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Review

Chemical aspects of hydrogen sulfide measurements in physiological samples ☆

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ABSTRACT

Background: Owing to recent discoveries of many hydrogen sulfide-mediated physiological processes, sulfide biology is in the focus of scientific research. However, the promiscuous chemical properties of sulfide pose complications for biological studies, which led to accumulation of controversial observations in the literature.
Scope of review: We intend to provide an overview of fundamental thermodynamic and kinetic features of sulfide redox- and coordination-chemical reactions and protonation equilibria in relation to its biological functions. In light of these chemical properties we review the strengths and limitations of the most commonly used sulfide detection methods and recently developed fluorescent probes. We also give a personal perspective on blood and tissue sulfide measurements based on proposed biomolecule–sulfide interactions and point out important chemical aspects of handling sulfide reagent solutions.
Major conclusions: The diverse chemistries of sulfide detection methods resulted in orders of magnitude differences in measured physiological sulfide levels. Investigations that were aimed to dissect the underlying molecular reasons responsible for these controversies made the important recognition that there are large sulfide reserves in biological systems. These sulfide pools are tightly regulated in a dynamic manner and they are likely to play a major role in regulation of endogenous-sulfide-mediated biological functions and avoiding toxic side effects.
General significance: Working with sulfide is challenging, because it requires considerable amounts of chemical knowledge to adequately handle reagent sulfide solutions and interpret biological observations. Therefore, we propose that a rigorous chemical approach could aid the reconciliation of the increasing number of controversies in sulfide biology. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

The discoveries that hydrogen sulfide[1] is produced endogenously [1] and that it is a potential neuromodulator [2] introduced a new era for sulfide biology, with exponentially increasing attention to its in vivo actions [3–9]. It is generated in virtually all studied organs during transsulfuration processes by *cystathionine-γ-lyase* (CSE) and *cystathionine-β-synthase* (CBS) [10] and via *3-mercaptopyruvate sulfurtransferase*-mediated (3MST) cysteine metabolism (see Scheme 1) [11]. On the other hand, sulfide catabolism is not well understood, but a major role for mitochondrial oxidation pathways is reported [12,13]. It is now well documented that sulfide is a modulator of pivotal physiological and pathophysiological functions in the gastrointestinal tract [14], brain [3], kidney [15] and vasculature [4] and its role is emerging in other organs too. Its physiological actions include regulation of inflammation [16–18], blood pressure [19], metabolic syndrome [20], energy production [21] and oxidative stress [5,22,23].

However, the promiscuous chemical properties of sulfide make it difficult to measure its physiological concentrations and to handle it as a reagent [24–26]. This resulted in huge discrepancies in reported sulfide levels in virtually all studied tissues and physiological fluids (see Tables S1 and S2) as well as in its biological functions. Therefore, major efforts are devoted to explain the increasing number of controversies that are accumulating in the sulfide literature. It is now accepted that significant amounts of "persulfide", "acid labile" and "alkaline labile" sulfide pools are available in biological systems [24–28]. In addition,

Abbreviations: 3MST, 3-mercaptopyruvate sulfurtransferase; CBS, cystathionine beta-synthase; CCO, cytochrome c oxidase; CSE, cystathionine gamma-lyase; Cys, L-cysteine; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); DTPA, diethylenetriamine-pentaacetic-acid; DTT, D,L-dithiotreitol; GC, gas chromatography; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; HSOH, sulfenic acid; MB, methylene blue method; MBB, monobromobimane method; PBS, phosphate buffered saline; RSOH, sulfenic acid derivative; RSSH, persulfide; ROS, reactive oxygen species; SDB, sulfide dibimane; TRIS, tris(hydroxymethyl)aminomethane

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[1] From now on we will use the term sulfide to refer to the sum of its different protonated forms that exist in solution, i.e. H_2S, HS^- and S^{2-}.

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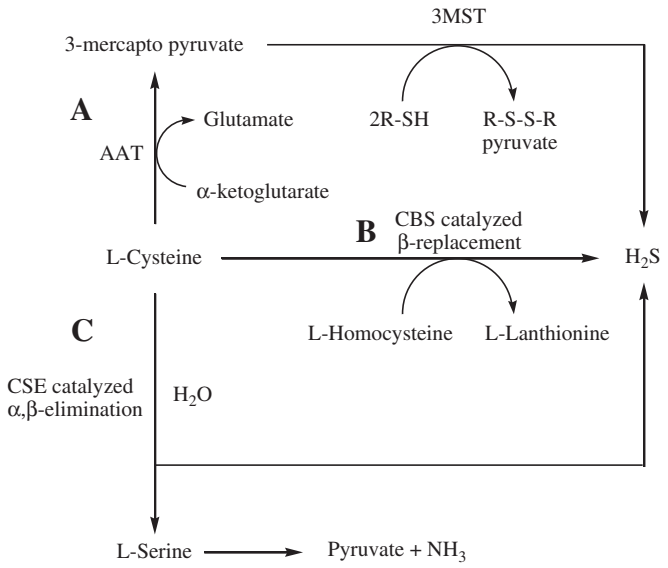


