Chapter 2

Chemical Tools for Studying Biological Hydrogen Sulfide

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Although hydrogen sulfide (H_2S) has historically been recognized as a toxic gas, recent studies have established H_2S as an important, endogenously-produced signaling molecule active in a wide array of (patho)physiological roles in biological systems. As the multifaceted biological roles of H_2S continue to emerge, tools to modulate, measure, and detect biological H_2S are needed. Here we highlight recent biocompatible advances in reaction-based H_2S detection methods and their associated benefits and pitfalls.

Biological Relevance of Hydrogen Sulfide

The study of signal-transducing gasotransmitters has evolved over the past twenty years based on the discovery that gaseous molecules can be both biorelevant and be produced endogenously. After the discovery in 1987 that biosynthetic nitric oxide (NO) was the endothelium-derived relaxing factor (EDRF) (*1*), two other endogenous gases, namely carbon monoxide (CO) and hydrogen sulfide (H_2S) (*2*, *3*), have garnered interest in the biomedical community. Hydrogen sulfide has emerged as the most recent biosynthetic gasotransmitter and is now accepted as an important signaling molecule with prominent (patho)physiological roles (*4–6*). Despite this interest, real-time detection methods compatible with biological systems are only beginning to emerge. As the field of H_2S detection progresses, development and implementation of new,

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sensitive, and robust H_2S detection and quantification methods are likely to greatly impact our current understanding of the basic science of biological H_2S as well as open avenues toward diagnostic techniques for different (patho)physiological conditions. Reaction-based methods of H_2S detection, which have emerged rapidly in the last two years, offer the first-generation solutions to this important problem. As the multifaceted biological roles of H_2S continue to emerge, the need for tools that modulate, measure, and detect its presence is paramount. To address these needs, chemists have devised a variety of H_2S delivery mechanisms using small molecule compounds that release H_2S at controlled rates, thereby mimicking enzymatic H_2S production more closely than adding exogenous sulfide sources such as H_2S, SH^-, or S^{2-} directly. Similarly, new biocompatible reaction-based chemical methods are emerging to detect and quantify H_2S. This review will focus on emerging strategies for H_2S detection, highlighting different sensing strategies as well as their associated benefits and pitfalls.

Hydrogen sulfide, much like NO and CO, meets the requirements of a gasotransmitter. It is a small, gaseous molecule that is produced enzymatically, and its production and metabolism are tightly regulated. Like NO, H_2S is readily oxidized, thus disfavoring long-range transport under normoxic conditions, and also suggesting the need for endogenous storage mechanisms, such as the formation of thiol hydropersulfides (RS-SH), which constitute a direct parallel to NO storage as nitrosothiols (RS-NO). Hydrogen sulfide is a weak acid (pK_{a1}: 6.76, pK_{a2}: 19.6) that exists primarily as SH^- (82%) rather than H_2S (18%) or S^{2-} (< 0.1%) under physiological conditions. This hydrosulfide anion is a more potent nucleophile than Cys or reduced glutathione (GSH) under physiological conditions due to the higher pK_a of these endogenous thiols by comparison to H_2S. Furthermore, the diprotic nature of H_2S allows for modulation between H_2S and SH^-, thus allowing for modulation of the water solubility, lipophilicity, and redox potential based on the local cellular environment.

Hydrogen-sulfide-generating enzymes produce the majority of H_2S in mammalian cells, although non-enzymatic H_2S production is also possible. Enzymes involved in transsulfuration pathways, such as cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), are the main H_2S-producing enzymes. Additionally, 3-mercaptopyruvate sulfurtransferase (3-MST) has recently been identified as an H_2S-generating enzyme in the mitochondria (Figure 1). Production of H_2S from CBS primarily arises from conversion of L-cysteine (Cys) to L-serine with concomitant release of H_2S. Similarly, CSE can also convert L-cysteine to H_2S directly. Alternatively, CSE reacts with L-cystine to generate thiocysteine, which, upon further reaction with a thiol, generates H_2S. CBS and CSE can also work in concert; for example, CBS-mediated condensation of homocysteine (Hcy) and L-serine forms L-cystathionine, which is a substrate for subsequent CSE-mediated H_2S production. In addition to CSE and CBS, 3-MST converts 3-mercaptopyruvate, which is generated from L-cysteine by cysteine aminotransferase (CAT), to H_2S. Non-enzymatic H_2S production pathways include the conversion of thiosulfate to H_2S under reducing conditions, typically by GSH, with concomitant formation of sulfate and oxidation of the thiol reducing agent to the corresponding disulfide. Once generated, H_2S can react with a variety of organic and inorganic biological targets, including heme irons, thiols, and other

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reactive oxygen/nitrogen species. Although the basic H_2S-producing pathways are known, the exact intercellular interplay between these enzymes, as well as crosstalk with other signaling molecules, remains an emerging arena.

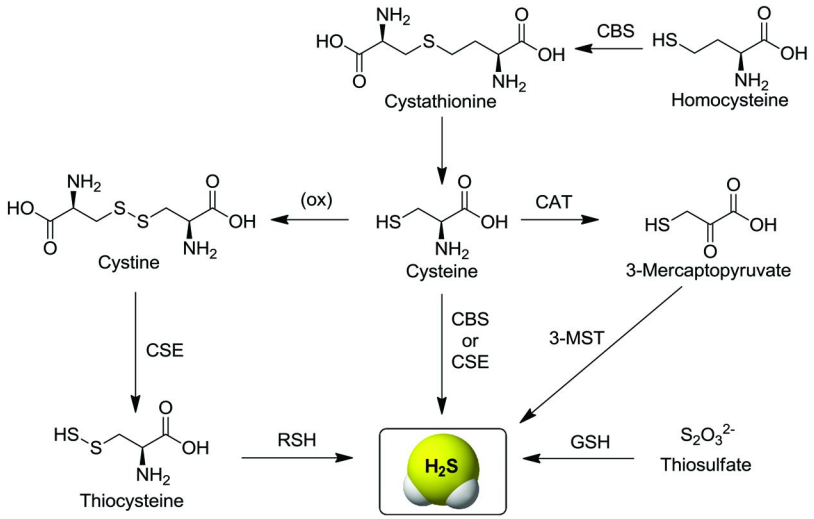

Figure 1. Biosynthetic pathways for H_2S formation in mammalian cells. CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; 3-MST: 3-mercaptopyruvate sulfurtransferase; CAT: cysteine aminotransferase.

