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TUTORIAL REVIEW
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A practical guide to working with H$_2$S at the interface of chemistry and biology
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Matthew D. Hartle and Michael D. Pluth*

Hydrogen sulfide (H$_2$S) is the most recently accepted endogenously produced gasotransmitter and is now implicated in a variety of physiological functions. In this tutorial review, our goal is to provide researchers new to the field of H$_2$S chemical biology with practical considerations, pitfalls, and best practices to enable smooth entry into investigations focused on biological H$_2$S. We present practical handling and safety considerations for working with this reactive biomolecule, and cover basic roles of H$_2$S biogenesis and action. Experimental methods for modulating H$_2$S levels, including enzymatic knockout, RNA silencing, enzymatic inhibition, and use of small molecule H$_2$S donors are highlighted. Complementing H$_2$S modulation techniques, we also highlight current strategies for H$_2$S detection and quantification.

Key learning points
- Basic properties of biological hydrogen sulfide (H$_2$S)
- The role of H$_2$S in the landscape of reactive sulfur, oxygen, and nitrogen species (RSONS)
- Practical safety considerations for handling H$_2$S and H$_2$S donors
- Methods for modulating biological H$_2$S: enzymatic knockout, RNA silencing, inhibition, stimulation, small molecule donors
- Methods for detecting and quantifying biological H$_2$S

Introduction

Hydrogen sulfide (H$_2$S) has emerged as an important biological signaling molecule that plays diverse roles in human health and physiology. Despite its long history as a toxic gas and environmental pollutant, H$_2$S now joins nitric oxide (NO) and carbon monoxide (CO) as a gasotransmitter – a small, enzymatically generated, gaseous molecule with a tightly regulated metabolism that impacts physiological functions.$^{1-3}$ The innate chemical properties of H$_2$S, including its redox activity, acidity, and high nucleophilicity, allow for reaction with different cellular targets as part of its signaling capacity. By comparison, disentangling this chemistry is in many ways more complicated than that of NO and CO.$^4$ Misregulation of endogenous H$_2$S is implicated in diverse physiological processes, including blood pressure regulation, immune response, and long term potentiation, as well as various diseases in the neuronal, gastrointestinal, circulatory, and endocrine systems.$^2$ Aligned with our rapidly expanding understanding of biological H$_2$S, new and impactful investigative tools for elucidating its genesis, translocation, and action have emerged and continue to be an active and fruitful research area.

By contrast to biological studies focused on NO and CO, H$_2$S-focused investigations are often faced with unique experimental challenges – some of which are due to the recent emergence of H$_2$S chemical biology and others from the innate physical properties of H$_2$S. For example, although CO, NO, and H$_2$S are all gasses, common gas detection techniques, such as gas chromatography (GC) and chemiluminescence detectors provide robust methods for detection and quantification for CO and NO, respectively, whereas similar techniques have not been widely developed for H$_2$S. The different protonation states of H$_2$S, and its interaction with the redox-active biological sulfur pool, make analysis by GC or other common analytical methods challenging. Similarly, reaction-based imaging techniques, as well as synthetic donor scaffolds, are often more complicated than similar systems for NO or CO. Despite these challenges, recent studies have suggested the potential interplay between the biological chemistry of H$_2$S and NO$^5$ as well as CO,$^3$ thus highlighting the interconnectivity of these three gaseous molecules. These interactions, as well as those with H$_2$S and reactive sulfur, oxygen, and nitrogen species (RSONS) highlight the
complexity of the biological landscape in which H₂S is involved, and the synergistic relationship of H₂S to other important and reactive small molecules in biology.

In this tutorial review, we highlight key points regarding H₂S chemical biology and provide a brief survey of accessible tools and methods for H₂S research at the interface of chemistry and biology. Our goal is not to provide a comprehensive overview of all investigative tools for H₂S research, but rather to highlight currently available classes of tools and to provide important considerations to help guide new researchers into the rapidly developing field of H₂S chemical biology.