Scheme 1. Proposed pathways for sulfide generation by cysteine metabolism via three different enzyme catalyzed pathways. **A** Aspartate/cysteine aminotransferase (AAT) catalyzes the transamination reaction between cysteine and α-ketoglutarate to form 3-mercaptopyruvate, from which the sulfur is transferred to an active site Cys residue of 3-mercaptopyruvate sulfurtransferase (3MST) to give a persulfide derivative. 3MST persulfide is reduced by thiols to give sulfide. **B** Among the sulfide producing catalytic reactions of cystathionine β-synthase (CBS), β-replacement between cysteine and homocysteine was proposed to be the most kinetically favorable under physiological conditions. **C** Sulfide production via cysteine metabolism by cystathionine γ-lyase (CSE) is most efficient by an α,β-elimination reaction, which generates pyruvate and ammonia (via serine) beside sulfide.

physiological sulfide concentrations were determined with a plethora of different techniques, (reviewed in [23–25,27,29]), which operate under very different experimental conditions and therefore liberate sulfide from these pools with different efficacies. Furthermore, polysulfides (that are the dominant sulfide oxidation products in aqueous solutions) are reported to be responsible for some of the observed biological actions of sulfide that are governed via protein sulfhydration reactions [30,31]. Although these recognitions provide some explanations, in order to adequately reconcile controversial biological observations, a better understanding of the chemical properties of sulfide is needed.

In this review we discuss the methodologies that are most frequently used to measure physiological sulfide levels and provide a summary of recently developed fluorescent probes from a rigorous chemical perspective. In addition, we discuss the chemical reactions of sulfide that are most likely to play important roles in its detection and biological actions and give practical advice on how to handle reagent sulfide solutions.

2. Solution chemistry of sulfide

Sulfur is a chalcogen element in group 16 of the periodic table, positioned right below oxygen with an electron configuration of $1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^4$. This configuration corresponds to 6 valence electrons and a vacant 3d orbital, which is the reason why sulfur can obtain oxidation states anywhere between −2 to +6. The oxidation state of the sulfide sulfur is −2 and therefore it is a reductant species that cannot be reduced further. The structure of H_2S is similar to that of H_2O, but the two molecules have very different chemical and physical properties. H_2S does not form H-bonds, therefore it is a gas at ambient conditions, is toxic at relatively high concentrations and has a distinct odor. H_2S is heavier than air and dissolves readily in water (solubility ~100 mM at 25 °C) [32]. Due to the strong nucleophilic character of its sulfur center sulfide engages in many different chemical reactions. The most well studied reactions that have already been shown (or proposed) to be important in its biological actions are: 1) reduction of reactive oxygen species (ROS) and disulfide bonds and 2) coordination to metal centers. In addition, its role in electrophile sulfhydration via nucleophilic addition is emerging [33].

2.1. Protonation equilibria

Sulfide solutions are mostly prepared via dissolving sulfide salts or via bubbling H_2S gas into aqueous media. Different sulfide salts of heavy metals with soft characters e.g. PbS and Ag_2S are sparingly soluble (for example at pH 7 solubility of HS^- in 1 mM Pb^{2+} is 6×10^{-20} M and of Ag_2S on an electrode surface is 6×10^{-15} M, respectively), while NaHS and Na_2S are very soluble in water. Therefore, the latter two are often used in biological studies to make reagent sulfide solutions. The aqueous solutions that are made by dissolving these salts are often called H_2S donors and some investigators even measured the rate of sulfide release by these molecules (in Ref. [34] a slow sulfide release was suggested, but Refs. [35,36] showed that H_2S forms upon crystal dissolution). From a chemical perspective the dissolution of these salts is accompanied by dissociation to give solvated Na^+ and HS^- or S^{2-} ions (with solvation shells that may consist of several layers of water molecules) and therefore this is the actual process that introduces sulfide into the solution. These anions are Brönsted bases (S^{2-} is an especially strong one) therefore, upon dissolution acid base reactions with water (e.g. $S^{2-} + H_2O \rightleftharpoons HS^- + OH^-$) take place, which can shift the pH of (even buffered) aqueous solutions. However, under similar conditions (pH, temperature, pressure, etc.), bubbling pure H_2S gas or dissolving high purity sulfide salts in well buffered aqueous solutions results in a similar distribution of HS^- and H_2S. On the other hand, partitioning of sulfide in its different protonation states (i.e. speciation) strongly depends on the applied conditions (especially on the pH). In water solution of sulfide the following protonation equilibria exist:

$$H_2S \rightleftharpoons HS^- + H^+ \quad pK_a^{H_2S} = 7.05[37] \tag{1}$$

$$HS^- \rightleftharpoons S^{2-} + H^+ \quad pK_a^{HS^-} > 14 \tag{2}$$

$$K_a^{H_2S} = [HS^-][H^+]/[H_2S] \tag{3}$$

$$K_a^{HS^-} = [S^{2-}][H^+]/[HS^-]. \tag{4}$$

The above equations (Eqs. (1)–(4)) determine the relative ratios of its protonation forms (might be called protonation isomers) at the actual pH, where the pK_a values change with temperature, pressure and ionic strength (the ionic strength is determined by the total concentrations of solvated ions including the buffer). Several values were reported for $pK_a^{HS^-}$ in the literature in the range of $13 > pK_a^{HS^-} > 19$. However, it is challenging (if not practically impossible) to measure pK_a values in this range, because the concentration of OH^- is already 1 M at pH 14. Fig. 1 shows the speciation of sulfide as a function of pH using $pK_a^{H_2S} = 7.05$ and $pK_a^{HS^-} = 15$. Under these conditions at pH 7.4 the percent distribution of H_2S:HS^-:S^{2-} will be 30:70:0.000002, respectively. Although, this shows that the equilibrium concentration of S^{2-} under physiological conditions is very low, it does not necessarily mean that it cannot be the actual reactive species (for an example see the detection of sulfide with sulfide selective electrodes, Section 3.3).

Due to the shift in $pK_a^{H_2S}$ by increasing the temperature from 20 °C to 37 °C, it has been demonstrated that the estimated amount of dissolved H_2S drops by as much as 30% (and the concentration of HS^- increases

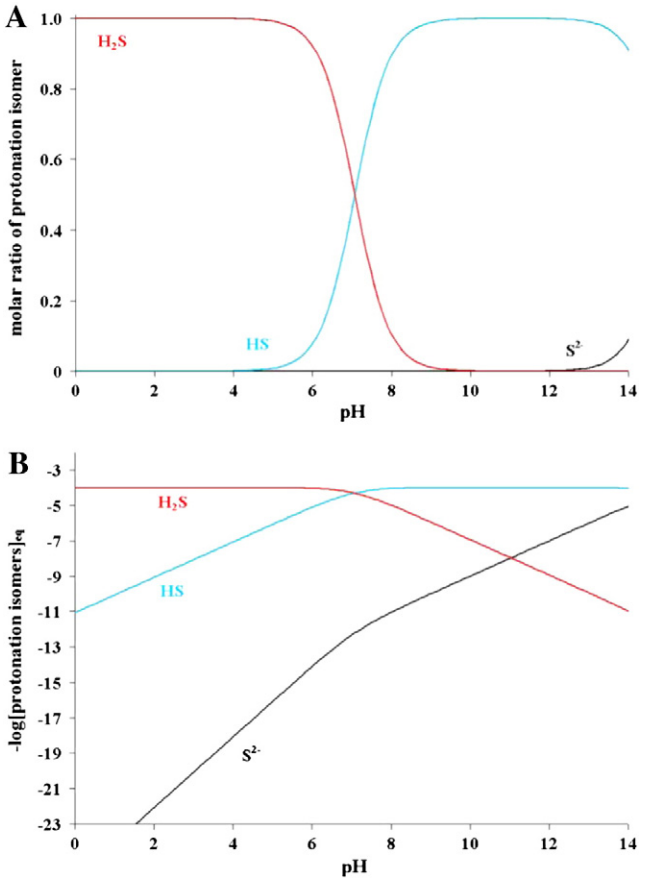


Fig. 1. Speciation in aqueous sulfide solutions. Calculated concentration distribution diagrams for the different protonation isomers as a function of pH shown in **A** molar ratio or — **B** log equilibrium concentration. Calculations were made for 100 μM total sulfide using $pK_a^{H_2S} = 7.05$ and $pK_a^{HS^-} = 15$.

by 30%) [25,27]. However, based on principal chemical grounds, this does not refer to the total available sulfide concentration, but only to a change in the distribution of the different protonation states. Because in some reactions the major player is H_2S (i.e. reaction (5)) and in others it is HS^- (i.e. reaction (6)), this shift in the protonation equilibrium will in fact change the steady-state concentration of the actual active form.