In addition to H_2S generation, storage of biological H_2S is an important, yet still poorly understood, aspect of H_2S homeostasis. Drawing parallels to other important bioinorganic analytes that exist in both free and bound pools, such as NO and Zn(II) (*7*), different H_2S storage mechanisms likely play important roles in releasing H_2S under different physiological conditions. For example, iron-sulfur clusters can be a source of acid-labile H_2S, although release of H_2S is only efficient under acidic conditions. Although these conditions are significantly removed from normal physiological pH, such acidities are accessible in different cellular locales, such as lysosomes. A likely more important pool of stored biological H_2S is sulfane-bound sulfur, resulting from reaction of H_2S with a thiol under oxidizing conditions (*8*, *9*). Such sulfane-bound sulfur sources include hydropersulfides (RS-SH) and polysulfides ($RS\text{-}S_{(n>1)}\text{-}R$), which release H_2S under reducing conditions or after transulfurization reactions with other reduced thiols. A variety of stored sulfur pools are likely required for ensuring H_2S homeostasis, but the release of biologically-stored sulfur from these sources complicates H_2S detection. For example, many classical methods of H_2S measurement require sample acidification or disruption of the pre-established redox balance prior to analysis (*vide infra*).

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Emerging biological and physiological roles of H_2S clearly establish that H_2S plays important and multifaceted roles in the cardiovascular, nervous, endocrine, and immune systems (*6*). One major challenge in establishing and understanding such roles is that the physiological response to H_2S is often dependent upon the method of H_2S administration or modulation. Despite these complications, the role of H_2S has been established in numerous biological processes. For example, H_2S functions as a vasorelaxant in the cardiovascular system with EC_{50} levels for induced vasorelaxation that correlate well with measured plasma H_2S levels (*10*), although the detection limit of the H_2S measurement method used in these studies has subsequently been revised (*11*). Additionally, high expression levels of CBS in the hippocampus and cerebellum, as well as the interaction with *N*-methyl-D-aspartate (NMDA) receptors, suggest important roles for H_2S in the central nervous system (CNS) in the modulation of neurotransmission and long term potentiation (LTP) (*12*, *13*). Furthermore, the existence of H_2S has been implicated in the endocrine system by influencing glucose metabolism homeostasis in islets through action on K_{ATP} channels in beta cells (*14*). Hydrogen sulfide plays important function in the immune system, displaying both pro- and anti-inflammatory effects depending on the mode and concentration of H_2S administration (*15*–*18*). Taken together, H_2S clearly plays diverse and important roles in various physiological systems. Although a complete description of the diverse biological roles of H_2S is beyond the scope of this review, the interested reader is referred to a recent, comprehensive summary of H_2S in biology (*6*). As the field continues to grow, revision and refinement of many of the current paradigms is likely as better tools for selectively delivering and measuring biological H_2S levels continue to emerge.

H_2S Detection Strategies

Classical instrumental methods of H_2S quantification include gas chromatography (GC), polarography, and sulfide-selective electrodes (*19*–*22*). For these techniques, samples are typically homogenized, and either the resultant solution or the gaseous headspace is analyzed. Polarographic and GC methods detect H_2S gas released from the solution and therefore require accurate pH measurements to correct for H_2S speciation under physiological conditions. Based on what is now known about acid-labile endogenous sulfur pools, such sample acidification may result in releasing bound sulfur, thereby resulting in total, rather than free, sulfide measurements. Most sulfide-selective electrodes also require sample homogenization followed by treatment of a sulfide antioxidant buffer containing sodium salicylate, ascorbic acid, and sodium hydroxide. Because the electrodes only measure S^{2-}, the least prevalent species in the H_2S acid-base equilibria, sulfide-sensitive electrodes are quite sensitive to small changes in sample pH. Additionally, commonly-used additives contain redox-active species, thereby increasing the possibility of perturbing the redox homeostasis of the sample and allowing for either release of or additional storage by sulfane-bound sulfur. Driven by the drawbacks of the instrumental methods of H_2S detection and quantification, biocompatible chemical analyses for H_2S are beginning to emerge.

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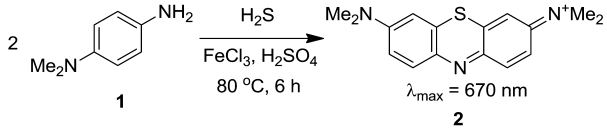
One of the most commonly-used chemical methods for H_2S quantification is the methylene blue assay (Scheme 1) (*23*, *24*). In this assay, H_2S from the desired sample is typically trapped initially with $Zn(OAc)_2$ to form ZnS. Sample acidification releases the trapped H_2S, and heating with *N,N*-dimethyl-*p*-phenylenediamine (**1**) and addition of $FeCl_3$ generates the methylene blue dye (**2**). After removal of precipitated proteins by centrifugation, the characteristic methylene blue absorbance at 670 nm is then measured and compared to a background sample and calibration curve. Despite the wide use of this quantification method, the revised detection limit (2 μM) is much less sensitive than the initially indicated detection limit (~10 nM) (*11*). Furthermore, although the methylene blue method has been used to detect and measure endogenously-produced H_2S, recent studies have demonstrated its inability to detect differential H_2S levels in mice deficient in H_2S-producing enzymes. Taking these limitations into account, many of the measured levels of H_2S may soon come under increased scrutiny as new, improved methods for H_2S measurement are developed.



*Scheme 1. Formation of methylene blue (**2**) to trap H_2S*

Common features of the H_2S detection and quantification methods described above include the requirement of sample homogenization and/or acidification prior to analysis. Such pre-treatments, as well as the time required to perform them, complicates the actual H_2S levels reported. Because H_2S is tightly regulated, lengthy or caustic workups, which often require pH changes, or incubation with metal salts, likely remove sulfur from other endogenous stores such as persulfides or iron sulfur clusters, thereby complicating detection and quantification. Such considerations highlight the highly controversial reported levels of H_2S in different tissues or cellular locales, partially because it is unclear whether free or total sulfide levels are being measured (*25*). To address the need for contemporary H_2S detection methods compatible with living tissues, numerous research groups, including our group, are investigating new methods of H_2S detection and quantification by utilizing the innate chemical reactivity of H_2S.

Although H_2S is often grouped with NO and CO as a gasotransmitter, its chemical reactivity is much different than either of these important gasses. Hydrogen sulfide is a weak reductant and is a weaker reducing agent than Cys or GSH (*26*). Similarly, H_2S, particularly SH^-, is a potent nucleophile able to react with electrophilic targets. Notably, the solubility of many metal-sulfur compounds is quite low, resulting in the use metal-sulfide precipitation as a classical gravimetric method for H_2S-mediated metal determination. These three properties have been exploited by chemists in the last few years to generate

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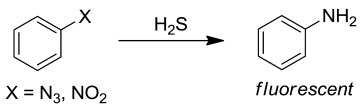
new detection methods for H_2S based on utilization of its physical and reactive properties. Consequently, reaction-based methods of H_2S detection have focused on exploiting the physical properties of H_2S to result in chemical sensing. These strategies can be broken down into three categories: (1) H_2S as a reductant, (2) H_2S as a nucleophile, and (3) H_2S as a metal precipitant. Recent advances using these strategies for H_2S detection are highlighted below.