Properties and enzymatic production of H₂S

H₂S is a weak acid with a first pKₐ of 7.0, and a second pKₐ, corresponding to the deprotonation of hydrosulfide anion (HS⁻) of >14.¹ Under physiological conditions, HS⁻ constitutes about 80% of the speciation, neutral H₂S about 20%, and diatomic S²⁻ less than 1%.¹ The HS⁻ and H₂S protonation states provide water-solubility and lipophilicity, respectively, and HS⁻ is a potent nucleophile that can react with different electrophilic cellular targets including RSONS. Additionally, the different protonation states afford different metal-ligation and redox properties. The complex redox landscape of biological sulfur, with oxidation states ranging from −2 in H₂S to +6 in SO₄²⁻, means that H₂S/HS⁻ can be readily oxidized to other biologically-relevant reactive sulfur species. Indeed, the redox-labile/sulfane-sulfur pool may provide an important method of sulfide storage and transport, but also may provide new signaling pathways distinct from those associated with H₂S alone (vide infra).⁶

Enzymatic H₂S biosynthesis stems primarily from cystathionine γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercapto-pyruvate sulfurtransferase (3-MST)/cysteine aminotransferase (CAT). In each of these pathways, the sulfur atom incorporated into H₂S is derived from the sulphydryl group of either homocysteine (Hey) or l-cysteine (l-Cys). Details of these pathways have been recently reviewed in significant detail and are summarized briefly here (Fig. 1).¹⁻³ CSE catalyzes the formation of H₂S and homolanthionine from 2 equiv. of Hey, but can also work in concert with CBS to convert Hey and Cys to H₂S and cystathionine. CBS can also catalyze the condensation of Hey with serine (Ser) to generate cystathionine and H₂O. Upon reaction with CSE, cystathionine is converted back to l-Cys with concomitant formation of homoserine. This generated Cys can participate in the above pathways, can be hydrolyzed to Ser and H₂S by CBS or CSE, or can be condensed with a second equivalent of Cys by the same two enzymes to form lanthionine and H₂S. Cysteine is also a viable substrate for CAT, which generates 3-mercapto-pyruvate (3-MP), which is then converted to pyruvate, NH₃, and H₂S by 3-MST. The 3-MST pathway also accounts for H₂S formed from l-Cys, which is first converted to 3-mercapto-pyruvate by DAO.

Practical handling and safety considerations

Hydrogen sulfide is a highly toxic gas, and adequate care must be given when handling H₂S gas, sulfide salts, and synthetic H₂S donors. Although the characteristic rotten egg odor is detectable as low as 0.01 ppm in air, odor alone should never be used as the primary method of safety monitoring. Rapid olfactory fatigue results upon exposure to concentrations greater than 100 ppm but also after continuous exposure to significantly lower levels. H₂S concentrations as low as 20 ppm cause eye and lung irritation, 300–500 ppm levels result in serious eye damage, and 700 ppm or higher levels can result in unconsciousness, respiratory failure, and death.⁷ The combination

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of high toxicity with rapid olfactory fatigue necessitates handling in a well ventilated fume hood equipped with a commercially-available H₂S alarm, which detects H₂S gas and typically reports in the 5–10 ppm range, especially when using H₂S gas directly or quantities of sulfide salts sufficient to provide a potentially toxic response. It is also practical, both from a safety and odor-mediation standpoint, to quench any H₂S-containing solutions after use. Such quenching can be readily accomplished by preparation and use of a Zn²⁺ quenching solution, which rapidly reacts with free sulfide to generate insoluble ZnS. This quenching slurry can be prepared by adding 30 g Zn(OAc)₂, 9 g sodium citrate, and 12 g NaOH to 1 L of H₂O.⁷