$$H_2S + \text{Reaction partner} \xrightarrow{k_5} \text{Products} \tag{5}$$

$$HS^- + \text{Reaction partner} \xrightarrow{k_6} \text{Products}. \tag{6}$$

For example, if sulfide is consumed in a nucleophilic (addition, substitution or redox) reaction than the better nucleophile HS^- will be the more reactive form and the reaction is expected to proceed via this species (reaction (6)). However, during the course of the reaction, more HS^- is supplied by the deprotonation of the available H_2S to attain equilibrium (1) (which together with Eq. (3) indicates that the relative ratio of H_2S and HS^- is constant in buffered solutions). Therefore, at an excess of the reaction partner (which is mostly the case in biological situations) the total amount of available sulfide will be consumed at both temperatures, albeit by different rates. For example, if the pre-equilibrium assumption[2] can be used for the protonation equilibrium (reaction (1)) and the reaction solely proceeds via HS^- (reaction (6)) than the rate law will have the following form:

$$d[\text{Product}]/dt = k_{app}[\text{Reaction partner}][\text{Sulfide}]_{tot} \quad \text{where } k_{app} = k_6 K_a^{H_2S}/\left(K_a^{H_2S} + [H^+]\right). \tag{7}$$

Eq. (7) clearly shows that the total amount of sulfide will be consumed at an excess of the reaction partner, but the apparent second order rate constant (k_{app}) at a given pH will depend on the $pK_a^{H_2S}$ value, which will change with the temperature.

Another good example for the interpretation of the shift in the protonation state of sulfide with the temperature is the penetration of sulfide into cells. It has been shown that the uncharged H_2S freely enters cellular membranes but HS^- does not [38]. The above shift in the protonation equilibrium of sulfide by the temperature does not mean that at 37 °C 30% less sulfide will enter the cell, but it means (based on similar grounds as discussed for the nucleophilic reactions) that the rate of membrane transport is expected to be slower at higher temperatures due to the lower steady state concentrations of the cell permeable H_2S form during the process (unless other rate enhancing factors come into play at higher temperatures).

2.2. Redox reactions

As noted above sulfide is a reducing agent. It readily engages in 1 and 2 electron redox reactions. Although many reports on radical mediated sulfide oxidation reactions are available in the chemistry literature, the mechanisms in most cases are still debated (for example its reaction with O_2 [39–41] or with intermediate radical species in its reaction with peroxynitrite [42,43]). This is due to the fact that radical species are highly reactive and they can initiate chain reactions, which complicates the kinetic analyses. The first sulfide derived intermediate that forms in $1e^-$ oxidation processes is the highly reactive HS• radical. The biological significance of these reactions or the HS• radical is not well studied, and therefore we do not discuss these reactions in detail. However, due to practical reasons in relation with the air oxidation of sulfide stock solutions it is worth mentioning that the $1e^-$ oxidation of sulfide by oxygen is thermodynamically unfavorable [40]. Although a two electron oxygen-mediated sulfide oxidation reaction would be thermodynamically favorable it has a serious kinetic barrier due to the spin pairing problem with O_2 [40]. Therefore, sulfide oxidation by O_2 only occurs at a reasonable time scale in the presence of transition metal ion catalysts [44].

Sulfide engages in $2e^-$ redox reactions with most of the biologically important ROS and an oxidant scavenging role was proposed for sulfide in various biological situations ranging from attenuation of hepatic or cardiac ischemia–reperfusion injury [45,46], through oxidative damage in the brain [47,48] and in the gastric mucosa [49], inhibition of atherogenic modifications by HOCl [50] or alleviation of peroxinitrite cytotoxicity [51]. However, to have an antioxidant role in vivo, these reactions need to be kinetically favored under biological conditions. Although the reaction of sulfide with HOCl is very fast (with a second order rate constant of $2 \times 10^9\ M^{-1}\ s^{-1}$that is close to the diffusion controlled limit [52]), sulfide does not show outstanding reactivities with other ROS [42,53,54]. In addition, biological sulfide concentrations (even at the largest reported values) are relatively low compared to protein thiol and reduced glutathione (GSH) concentrations and therefore it is unlikely that sulfide will have a direct protecting role against oxidative stress in an in vivo situation. On the other hand we propose that oxidants could modulate the actions of sulfide via redox reactions to give a range of bioactive products. This is highlighted by the fact that a major role is proposed for persulfides (that are sulfide oxidation products) in sulfide mediated signaling events [55–59]. In relation to protein sulfhydration it is important to emphasize that the formation of a persulfide moiety on Cys residues