H_2S Detection Methods Based on Chemical Reduction

Thiols play an important role in cellular redox chemistry, with GSH playing a central role in maintaining cellular redox homeostasis. Drawing parallels to biologically-relevant thiols, H_2S is also a reducing agent, although its reduction potential is lower than GSH or Cys (*26*). Although the reduction of azide and nitro groups by H_2S has been known for over 30 years (*27–30*), it has only recently been applied to reaction-based chemical methods for H_2S detection (Scheme 2). Empirically, even though H_2S is a weaker reductant than GSH, it reduces azides and other oxidized nitrogen species faster than GSH or other thiols, resulting in a viable H_2S detection method. This reduction-based strategy has emerged as a general method for H_2S detection, with various fluorescent probes being reported in the last two years. The selectivity of these probes for H_2S over GSH is typically moderate to good, although variable conditions and concentrations under which the different probes were evaluated make direct comparison difficult. For example, although cellular levels of GSH are typically in the millimolar concentration range, many of the reported azide-based H_2S probes only test micromolar levels of GSH to compare with H_2S, thereby limiting the relevance to typical biological conditions. Despite these limitations, a palette of different excitation and emission wavelengths are possible with currently developed probes, ranging from standard blue, green, yellow, and red channels (Figure 2). One important note on reduction-based probes is that the reaction product from H_2S- and thiol-mediated reduction is identical, thus precluding accurate quantification of H_2S unless exact thiol concentrations are known for the sample of interest. Furthermore, because the reaction rates may depend on pH, ionic strength, or local protein environment, drawing definitive quantitative conclusions remains difficult. Although many of the developed probes demonstrate a highly-linear response to H_2S, caution should be taken when using these probes to quantify H_2S unless adequate control experiments are performed. Recent advances in reduction-based probes for H_2S will be outlined below, although not all probe features are discussed in depth.



Scheme 2. Reduction of an oxidized amine with H_2S regenerates the parent amine. Translation of this detection mechanism to fluorophores with fluorogenic amines provides a viable strategy for H_2S detection.

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A flurry of fluorescent probes using H_2S-mediated azide reduction as the turn-on mechanism were reported in late 2011 and early 2012 by a variety of research groups. For example, Chang and co-workers initially reported functionalized rhodamine azides SF1 (**3**) and SF2 (**4**) in which reduction of the azide to the parent amine resulted in regeneration of the rhodamine platform and concomitant fluorescence turn-on (*31*). When tested in buffer, SF1 and SF2 demonstrated a 6-8 fold turn-on with H_2S after 60 min with 2-5 fold selectivity over other reactive sulfur, oxygen, and nitrogen species (RSONS). Furthermore, both SF1 and SF2 were used to detect exogenous H_2S in HEK293T cells (*31*). A diazido rhodamine-based H_2S sensing platform was later reported by Chang and co-workers to generate SF4, and SF5-AM to SF7-AM (**5-8**), which feature different ester functionalization to impart cell trappability (*32*). These later probes offer higher fluorescence turn-on than that of SF1 and SF2; for example SF7-AM results in a 20-fold fluorescence turn-on after 60 min with 8-10 fold selectivity over other RSONS. SF-7-AM was used to detect endogenous H_2S produced in human umbilical vein endothelial cells treated with vascular endothelium growth factor (VEGF), establishing a role for cellular crosstalk between H_2S and H_2O_2 (*32*).

Azide reduction has also been used for ratiometric H_2S detection as reported by Han and co-workers (*33*). Using the infrared chromophore cyanine to generate Cy-N_3 (**9**), which upon reduction to the parent Cy-NH_2 amine by H_2S generates new absorption and emission bands centered at 660 nm and 750 nm, respectively. Ratiometric H_2S detection based on the fluorescent signals of the Cy-N_3 and Cy-NH_2 signals (F_{750}/F_{710}) resulted in a ratio of 2.0 for H_2S and signals of less than 0.7 for other RSONS. The response of Cy-N_3 probe for both H_2S and ADT-OH, a common organic-based H_2S donor, was also demonstrated. Additionally, the Cy-N_3 probe was used to image exogenous H_2S in Raw 264.7 cells using ratiometric imaging (*33*).

Our group reported the naphthalimide-based azide HSN-2 (**11**) that reacts with H_2S to afford the parent naphthalimide amine (*34*). This sensing scaffold benefits from a large Stokes shift of the naphthalimide dye and a large fluorescence turn-on. Reaction of the azide-based HSN-2 with H_2S results in a 60-fold turn-on while maintaining high selectivity at large (2,000-fold, 10 mM) excesses of Cys and GSH. In addition to azide reduction, H_2S-reduction of NO_2 groups was demonstrated for H_2S detection in HSN-1 (**10**). Although HSN-1 is selective for H_2S over equimolar RSONS, the selectivity eroded when challenged with high (10 mM) GSH concentrations (*34*). Additionally, H_2S-mediated reduction of the azide is faster than reduction of the nitro group. Nevertheless, both HSN-1 and HSN-2 were demonstrated to detect exogenous H_2S in live HeLa cells.

Use of naphthalimide-based azide and nitro compounds for H_2S detection has been further elaborated by the Zhang and Wu groups, which have reported micelle-based and carbon-dot-based probes, respectively. By appending hydrophobic groups to the diimide of the naphthalimide azide scaffold, the highly-hydrophobic probes could be encapsulated in CTAB micelles, which were used to detect H_2S in buffer and in fetal bovine serum (FBS) (*35*). Alternatively, appending a naphthalimide azide to carbon dots allows for coupling of the naphthalimide and carbon dot absorptions/emission profiles, resulting

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in ratiometric H_2S detection (*36*). The resultant scaffold was used to detect exogenous H_2S in HeLa and L929 cells. Additionally, Wang and co-workers reported a naphthalimide hydroxylamine for H_2S sensing, since hydroxylamines are proposed intermediates in NO_2 group reduction (*37*). The hydroxylamine naphthalimide scaffold resulted in a ~9-fold turn-on with H_2S, a 3-fold selectivity over other reactive biological species including Cys and GSH, and was used to image exogenous H_2S in astrocyte cells.

Although the majority of azides used for H_2S detection have been aryl azides, Wang and co-workers reported the use of dansyl azide (Ds-N_3, **12**), a sulfonyl azide, for H_2S detection (*38*). The dansyl azide reacts quickly with H_2S to afford the fluorescent dansyl amine product. This probe is selective for SH^- over other anions although only moderate selectivity over low concentrations of thiols such as thiophenol, benzylthiol, or Cys is reported. The Ds-N_3 probe showed linear H_2S detection both in Tween buffer and in FBS. Furthermore, Ds-N_3 was also used to detect and quantify H_2S in mouse blood, providing concentrations (32 ± 9 μM) that agreed well with previously-determined concentrations (*38*).