For experiments involving aqueous sulfide solutions, it is often more practical to use sulfide salts, such as NaSH or Na₂S, rather than H₂S gas directly. Despite their convenience, the commercial purity of NaSH or Na₂S is often poor, and many samples contain significant quantities of elemental sulfur or polysulfides.⁴,⁶,⁷ In general, sulfide salts should be free-flowing white powders – any inclusion of yellow or other highly colored impurities should signal to the user that the sample does not have the appropriate purity and should be discarded. Because of the propensity of H₂S/HS⁻ to oxidize, especially in the presence of trace metal ions and oxygen, H₂S solutions should be prepared in anaerobic buffer under a blanket of nitrogen or argon in clean, metal-free, glass- or plastic-ware. Such solutions should also be prepared in septum-sealed vials to prevent significant loss from H₂S volatilization and also to enable easy transfer of sulfide solutions via gas-tight syringe. Because of this volatility and potential for oxidation, it is most practical to prepare sulfide-containing solutions immediately prior to use rather than to re-use solutions from previous experiments.

Interactions with the sulfane-sulfur pool

Complementing the signaling roles of biological H₂S, significant evidence suggests that sulfane-sulfur compounds, such as polysulfides, persulfides, and other sources of reductant-labile sulfur, play important biochemical roles.⁵,⁶,⁷ The term “sulfane
sulfur" refers to a sulfur atom with formally six valence electrons and no charge (S⁰), which is bound to one or more sulfur atoms.¹⁰ Upon reaction with cellular reductants or thiols, sulfane-sulfur compounds can release sulfide, thus providing a convenient source of H₂S storage. Additionally, sulfane-sulfur species have distinct chemical reactivities that may contribute to additional modes of action. For example, persulfides (RSSH) are more nucleophilic than the corresponding thiols, and S'-persulfidation of nucleophilic Cys residues in enzymes is known to modify enzymatic activity.¹¹ As completely inorganic species, hydropolysulfides (HSS₉,H) are also important sulfane-sulfur species, as evidenced by a recent report demonstrating that 3-MST not only generates H₂S, but also H₂S₉.¹² The use of small molecule persulfides and persulfide-releasing motifs as H₂S donors (vide infra) and as model systems is quickly providing more information on persulfide reactivity and its role in the intricate chemistry of RSONS.¹³ Although isolated persulfides are typically unstable in solution and have not yet found utility in biochemical investigations, caged persulfides, which release persulfides upon hydrolysis or reaction with nucleophiles, or persulfides generated in situ from reaction of GSSG with HS⁻, have been used to investigate different roles of persulfides in reactive sulfur species regulation and action.¹²

One of the most basic methods for sulfane-sulfur detection and quantification is the cold cyanolysis assay, which utilizes the reaction of sulfane sulfur atoms with CN⁻ at basic pH to form thiocyanoate (SCN⁻). Addition of excess ferric iron results in the formation of [Fe(SCN)(H₂O)₅]²⁺, which can be readily detected and quantified by the characteristic absorbance at 460 nm (Scheme 1a).¹¹ In practice, the cold cyanolysis method is usually used for purified proteins or samples in simple matrices and cannot differentiate between individual components, such as persulfides and polysulfides, in the sulfane-sulfur pool. One significant limitation of the cold cyanolysis method is that persulfides are not differentiated from polysulfides or other sulfane-sulfur sources. As a step toward addressing this challenge, a tag-switch method to detect and assay persulfides was recently reported (Scheme 1b).¹⁴ In this method, all sulfhydryl groups, both from thiols (−SH) and persulfides (−SSH) are labelled with an electrophile, such as methylsulfonyl benzothiazole (MSBT), to generate the corresponding benzothiazole thioether and disulfide, respectively. Importantly, the resultant benzothiazole disulfide remains reactive toward specific carbon-based nucleophiles, allowing for conjugation with biotin tags. The biotinylated proteins can then be subjected to streptavidin pulldown and detection by standard Western blot or mass spectrometric techniques.¹⁴ Complementing these strategies, selective reaction-based fluorescent probes for polysulfides and sulfane sulfur have been reported and are the subject of a recent review.¹⁵ These, as well as other emerging tools, are poised to help differentiate between the genesis of H₂S and other reactive polysulfides.

