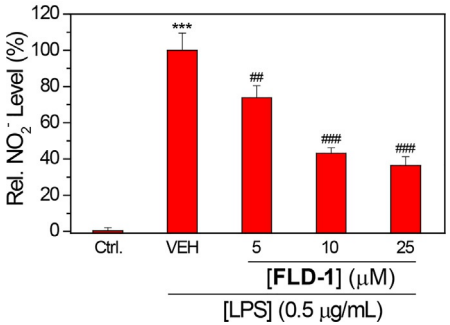


Fig. 5 Effects of FLD-1 on LPS-induced NO_2^- accumulation. Raw 264.7 cells were pretreated with FLD-1 (0–25 μM) for 2 h, followed by a 24-h treatment of LPS (0.5 μg/mL). Results are expressed as mean ± SD ($n = 4$). *** $P < 0.001$ vs the control group; ## $P < 0.01$ vs vehicle-treated group; ### $P < 0.001$ vs vehicle-treated group. *Modified from Zhao, Y., Cerda, M. M., & Pluth, M. D. (2019). Fluorogenic hydrogen sulfide (H_2S) donors based on sulfenyl thiocarbonates enable H_2S tracking and quantification.* Chemical Science, 10*(6), 1873–1878 with permission from the R.S.C.*

3.1 Materials

- HeLa cells (ATCC: CCL-2).
- Raw 264.7 cells (ATCC: TIB-71).
- Dulbecco's modified Eagle's medium.
- Fetal bovine serum (FBS).
- Penicillin.
- Streptomycin.
- Cell incubator with 5% CO_2.
- Poly-D-lysine coated plates (MatTek).
- 7-Azido-4-methylcoumarin (C7-Az); commercially available from Santa Cruz Biotech. or readily prepared by previously published methods (Thorson, Majtan, Kraus, & Barrios, 2013).
- Inverted fluorescence microscope with filter cubes for blue (DAPI) and green (GPF, FITC) channels.
- Lipopolysaccharide (LPS).
- Griess nitrite assay kit for nitrite quantification (ThermoFisher, G7921).

3.2 Procedure

3.2.1 Cell culture

1. Culture HeLa cells (ATCC) in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2.

2. Plate HeLa cells in poly-D-lysine coated plates (MatTek) containing 2.00 mL of DMEM and incubated at 37 °C under 5% CO_2 for 24 h.
3. Wash the cells with PBS and then add C7-Az (50.0 μM) and FLD (50.0 μM) and incubate for 30 min.
4. Wash the cells with PBS and bathe in 2.0 mL of PBS for imaging.
5. Collect fluorescence images for using an inverted fluorescence microscope. The excitation and emission profiles for C7-Az ($\lambda_{ex} = 365$ nm; $\lambda_{em} = 400$–550 nm) and FLD-1 ($\lambda_{ex} = 490$ nm; $\lambda_{em} = 500$–650 nm) match common filter sets used for blue/DAPI and green/GFP, respectively.

3.3 Anti-inflammatory activities of FLD-1 and control compounds

1. Seed Raw 264.7 macrophage cells (ATTC) in a 24-well plate (5×10^5 cells/well) containing 500 μL of DMEM and incubate at 37 °C under 5% CO_2 for 24 h.
2. Wash the cells with PBS and incubate with the FLD donor (0–25.0 μM) at 37 °C for 2 h.
3. Wash the cells with PBS to remove excess FLD donor.
4. Incubate the FLD-treated cells with FBS-free DMEM containing LPS (0.500 μg/mL) for 24 h.
5. Measure NO_2^- levels were measured by using a Griess Reagent Kit.

4. Concluding remarks

The delivery of small molecules from donor platforms provides a key tool for perturbing levels of biological analytes in complex systems. By being able to monitor the release in real time using non-invasive spectroscopic techniques, researchers now have an additional methods monitor experiments under different conditions. In addition to the FLD-1 and FLD-3 donors described in this chapter, additional optically-responsive H_2S-releasing donors have recently been reported. Select examples include colorimetric donors (Zhao et al., 2018a), photoactivatable fluorescent donors (Venkatesh et al., 2018), and other fluorescent donor motifs (Hu et al., 2019; Kim et al., 2019). In addition, fluorescent donors are also available for NO donors (Hibbard & Reynolds, 2019; Ravikumar, Bagheri, Saini, & Chakrapani, 2017) and CO donors (De La Cruz et al., 2018; Popova, Soboleva, Ayad, Benninghoff, & Berreau, 2018), which provide a diverse palette for monitoring the release of these important small gaseous signaling molecules in complex environments.

Acknowledgments

This work was supported by the NIH (R01GM113030) and Dreyfus Foundation. NMR, fluorescence microscopy, and MS instrumentation in the UO CAMCOR facility that enabled this work is supported by the NSF (CHE-1427987, CHE-1531189, and CHE-1625529).

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