Coumarin-derived azides for H_2S detection have been reported by both the Tang and Li groups. Tang reported the use of both 6- and 7-azido coumarin for H_2S detection; however, only the 7-isomer (C7-Az, **13**) is reactive toward H_2S (*39*). Subsequent DFT calculations demonstrated that the orbital density on the azide is much lower for the non-reactive 6-azidocoumarin than for the 7-isomer, thereby suggesting that differences in electronic structure may be responsible for the different reactivity. The 7-azido coumarin reacts selectively with H_2S over other anions and reactive biological species and displays a linear response to H_2S both in buffer and in FBS (*39*). The resultant probe was used to visualize exogenous H_2S in HeLa cells using two-photon laser scanning confocal imaging. Similarly, Li reported ethylamino coumarin azide (**14**) for H_2S sensing (*40*). The resultant probe demonstrated ~40-fold turn-on with H_2S but only 2-fold selectivity for H_2S over GSH. Despite this low selectivity, the resultant probe was used to detect H_2S in rabbit plasma as well as exogenous H_2S in PC-3 cells.

Other azide/nitro group reduction chromophores have also been used for H_2S detection, many of which address specific applications for common fluorescent probes. For example, Ai and co-workers reported the genetically-encoded azide in cpGFP-Tyr66*p*AzF (**15**) to allow for direct incorporation of the azide-reduction method in biomolecules (*41*). Reaction of the resultant azide with NaSH results in a 0.6-fold turn-on with H_2S and was used to detect exogenous H_2S in HeLa cells. For applications in which high water solubility is important, Hartman and co-workers prepared 8-azidopyrene-1,3,6-trisulfonic acid (N_3-PTS, **16**) which, due to the anionic sulfonate groups, maintains water solubility of ~100 mM (*42*). The trianionic probe reacts with NaSH to afford the parent amine, resulting in a linear fluorescent response to H_2S in buffer and FBS as well as moderate selectivity over other RSONS. Aliphatic azides have received significantly less attention for H_2S sensing than aryl azides, although Han and co-workers reported the use of the *o*-azidomethylbenzoyl group appended to coumarin in AzMB-Coumarin (**17**) (*43*). When treated with equimolar thiols or H_2S, AzMB-Coumarin resulted in 6-fold selectivity for H_2S over other thiols. Although this probe was demonstrated to detect exogenous H_2S in HeLa cells, very high

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probe (1 mM) and NaSH (1 mM) concentrations were required, thus limiting the potential biological applications of this method. Additionally, H_2S probes based on phenanthroimidazole (**18**) (*44*), resorufamine (**19**) (*45*), nitrobenzofurazan (**20**) (*46*), benzathiazole fluorine (**21**) (*47*), cresyl violet (**22**) (*48*), as well as constructs based on cleavage of dinitrophenyl esters (*49*) or modulation of nitrophenyl fluorescence quenching groups (*50*), have also been reported.

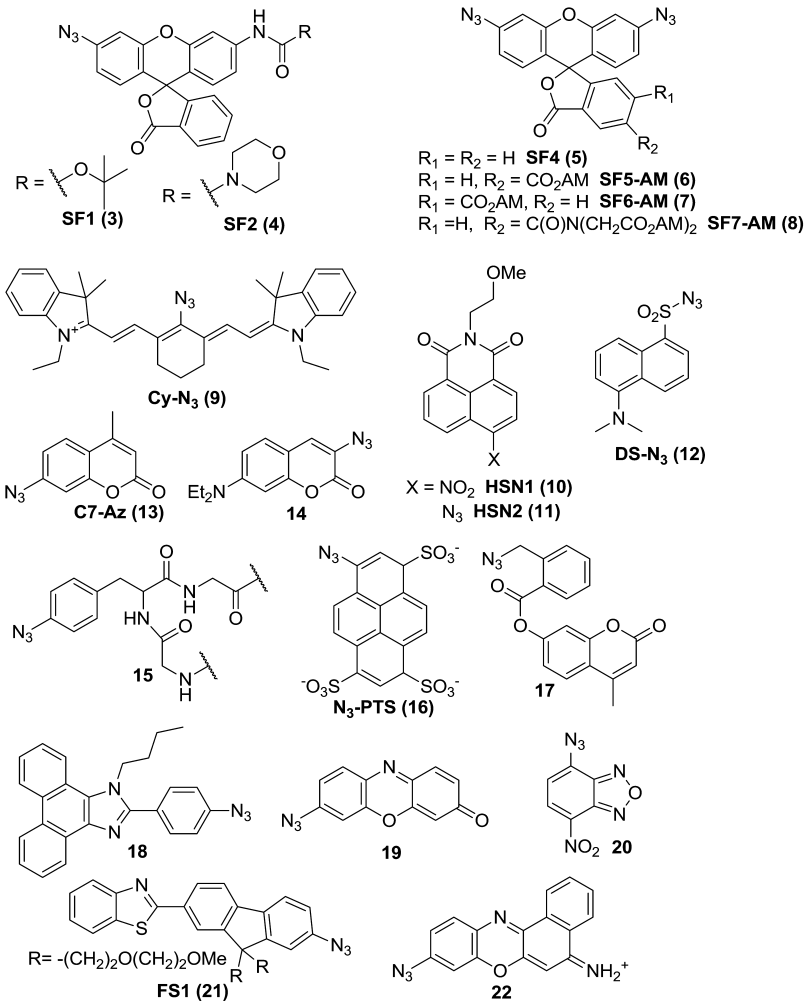

Figure 2. Highlighted reduction-based methods of H_2S detection.

Taken together, reduction-based H_2S detection methods offer a rapidly-evolving class of H_2S detection methods amenable to a wide array of fluorophores. Based on the available probes, many absorption/emission wavelengths are accessible with many probe alternatives available. One major challenge in comparing the potential biological efficacy of currently-available probes is that

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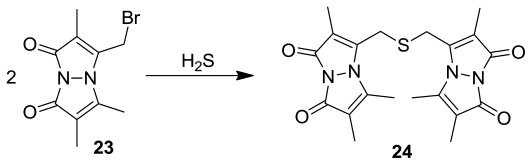
most of the probes have been investigated under significantly different conditions, including probe and analyte concentration as well as solvent/buffer composition. Similarly, because H_2S is well known to undergo redox chemistry with molecular oxygen, different procedures in handling the probe and H_2S under an ambient or inert atmosphere may influence the observed response. Although most current probes display moderate to good selectivity for H_2S over other reactive species, the fact that the final product after reaction with H_2S is identical to that generated from unwanted side reactions with thiols makes quantification difficult unless the exact thiol concentrations are known in the sample of interest. Despite these current limitations, azide/nitro-based H_2S detection methods have emerged as a viable method for observation of biological H_2S and will likely serve as a robust platform for future probe development and refinement.