Methods for modulating H₂S levels

The ability to modulate cellular H₂S levels provides a cornerstone for investigating the actions of H₂S in biology. Such control can be achieved by selective knockout and/or knockdown of H₂S-producing enzymes, use of small-molecule competitive inhibitors or stimulators, or by administration of synthetic H₂S donors. In model cell lines and organisms, supplementation with exogenous H₂S can often provide protection (or rescue) from various (patho-)physiological disease states associated with abnormal H₂S biosynthesis. These insights suggest that synthetic H₂S donors may not only provide important chemical tools for understanding biological H₂S, but may also offer viable therapeutic potential for diseases associated with H₂S misregulation.

KO mouse models

Selective knockout (KO) of CSE, CBS, or 3-MST in different cell lines has been utilized in specific investigations, but the development of KO mouse models has provided a more broad platform on which to study the impacts of reduced enzymatic H₂S generation.¹⁶ Homozygous CBS⁻/⁻ KO in mice results in severe developmental growth problems, and few of the mice live past 4 weeks of age.¹⁷ The heterozygous CBS⁺/⁻ KO mice exhibit better viability and present hyperhomocysteinemia (high levels of homocysteine in the blood) but have not been used extensively in H₂S-related investigations.¹⁷ By contrast, the CSE⁻/⁻ mouse model¹⁸ has found significantly more utility in H₂S-related investigations, including studies on the role of H₂S in blood pressure regulation, angiogenesis, and neurodegeneration, as well as many other studies. A second CSE⁻/⁻ mouse model exhibiting somewhat different phenotypes was also subsequently developed and used as an animal model for cystathioninemia.¹⁹ Homozygous 3-MST⁻/⁻ mice have been developed more recently and may also provide a useful animal model for H₂S-related investigations.²⁰ As a whole, the availability of KO mouse models provide a key tool in studying the impacts of H₂S in contextually-rich biological environments and overcome some of the limitations of small molecule inhibitors or sulfide donors (vide infra).

RNA knockdown methods

In addition to enzyme KO methods, gene knockdown by RNA interference (RNAi) methods has been used to silence CSE, CBS, and 3-MST. Importantly, these methods often provide a
more simple approach to overcome the limitation of animal models or small-molecule inhibitors, and also offer a complementary approach for other enzyme modulation experiments. For example, small interfering RNA (siRNA) methods have been used to silence CSE and CBS. Unlike small-molecule inhibitors for 3-MST (*vide infra*), siRNA methods aimed at this enzyme have proven efficacious in reducing H$_2$S production, leading to important insights into mitochondrial electron transport and cellular bioenergetics.

**Small-molecule inhibitors**

Small-molecule competitive inhibitors of H$_2$S-producing enzymes are commonly used to decrease H$_2$S synthesis in studies using isolated enzymes or cell culture models. On the basis of this widespread use, significant efforts have been made to discover or develop potent enzymatic inhibitors for CBS, CSE, and 3-MST. Although these studies have provided a useful suite of compounds for partial inhibition of H$_2$S-producing enzymes, identifying inhibitors with specificity for one enzyme over another and low inhibitory constant ($K_i$) values remains a significant challenge.

Commonly-used CSE inhibitors with $IC_{50} < 1$ mM are L-aminoethoxyvinylglycine (AVG, 1 mM), β-cyano-L-alanine (BCA, 14 mM), and propargylglycine (PAG, 40 μM) (Fig. 2). Each of these inhibitors also affect other proximal phosphate (PLP)-dependent enzymes at mM concentrations, making it likely that off-target inhibition is observed at practical concentrations. Inhibition of CBS has proven similarly more challenging, with fewer inhibitors with $IC_{50} < 1$ mM identified. Hydroxylamine (HA, 278 μM), aminoxyacetic acid (AOAA, 8.52 μM), and trifluoroalanine (66 μM) are among the most commonly used competitive inhibitors for CBS, but none of these show marked selectivity for CBS over CSE. Recent studies have used high-throughput screening methods, often in combination with H$_2$S fluorescent probes (*vide infra*), to identify CBS inhibitors. Such screens have identified new lead compounds with good $IC_{50}$ values in the low micromolar range, but many hits have not been compared directly with commonly-used inhibitors and their selectivity for CBS over CSE remains to be fully elucidated. Similar to the challenges with developing selective CBS inhibitors, selective inhibition of 3-MST has been unsuccessful to date.