H_2S Detection Methods Based on Nucleophilic Attack

In addition to its redox chemistry, H_2S is also a potent nucleophile. Hydrogen sulfide primarily exists as SH^- at physiological pH, whereas thiols are primarily in the neutral RSH form. This difference in protonation state results in increased nucleophilicity of H_2S by comparison to thiols under typical physiological conditions. The nucleophilic nature of H_2S, as well as its diprotic nature and ability to undergo two sequential nucleophilic attacks, has been exploited to both detect and quantify H_2S. For example, upon reaction with two equivalents of monobromobimane (**23**), H_2S can be trapped as the fluorescent thioether product (**24**) (*11*, *51*) (Scheme 3). For this system, separation by HPLC is required to determine H_2S content because both the H_2S-derived thioether product, as well as unwanted side reactions of monobromobimane with thiols to form bimane-SR, produce fluorescent products. Although the monobromobimane method cannot be used to detect H_2S in real-time, it has emerged as one of the preferred methods for H_2S quantification with detection limits as low as 2 nM (*11*). Recent advances in the use of H_2S as a nucleophile for H_2S detection have focused on attack by H_2S on an activated electrophile to generate a thiol followed by a second intramolecular nucleophilic attack on a second electrophile to generate a fluorescent response (Figure 3).



*Scheme 3. Monobromobimane can trap H_2S to form fluorescent bimane thioether (**24**), allowing for H_2S quantification by HPLC*

Building on the doubly-nucleophilic nature of H_2S, He and co-workers reported H_2S fluorescent probes SFP-1 (**25**) and SFP-2 (**26**), in which initial attack of H_2S on an aldehyde results in formation of a thiol that undergoes a

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second attack on an α-β-unsaturated olefin (*52*). Upon addition of H_2S to the olefin, the fluorescence quenching mechanism is abrogated, thereby resulting in fluorescence turn-on. This detection strategy was used in SFP-1 to generate a 16-fold fluorescence enhancement upon H_2S addition with a 3-4 fold selectivity over Cys and GSH. Translation of the same sensing strategy to a BODIPY scaffold generated SFP-2, which was used to detect H_2S generated from purified CBS as well as exogenous H_2S in live HeLa cells (*52*). Although the rate of fluorescence turn-on was slow and required 3-4 hours for complete reaction, increasing the electrophilicity of the olefin in second generation scaffolds resulted in a more active probe. The more highly-reactive SFP-3 (**27**) exhibited complete reaction with H_2S within 30 minutes, thereby greatly enhancing the temporal resolution of the sensing platform (*53*). This probe was further used to detect and measure H_2S levels in blood plasma and brain tissue of mice.

Similarly, electrophilic olefins have been used for H_2S detection. For example, Xian and co-workers reported two probes for H_2S detection exploiting acrylates substituted with either nitrile or ester groups to enhance their electrophilicity (**28**) (*54*). These probes rely on initial addition of H_2S to the activated olefin, followed by intramolecular attack on an ester linkage to the fluorophore. This strategy allows for fluorophore liberation after the double nucleophilic attack by H_2S and a fluorescence turn on-of ~160-fold. Additionally, these Michael acceptor olefins can react with thiol nucleophiles but may do so reversibly, thereby enhancing the selectivity of these probes for H_2S over other biological nucleophiles (*54*).

Electrophilic disulfides have also been used to generate H_2S-selective fluorescent probes. By appending such a disulfide on a fluorophore protecting group, Xian and co-workers reported the generation of an esterified methylfluorescein platform (**29**) which, upon reaction with H_2S, generated a nucleophilic bound hydropersulfide that cleaves the ester bond ligating the protecting group to the fluorophore (*55*). This strategy was selective for H_2S over equimolar Cys or GSH and was used to detect exogenous H_2S in COS7 cells. A similar disulfide exchange reaction was later used by Qian and co-workers by appending a pyridyl disulfide onto a benzathiazole platform to generate the H_2S probe E1 (**30**) (*56*). The resultant platform reacted quickly with H_2S, was selective for H_2S over Cys, Hcy, and GSH, and was used to detect exogenous H_2S in HeLa cells.

In addition to scaffolds exploiting the doubly-nucleophilic nature of H_2S highlighted above, the high nucleophilicity of H_2S has also been utilized to disrupt the π-system of a conjugated fluorophore, resulting in modulation of the fluorescent properties of the dye. For example, Guo and He reported the development of CouMC (**31**), which contains a diethylaminocoumarin fluorophore appended to an indolenium group (*57*). Nucleophilic attack by H_2S on the electrophilic carbon of the indolenium ring breaks the conjugation of the system and changes the emission characteristics of the probe. This chemistry results in a ratiometric response to H_2S with high selectivity for H_2S over other RSONS. The reaction of CouMC with H_2S occurs within minutes, and ratiometric imaging of exogenous H_2S in MCF-7 cells was demonstrated (*57*). Similarly, Guo and co-workers synthesized a flavylium derivative (**32**), in which attack of H_2S

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on the electrophilic benzopyrylium moiety disrupts conjugation, thereby leading to a decrease in fluorescence in the red channel and increase in the green channel (*58*). This shift in fluorescence was used to detect exogenous H_2S in HeLa cells.

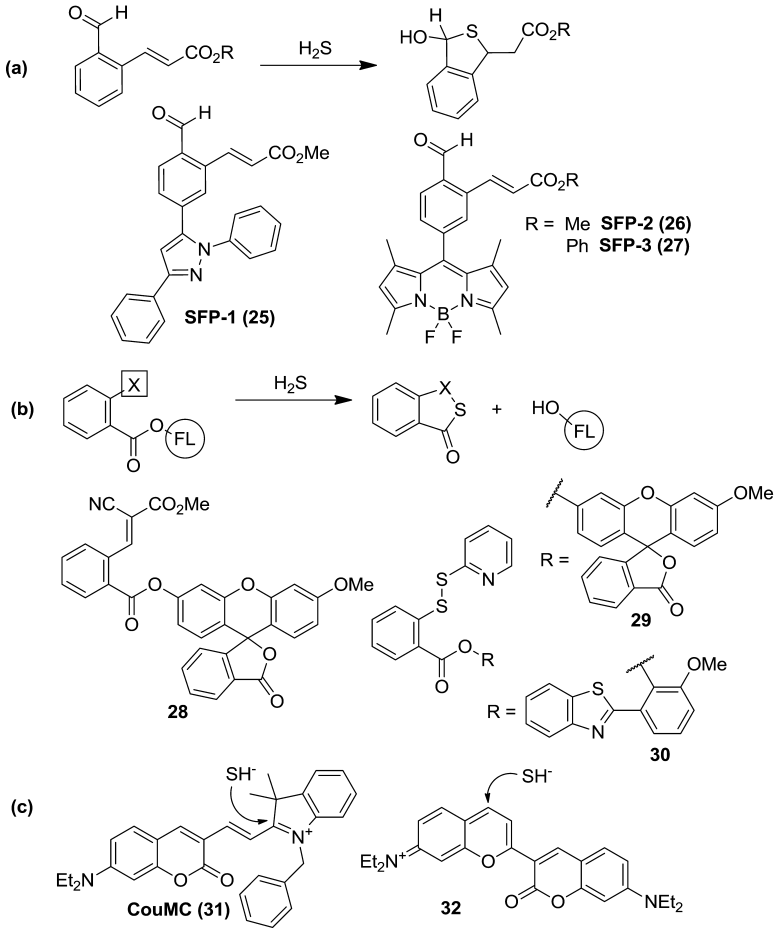

Figure 3. Selected method of H_2S detection based on nucleophilic attack. (a) Addition to an aldehyde followed by intramolecular attack on an olefin; (b) Attack on an activated electrophile followed by attack on an ester-bound fluorophore; (c) Attack on an electrophilic center to disrupt fluorophore conjugation.