Given the overall difficulty in selectively inhibiting individual H$_2$S producing enzymes, the identification and development of new inhibitors for H$_2$S-producing enzymes is needed. Such inhibitors, if selective and functional at pharmacologically-reasonable concentrations, would provide important research tools for modulating cellular H$_2$S levels.

**Enzymatic stimulators**

Complementing methods to reduce H$_2$S synthesis using small-molecule inhibitors, H$_2$S synthesis can also be increased by use of different enzymatic stimulators. The most common of such methods is use of S-adenosylmethionine (AdoMet), an allosteric activator of CBS, to increase enzymatic H$_2$S production. In addition to CBS, CSE stimulation has also been demonstrated using cytokines and endotoxins, such as tumor necrosis factor α (TNF-α) or lipopolysaccharides (LPS), involved in immune response. By contrast to CBS and CSE stimulation, common stimulators of 3-MST are not readily available and remain an active area of research.

**Small-molecule H$_2$S donors**

Complementing methods to modulate endogenous H$_2$S synthesis, the ability to increase endogenous H$_2$S levels using exogenous sulfide sources provides an important research and pharmacological tool for studying the roles of biological H$_2$S. Commonly-used sources of exogenous H$_2$S include inorganic salts, such as NaSH and Na$_2$S, as well as small-molecule synthetic donors. Although NaSH and Na$_2$S provide convenient sources of sulfide, the large dose of H$_2$S released upon addition to buffer does not match the slower, continuous, enzymatic production characteristic of endogenous H$_2$S synthesis. Additionally, the large bolus of H$_2$S often results in a toxicological response and is quickly oxidized/metabolized by the cellular environment. Motivated by these limitations, researchers have developed slow-release sulfide donors that better mimic the gradual H$_2$S release of enzymatic synthesis. As our understanding of sulfide biology continues to evolve, one common observation is that slow-releasing H$_2$S donors often elicit different cellular responses than inorganic sulfide salts. For example, sulfide salts and slow-releasing donors have been shown to be pro- and anti-inflammatory, respectively, in different models of inflammation, including sepsis. These differences highlight the importance of experimental design when using different sulfide sources, but also highlight the potential pharmacological importance of such slow-releasing donors. Here we provide a brief overview of the most commonly-used donor motifs along with their sulfide release mechanisms when known. We also refer interested readers to recent reviews on this topic.

Some of the most simple sulfide-donating motifs are organic polysulfides, which are often found in natural products. For example, diallyltrisulfide (DATS), isolated from garlic and other alliums, is a commonly used donor molecule, and other organic polysulfides such as varacin have also been implicated in H$_2$S release. Because polysulfides are electrophilic, attack by GSH or other thiols results in formation of an intermediate persulfide, which after a second reaction with GSH releases H$_2$S (Scheme 2a).
Although DATS is the most commonly-used polysulfide donor, it is likely that other small molecule polysulfides are also potential platforms for H₂S release.

Inspired by disulfide exchange chemistry, chemists have also developed H₂S donors that are activated by nucleophilic attack by endogenous thiols. For example, protection of the thiol in penicillamine derivatives with an acyldisulfide results in persulfide formation after nucleophilic attack by Cys (Scheme 2b). Once the persulfide is released, reaction with a second thiol generates a stable disulfide and extrudes H₂S. Application of such donors to the treatment of myocardial ischemia/reperfusion (MI/R) injury in murine models results in reduced circulating levels of MI/R biomarkers suggesting these and similar donor motifs exhibit cardiac protection and may have potential therapeutic applications.