Nucleophilic attack by SH^- is an attractive strategy for H_2S detection that utilizes the ability of H_2S to participate in two sequential nucleophilic attacks, thus providing a clear pathway to differentiate H_2S from endogenous thiols. One major challenge, however, is ensuring that the developed constructs are not inactivated by thiols. Although attack by a thiol typically does not result in a fluorescence response, it often deactivates the probe and prevents future reaction with H_2S. Emerging strategies based on electrophiles able to react reversibly with thiols offer

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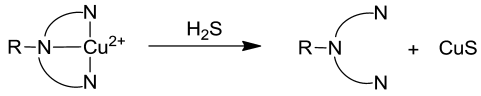
an attractive platform on which this problem can be addressed, but fine-tuning of the reactivity of such compounds to react reversibly with thiols and quickly with H_2S remains challenging (*59*).

H_2S Detection Methods Based on Metal Precipitation

A third strategy for reaction-based H_2S detection relies on modification of the classic gravimetric method for Cu(II) detection using H_2S to precipitate solid CuS (Scheme 4). By appending a Cu(II)-binding ligand to a fluorophore, the proximity of the unpaired electron from Cu(II) can potentially quench fluorescence from the fluorophore due to photoinduced electron transfer (PET). Such Cu(II)-mediated fluorescence quenching has been used to generate a variety of nitric oxide and nitroxyl-detecting probes (*60–63*). By judicial choice of Cu(II)-binding scaffold, H_2S can precipitate CuS without unwanted reduction of Cu(II) to Cu(I). Because Cu(II) and Cu(I) have different preferred coordination geometries, reduction to Cu(I) would likely eject the metal and result in fluorescence turn-on from the probe. Minimizing such unwanted reduction from thiols is a key requirement in maintaining high selectivity for H_2S over other biologically relevant thiols. To date, the H_2S-mediated CuS precipitation coupled with fluorescence appears to be predominantly empirical, with small modifications of the ligand resulting in large changes in thiol versus H_2S selectivity (Figure 4).



Scheme 4. Treatment of a Cu(II)-bound ligand with H_2S can result in precipitation of the insoluble CuS thus releasing the fluorophore

The initial example of H_2S detection using the CuS precipitation strategy was reported by Chang and co-workers using a dipicolylamine-appended fluorescein derivative (**33**) (*64*). Complexation of the fluorescein construct with Cu(II) resulted in fluorescent quenching. Upon treatment with S^{2-}, and concomitant precipitation of CuS, the fluorescence of the fluorescein platform was restored. This method for sulfide detection was selective for H_2S over other anions but was not selective over biologically-relevant thiols such as GSH.

Nagano and co-workers expanded on this sulfide detection manifold by preparing a cyclen-containing fluorescein derivative (HSip-1, **34**) to bind Cu(II) (*65*). This scaffold allows for the fast detection of H_2S, thus generating a fluorescence turn-on. Additionally, the fluorescence response of HSip-1 was selective for H_2S over RSONS including potential reducing agents. In addition to detecting H_2S from NaSH and a Cys-activated H_2S donor (*66*), HSip-1 was also used to detect exogenous H_2S in HeLa cells.

Similar platforms utilizing different Cu(II) binding ligands have also been used for H_2S detection. For example, Ramesh, Das, and co-workers reported the use of a FRET-based indole-ligated rhodamine (**35**) for H_2S detection. This

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scaffold resulted in a turn-on NIR fluorescence response upon treatment with H_2S and was selective for sulfide over other anions or transition metals, but the probe was not tested with thiols (*67*). Similarly, Bai, Zeng, and co-workers reported a 8-hydroxyquinoline-appended fluorescein derivative (**36**) able to bind Cu(II) (*68*). Treatment with H_2S resulted in a 5-fold fluorescence turn-on and the probe maintained selectivity over other anions. Again, this complex was not tested with other thiol-containing reactive species. Both **35** and **36** were used to detect exogenous H_2S in HeLa cells. The same general CuS precipitation strategy has been used to generate colorimetric H_2S detection methods based on a Cu(II)-chelated azo dye (**37**) (*69*) and a quinoline-dipicolyl amine BODIPY platform (**38**) (*70*).

The method of CuS precipitation has generated a highly-modifiable strategy for H_2S detection. This method does have practical limitations, however, because the usable probe concentration ranges depend on the binding constant of Cu(II) to the ligand scaffold. Additionally, high probe concentrations are disfavored due to the stoichiometric precipitation of solid CuS. To demonstrate biocompatibility for future probes based on CuS precipitation, such scaffolds need to be tested with both biologically relevant thiols and reductants to ensure that reduction of Cu(II) to Cu(I) does not compete with CuS precipitation. Similarly, such scaffolds must be tested with other biologically-relevant metal ions such as Zn(II) to ensure that metal exchange is not occurring under physiological conditions.

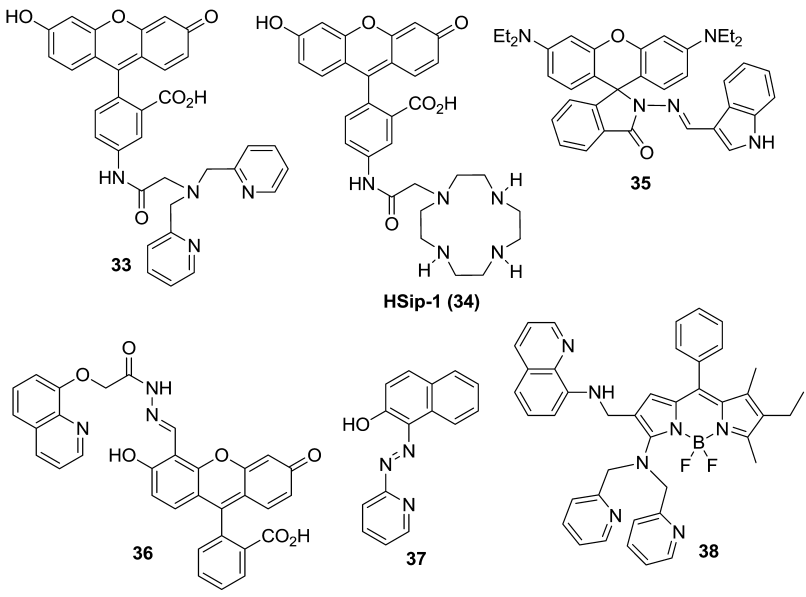

Figure 4. Highlighted metal precipitation-base methods of H_2S detection.