Operating by similar Cys-activated H₂S release mechanisms, donors containing N-mercapto (N-SH) motifs also generate a persulfide intermediate en route to H₂S release (Scheme 2c). In these donors, thiol exchange between Cys and the donor generates an S-acylated Cys intermediate, which undergoes native chemical ligation to rearrange to N-acylated Cys and an N-mercaptobenzamide intermediate. This N-SH compound reacts with Cys to generate Cys persulfide, which reacts with a second equivalent of thiol to release H₂S. The rate of H₂S release from these scaffolds can be tuned by the addition of electron donating or withdrawing groups on the scaffold. Additionally, new H₂S-donating materials activated by thiol activation are also emerging, indicating the potential viability of this strategy for incorporation into therapeutics such as wound dressing to encourage angiogenesis.

In addition to donor activation by nucleophilic attack of thiols, other common donor constructs are activated by hydrolysis. For example, one of the most commonly used synthetic donors, GYY4137, is a phosphino-dithioate derived from Lawson’s reagent. GYY4137 undergoes slow hydrolysis in water to release H₂S (Scheme 2d), although the efficiency of sulfide release remains low. The rate of hydrolysis is pH dependent and slow at physiological pH, contrasting the rapid release of H₂S by inorganic salts.

For example, treatment of precontracted aortic rings with NaSH results in relaxation in 20 to 30 seconds, whereas GYY4137 treatment requires nearly 10 minutes to achieve similar relaxation.

Another common class of H₂S donors is based on 1,2-dithiole-3-thiones, with the most often used being 3-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH) (Scheme 2e). Although the mechanism of H₂S release from these scaffolds remains to be fully elucidated, one benefit of this donor motif is the ease with which it can be tethered to different molecules through ester or hydrolytically-stable amide linkages. For example, linkage of a triphenylphosphonium cation imparts mitochondrial targeting for AP39, whereas ligation to common non-steroidal anti-inflammatory drugs (NSAIDs) has resulted in significant reduction in GI damage scores. The use of ADT derivatives in NSAID applications highlight the potential therapeutic action of H₂S donors; however, insights into the H₂S release mechanism from such donors is poised to make significant impacts into our understanding of the therapeutic action of these donor motifs.

All of the above donor motifs provide sources of sulfide in aqueous solution, but researchers interested in H₂S/HS⁻ using biomimetic compounds in organic solvents also have access to organic-soluble sources of sulfide. In practice, H₂S gas is sufficiently soluble in organic solutions for most investigations; however, NaSH or Na₂S are generally insoluble in organic solvents. To aid in such investigations, we recently reported a simple method to prepare analytically-pure NBu₄SH, which provides access to an organic-soluble form of HS⁻. Such compounds offer a convenient source of HS⁻ in organic solution, enabling the separation of HS⁻ from H₂S in different bio(in)organic model studies, which is otherwise not possible in aqueous solution.

**H₂S detection and quantification**

Coincident with the increased biological importance of H₂S, new methods for H₂S quantification and detection are rapidly
emerging. Ranging from binary presence/absence tests based on the formation of PbS from Pb salts to complex molecular architectures aimed at real-time H$_2$S detection, analytical methods of H$_2$S determinations comprise an important area of H$_2$S research. We highlight here general classes of analytical tools for H$_2$S research and describe their general benefits and pitfalls. On the basis of the rapid expansion of new methods for H$_2$S detection and quantification, as well as revisions and refinements of current methods, we refer the interested reader to a number of recent reviews on these topics.$^{15,43}$

Quantification methods

Commonly used sulfide quantification methods typically require sample homogeneity, which is straightforward for biological fluids such as blood or serum, but significantly more complicated for naturally heterogeneous samples such as those from cell or tissue culture experiments. Spectrophotometric methods such as the methylene blue (MB) assay have constituted one of the classical methods of H$_2$S quantification. This method leverages the FeCl$_3$-catalyzed electrophilic aromatic substitution of p-dimethylamino aniline with sulfide to form MB, which has a characteristic absorbance at 670 nm and allows for sulfide quantification (Scheme 3a). Despite its widespread use, recent studies have demonstrated that the detection limit of the MB assay for sulfide is only 2 µM rather than previously-reported lower values, making it insufficiently sensitive to differentiate between sulfide levels in normal versus CSE$^{-/}$ mice.$^{44}$ Furthermore, the MB method is performed under highly acidic conditions, which can result in sulfite extraction from other biological sources and diminish the accuracy of sulfide levels measured with this method. Researchers new to the field of sulfide quantification will notice the large number of past studies using the MB method but are cautioned when comparing H$_2$S levels measured with this method, especially when more accurate contemporary methods are available. For example, the monobromobimane (mBB) method, which utilizes the reaction of two equivalents of mBB with H$_2$S under basic conditions to generate fluorescent sulfide dibimane (SdB), enables quantification of H$_2$S by fluorescence HPLC with a detection limit of 2 nM (Scheme 3b).$^{44}$ Because mBB reacts with both H$_2$S and thiols, separation of the fluorescent signals by HPLC is required for quantification. Despite this limitation, a significant benefit of this method is that workflows have been developed to analytically separate the free, reductant-labile/sulfane-sulfur, and acid-labile sulfide pools, enabling more detailed investigations across distributions in complex systems.$^{44}$ Additionally, the mBB method has been used more recently to also detect and quantify different poly- and persulfides, providing analytical information about these important reactive sulfur species.$^{45}$

Complementing spectrophotometric H$_2$S quantification methods are other analytical tools, such as sulfide selective electrodes. These tools can provide both the ability to quantify H$_2$S, based on a calibration curve, and provide real-time H$_2$S detection in solution. Readers interested in the differences in available sulfide electrodes are referred to a recent review focused on this topic.$^{43}$ In general, sulfide electrodes employ an ion-selective membrane that allows H$_2$S permeability for solution-based measurements. In the solution phase of the electrode, a strongly basic environment allows the dianionic sulfide ion to reduce Fe(CN)$_6^{3-}$ to Fe(CN)$_6^{4-}$, which is subsequently re-oxidized at the platinum electrode to produce a current relative to H$_2$S concentration. One advantage of sulfide electrodes is that they can be used directly in mammalian tissues; however, these electrodes cannot provide sub-cellular resolution of sulfide generation, storage, or transport. Even so, sulfide electrodes provide a convenient, time-resolved method for sulfide quantification or detection.$^{43}$

Detection methods

The development, refinement, and application of reaction-based probes for H$_2$S detection has expanded rapidly in the last few years. Such scaffolds typically couple various H$_2$S-selective reactions with different chromophores to generate a colorimetric or fluorescence response. Because of this design strategy, such methods are irreversible and the resultant methods measure accumulated probe activation rather than real-time H$_2$S dynamics. Despite these limitations, such constructs offer the potential to provide significantly higher spatiotemporal resolution than current H$_2$S quantification methods and also provide access to live cell and tissue imaging experiments. The predominant strategy for developing reaction-based probes for H$_2$S has been to utilize a fluorescence-quenching group on a fluorophore that can be modified or removed selectively by H$_2$S. As is common with most small molecule fluorescent probes, different detection strategies exhibit somewhat different selectivity patterns and each strategy may be better suited for certain types of investigations. The interested reader is referred to recent reviews on different aspects of reaction-based H$_2$S probe development.$^{15}$

The most common reaction-based detection strategy to date is H$_2$S-mediated azide (R-N$_3$) reduction (Scheme 4a)$^{17}$ Based on the ease of appending azides onto fluorogenic scaffolds, over 70 papers in the last few years described H$_2$S-detecting scaffolds using an azide to generate turn-on fluorescent probes (Scheme 4a)$^{46-48}$ Reduction of other oxidized nitrogen functional groups, such as nitro groups, has also been utilized for H$_2$S detection.$^{48}$ For most fluorophores, the azide moiety quenches fluorescence by a push–pull energy transfer mechanism although other quenching mechanisms are also possible. Although the selectivity of azides for reduction by H$_2$S rather than biological thiols