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Conclusion

The field of H_2S sensing has grown rapidly in the last two years based on three general detection strategies: reduction of azide or nitro groups, nucleophilic attack on activated electrophiles, and precipitation of CuS from Cu(II)-ligated fluorophores. These detection strategies have provided an array of tools available to scientists interested in understanding the multifaceted roles of H_2S in biology. In addition to the above highlighted examples, other reaction-based detection methods have emerged, typically revolving around the reaction of H_2S with various metals or metals housed in biomimetic scaffolds (*71–74*). One of the major challenges in characterizing new probes remains drawing definitive conclusions about reaction rates or absolute selectivity over other reactive biological species because of the disparate conditions in which current probes have been tested. One clear conclusion from currently-available probes is that each of the developed strategies offers its own advantages and disadvantages, suggesting that specific properties of individual probes are likely to dictate the suitability of that platform to answer a specific biological question.

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References

1. Palmer, R. M.; Ferrige, A. G.; Moncada, S. *Nature* **1987**, *237*, 524–526.
2. Benavides, G. A.; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17977–17982.
3. Qu, K.; Lee, S. W.; Bian, J. S.; Low, C. M.; Wong, P. T. H. *Neurochem. Int.* **2008**, *52*, 155–165.
4. Pryor, W. A.; Houk, K. N.; Foote, C. S.; Fukuto, J. M.; Ignarro, L. J.; Squadrito, G. L.; Davies, K. J. A. *Am. J. Physiol-Reg I* **2006**, *291*, R491–R511.
5. Olson, K. R. *Antioxid. Redox Signaling* **2012**, *17*, 32–44.
6. Wang, R. *Physiol. Rev.* **2012**, *92*, 791–896.
7. Pluth, M. D.; Tomat, E.; Lippard, S. J. *Annu. Rev. Biochem.* 2011, 80, 333−355.
8. Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signaling* **2009**, *11*, 205–214.
9. Toohey, J. I. *Biochem. J.* **1989**, *264*, 625–632.
10. Hosoki, R.; Matsuki, N.; Kimura, H. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 527–531.
11. Shen, X.; Pattillo, C. B.; Pardue, S.; Bir, S. C.; Wang, R.; Kevil, C. G. *Free Radical Biol. Med.* **2011**, *50*, 1021–1031.
12. Abe, K.; Kimura, H. *J. Neurosci.* **1996**, *16*, 1066–1071.

13. Kimura, H. *Mol. Neurobiol.* **2002**, *26*, 13–19.
14. Yang, W.; Yang, G. D.; Jia, X. M.; Wu, L. Y.; Wang, R. *J. Physiol. (London)* **2005**, *569*, 519–531.
15. Collin, M.; Anuar, F. B. M.; Murch, O.; Bhatia, M.; Moore, P. K.; Thiemermann, C. *Br. J. Pharmacol.* **2005**, *146*, 498–505.
16. Zhang, H. L.; Moochhala, S. M.; Bhatia, M. *J. Immunol.* **2008**, *181*, 4320–4331.
17. Elrod, J. W.; Calvert, J. W.; Morrison, J.; Doeller, J. E.; Kraus, D. W.; Tao, L.; Jiao, X. Y.; Scalia, R.; Kiss, L.; Szabo, C.; Kimura, H.; Chow, C. W.; Lefer, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 15560–15565.
18. Hu, L. F.; Wong, P. T. H.; Moore, P. K.; Bian, J. S. *J. Neurochem.* **2007**, *100*, 1121–1128.
19. Brunner, U.; Chasteen, T. G.; Ferloni, P.; Bachofen, R. *Chromatographia* **1995**, *40*, 399–403.
20. Doeller, J. E.; Isbell, T. S.; Benavides, G.; Koenitzer, J.; Patel, H.; Patel, R. P.; Lancaster, J. R.; Darley-Usmar, V. M.; Kraus, D. W. *Anal. Biochem.* **2005**, *341*, 40–51.
21. Esfandyarpour, B.; Mohajerzadeh, S.; Khodadadi, A. A.; Robertson, M. D. *IEEE Sens. J.* **2004**, *4*, 449–454.
22. Kuhl, M.; Steuckart, C.; Eickert, G.; Jeroschewski, P. *Aquat. Microb. Ecol.* **1998**, *15*, 201–209.
23. Fogo, J. K.; Popowsky, M. *Anal. Chem.* **1949**, *21*, 732–734.
24. Lawrence, N. S.; Davis, J.; Compton, R. G. *Talanta* **2000**, *52*, 771–784.
25. Shen, X. G.; Peter, E. A.; Bir, S.; Wang, R.; Kevil, C. G. *Free Radical Biol. Med.* **2012**, *52*, 2276–2283.
26. Kabil, O.; Banerjee, R. *J. Biol. Chem.* **2010**, *285*, 21903–21907.
27. Gronowitz, S.; Westerlund, C.; Hornfeldt, A. B. *Acta Chem. Scand., Ser. B* **1975**, *B29*, 224–232.
28. Huber, D.; Andermann, G.; Leclerc, G. *Tetrahedron Lett.* **1988**, *29*, 635–638.
29. Lin, Y.; Lang, S. A. *J. Heterocycl. Chem.* **1980**, *17*, 1273–1275.
30. Nickson, T. E. *J. Org. Chem.* **1986**, *51*, 3903–3904.
31. Lippert, A. R.; New, E. J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 10078–10080.
32. Lin, V. S.; Lippert, A. R.; Chang, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7131–7135.
33. Yu, F.; Li, P.; Song, P.; Wang, B.; Zhao, J.; Han, K. *Chem. Commun.* **2012**, *48*, 2852–2854.
34. Montoya, L. A.; Pluth, M. D. *Chem. Commun.* **2012**, *48*, 4767–4769.
35. Tian, H.; Qian, J.; Bai, H.; Sun, Q.; Zhang, L.; Zhang, W. *Anal. Chim. Acta* **2013**, *768*, 136–142.
36. Yu, C.; Li, X.; Zeng, F.; Zheng, F.; Wu, S. *Chem. Commun.* **2013**, *49*, 403–405.
37. Xuan, W.; Pan, R.; Cao, Y.; Liu, K.; Wang, W. *Chem. Commun.* **2012**, *48*, 10669–10671.
38. Peng, H.; Cheng, Y.; Dai, C.; King, A. L.; Predmore, B. L.; Lefer, D. J.; Wang, B. *Angew. Chem., Int. Ed.* **2011**, *50*, 9672–9675.