![](image)
is predominantly empirical, most azide-based probes provide
good to excellent selectivity for \( \text{H}_2\text{S} \) over other RSONS. A recent
mechanistic investigation established that \( \text{HS}^- \) is the active
species involved in \( \text{H}_2\text{S} \)-mediated azide reduction, and that
sulfide is oxidized to sulfane sulfur, such as \( \text{HSS}^- \), during probe
activation.\(^{49}\)

Further leveraging the high nucleophilicity of \( \text{HS}^- \), a variety
of \( \text{H}_2\text{S} \) sensing strategies have been developed in which sulfide
attacks an electrophilic, fluorescence-quenching group appended
on a fluorophore. Such scaffolds generally rely on the ability of
\( \text{H}_2\text{S} \) to participate in two sequential nucleophilic reactions to
remove or modify the protecting group. One benefit of this
strategy is that it imparts inherent selectivity because thiols,
which can only participate in one nucleophilic attack, are unable
to remove the protecting group.\(^{15}\) One consequence of this
selectivity, however, is that in many cases thiols can react with
the electrophilic scaffolds, thus effectively consuming the probe
prior to reaction with \( \text{H}_2\text{S} \). This general strategy for \( \text{H}_2\text{S} \) sensing
has been used in activated disulfides, \( \alpha\beta \)-unsaturated ketones,
and other activated electrophiles (Scheme 4b).\(^{50-52}\)

In addition to exploiting the high nucleophilicity of \( \text{HS}^- \) for
sensing, the high metallophilic nature of sulfur has also been
exploited.\(^{53}\) For example, ligation of a paramagnetic metal ion,
such as \( \text{Cu}^{2+} \), to a fluorescent molecule results in fluorescence
quenching. Subsequent reaction with \( \text{H}_2\text{S} \) results in \( \text{CuS} \)
formation and precipitation, thus releasing the fluorophore and
producing a turn-on response. This strategy is inherently
selective for sulfide over other biological thiols, but depending
on the ligand characteristics may also be prone to metal
reduction and release by cellular reductants or thiols, or
exchange of the quenching paramagnetic metal ion with more
prevalent diamagnetic ions like \( \text{Zn}^{2+} \).

Beyond \( \text{H}_2\text{S} \), polysulfides and persulfides are quickly becom-
ing reactive sulfur species of interest. In addition to contributing
to the biological chemistry associated with \( \text{H}_2\text{S} \), it is likely that
these sulfane-sulfur species provide signaling pathways distinct
to those associated with sulfide. Aligned with this importance,
reaction-based probes for polysulfides and other sulfane-sulfur
sources are now beginning to emerge.\(^{54,55}\) The development and
refinement of these, as well as other, tools appear poised to
provide complementary information on the different pools of
biological sulfide and further inform on the role of \( \text{H}_2\text{S} \) and
sulfane-sulfur in the RSONS landscape.

Conclusions and outlook

Hydrogen sulfide is an increasingly important biological molecule
joining NO and CO as a key signaling agent and adding a new level of
complexity to the RSONS landscape. Importantly, many of the
unanswered questions in the field lay at the interface of chemistry
and biology, thus requiring collaborative investigations and innova-
tion to develop, refine, and apply new investigative tools in this
rapidly expanding area of research. Key challenges include develop-
ing selective inhibitors of \( \text{H}_2\text{S} \) producing enzymes, generating new
classes of sulfide donors that do not consume cellular thiols for
activation or that can be programmed to release \( \text{H}_2\text{S} \) in response to
certain stimuli, and refining \( \text{H}_2\text{S} \) imaging methods so that they do
not irreversibly consume sulfide. As researchers progress toward
these goals, the toolbox of available methods for studying biological
\( \text{H}_2\text{S} \) will continue to grow and become more accessible to researchers
in adjacent fields (such as NO or CO biochemistry, redox biology, and
pharmacology), thus broadening the potential impact of \( \text{H}_2\text{S} \)-related
investigations in these diverse scientific communities.

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Scheme 4

Common motifs in reaction-based probes for \( \text{H}_2\text{S} \), including reactions based on (a) reduction, (b) nucleophilic attack, and (c) metal precipitation.
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Notes and references

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