In Biochalcogen Chemistry: The Biological Chemistry of Sulfur, Selenium, and Tellurium; Bayse, Craig A., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

39. Chen, B.; Li, W.; Lv, C.; Zhao, M.; Jin, H.; Jin, H.; Du, J.; Zhang, L.; Tang, X. *Analyst* **2013**, *138*, 946–951.
40. Li, W. H.; Sun, W.; Yu, X. Q.; Du, L. P.; Li, M. Y. *J. Fluoresc.* **2013**, *23*, 181–186.
41. Chen, S.; Chen, Z.-J.; Ren, W.; Ai, H.-W. *J. Am. Chem. Soc.* **2012**, *134*, 9589–9592.
42. Hartman, M. C. T.; Dcona, M. M. *Analyst* **2012**, *137*, 4910–4912.
43. Wu, Z.; Li, Z.; Yang, L.; Han, J.; Han, S. *Chem. Commun.* **2012**, *48*, 10120–10122.
44. Zheng, K.; Lin, W.; Tan, L. *Org. Biomol. Chem.* **2012**, *10*, 9683–9688.
45. Chen, B.; Lv, C.; Tang, X. *Anal. Bioanal. Chem.* **2012**, *404*, 1919–1923.
46. Zhou, G.; Wang, H.; Ma, Y.; Chen, X. *Tetrahedron* **2013**, *69*, 867–870.
47. Das, S. K.; Lim, C. S.; Yang, S. Y.; Han, J. H.; Cho, B. R. *Chem. Commun.* **2012**, *48*, 8395–8397.
48. Wan, Q.; Song, Y.; Li, Z.; Gao, X.; Ma, H. *Chem. Commun.* **2013**, *49*, 502–504.
49. Cao, X.; Lin, W.; Zheng, K.; He, L. *Chem. Commun.* **2012**, *48*, 10529–10531.
50. Wang, R.; Yu, F.; Chen, L.; Chen, H.; Wang, L.; Zhang, W. *Chem. Commun.* **2012**, *48*, 11757–11759.
51. Wintner, E. A.; Deckwerth, T. L.; Langston, W.; Bengtsson, A.; Leviten, D.; Hill, P.; Insko, M. A.; Dumpit, R.; VandenEkart, E.; Toombs, C. F.; Szabo, C. *Br. J. Pharmacol.* **2010**, *160*, 941–957.
52. Qian, Y.; Karpus, J.; Kabil, O.; Zhang, S.-Y.; Zhu, H.-L.; Banerjee, R.; Zhao, J.; He, C. *Nat. Commun.* **2011**, *2*, 495.
53. Qian, Y.; Zhang, L.; Ding, S.; Deng, X.; He, C.; Zheng, X. E.; Zhu, H.-L.; Zhao, J. *Chem. Sci.* **2012**, *3*, 2920–2923.
54. Liu, C.; Peng, B.; Li, S.; Park, C.-M.; Whorton, A. R.; Xian, M. *Org. Lett.* **2012**, *14*, 2184–2187.
55. Liu, C.; Pan, J.; Li, S.; Zhao, Y.; Wu, L. Y.; Berkman, C. E.; Whorton, A. R.; Xian, M. *Angew. Chem., Int. Ed.* **2011**, *50*, 10327–10329.
56. Xu, Z.; Xu, L.; Zhou, J.; Xu, Y.; Zhu, W.; Qian, X. *Chem. Commun.* **2012**, *48*, 10871–10873.
57. Chen, Y.; Zhu, C.; Yang, Z.; Chen, J.; He, Y.; Jiao, Y.; He, W.; Qiu, L.; Cen, J.; Guo, Z. *Angew. Chem., Int. Ed.* **2013**, *52*, 1688–1691.
58. Liu, J.; Sun, Y.-Q.; Zhang, J.; Yang, T.; Cao, J.; Zhang, L.; Guo, W. *Chem.-Eur. J.* **2013**, *19*, 4717–4722.
59. Montoya, L. A.; Pearce, T. F.; Hansen, R. J.; Zakharov, L. N.; Pluth, M. D. *J. Org. Chem.* **2013**, *78*, 6550–6557.
60. Lim, M. H.; Xu, D.; Lippard, S. J. *Nat. Chem. Biol.* **2006**, *2*, 375–380.
61. McQuade, L. E.; Ma, J.; Lowe, G.; Ghatpande, A.; Gelperin, A.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 8525–8530.
62. Pluth, M. D.; Chan, M. R.; McQuade, L. E.; Lippard, S. J. *Inorg. Chem.* **2011**, *50*, 9385–9392.
63. Rosenthal, J.; Lippard, S. J. *J. Am. Chem. Soc.* **2010**, *132*, 5536–5537.
64. Choi, M. G.; Cha, S.; Lee, H.; Jeon, H. L.; Chang, S. K. *Chem. Commun.* **2009**, 7390–7392.

65. Sasakura, K.; Hanaoka, K.; Shibuya, N.; Mikami, Y.; Kimura, Y.; Komatsu, T.; Ueno, T.; Terai, T.; Kimura, H.; Nagano, T. *J. Am. Chem. Soc.* **2011**, *133*, 18003–18005.
66. Zhao, Y.; Wang, H.; Xian, M. *J. Am. Chem. Soc.* **2011**, *133*, 15–17.
67. Kar, C.; Adhikari, M. D.; Ramesh, A.; Das, G. *Inorg. Chem.* **2013**, *52*, 743–752.
68. Hou, F. P.; Huang, L.; Xi, P. X.; Cheng, J.; Zhao, X. F.; Xie, G. Q.; Shi, Y. J.; Cheng, F. J.; Yao, X. J.; Bai, D. C.; Zeng, Z. Z. *Inorg. Chem.* **2012**, *51*, 2454–2460.
69. Zhang, D. Q.; Jin, W. S. *Spectrochim. Acta, Part A* **2012**, *90*, 35–39.
70. Gu, X.; Liu, C.; Zhu, Y.-C.; Zhu, Y.-Z. *Tetrahedron Lett.* **2011**, *52*, 5000–5003.
71. Quek, Y.-L.; Tan, C.-H.; Bian, J.; Huang, D. *Inorg. Chem.* **2011**, *50*, 7379–7381.
72. Strianese, M.; De Martino, F.; Pellecchia, C.; Ruggiero, G.; D'Auria, S. *Protein Pept. Lett.* **2011**, *18*, 282–286.
73. Strianese, M.; Palm, G. J.; Milione, S.; Kühl, O.; Hinrichs, W.; Pellecchia, C. *Inorg. Chem.* **2012**, *51*, 11220–11222.
74. Wang, B.; Li, P.; Yu, F.; Song, P.; Sun, X.; Yang, S.; Lou, Z.; Han, K. *Chem. Commun.* **2013**, *49*, 1014–1016.