[2] The rates of protonation and deprotonation reactions are usually fast and therefore the pre-equilibrium assumption is often used for protonation equilibria in kinetic studies. This basically means that the equilibrium readily responds to a change in the conditions (e.g. loss of H_2S via volatilization or loss of HS^- in a chemical reaction; change in the pH or temperature) and proton exchange reactions are seldom rate determining in aqueous media.

cannot be due to the interaction of sulfide and the Cys thiol, because sulfur is in its lowest oxidation state (of −2) in both species. Therefore, sulfide-mediated sulfhydration requires one molar oxidant equivalent [52]. Several mechanisms were proposed for protein persulfide formation but the available experimental data is very limited, and therefore further mechanistic studies are highly desired in order to better understand sulfide mediated redox signaling [60]. On Scheme 2 we summarize what we consider the most feasible reaction pathways for protein persulfide formation and sulfide liberation from persulfide species. We suggest that sulfide-mediated redox signaling events can occur via three major reaction cycles: A) direct oxidation of Cys residues (the kinetics and mechanisms of thiol oxidation reactions are reviewed in [61]), B) reduction of protein-disulfides by sulfide or C) via polysulfides (that are oxidation products of sulfide). In addition, persulfides are in equilibrium with trisulfide and possibly longer polysulfide chain containing Cys-derivatives [62]. Although the underlying molecular mechanisms of sulfide oxidation processes are not well understood, we mention a few general chemical considerations in relation to the reported biological actions of sulfide:

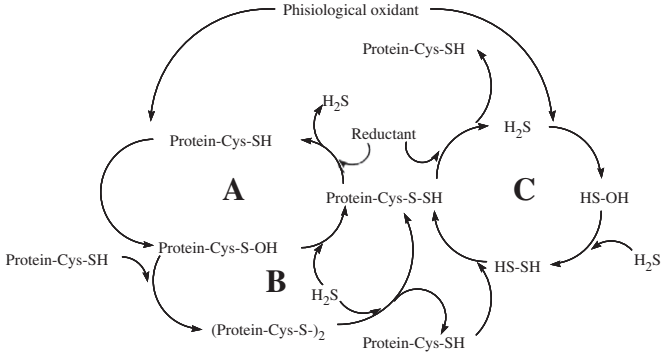
In analogy with cysteine, it is expected that the first product of $2e^-$ sulfide oxidation reactions will be the sulfenic acid (HSOH) [61,63]. Owing to its high reactivity, this species has not been detected in solution (to the best of our knowledge), but extensive studies were carried out for its characterization in the gas phase [64,65]. Due to the intermediate "0" oxidation state of sulfur in HSOH, it can serve as either an oxidant or a reductant species. Therefore, the fate of HSOH can either be reaction with another nucleophile (e.g. Cys or HS^-) or with another oxidant molecule (depending on the relative reactivities and concentrations of the reactants). The former reactions will give rise to a persulfide (RSS^-; reaction (8)) or a disulfide (HSS^-; reaction (9)) species, respectively. HSSH (or HSS^-) is not stable under physiological conditions [66], and it rapidly disproportionates to polysulfides (reaction (10); Nagy et al. unpublished results and Ref. [52]).

$$HSOH + RS^- \rightleftharpoons RSS^- + H_2O \tag{8}$$

$$HSOH + HS^- \rightleftharpoons HSS^- + H_2O \tag{9}$$

$$nHSS^- \rightleftharpoons HS^-_{(n+1)} + (n-1)HS^- (n = 1-9). \tag{10}$$

Distribution of polysulfide derivatives mostly depends on the pH, and the total sulfide vs. oxidant concentration ratios. In general, higher pH and higher sulfide to oxidant ratios favor the formation of polysulfides with lower numbers of zerovalent sulfur atoms [66].



Scheme 2. Three potential redox cycles for protein persulfide formation in relation to sulfide-mediated cell signaling. The persulfide species can be generated by **A** direct oxidation of the protein Cys, **B** disulfide exchange or **C** direct oxidation of sulfide. The depicted reactions are likely to represent a dynamic equilibrium system where the distribution of the oxidizing equivalents in equilibrium will depend on thermodynamic parameters such as the relative reductant to oxidant ratios, the redox potentials of the appropriate redox couples, pH and the concentrations of thiol derivatives (Cys residues and H_2S) relative to each other.

Two recent publications demonstrate important roles for polysulfides in sulfide-mediated biological functions, and suggest a major mechanism for persulfide formation via these species [30,31]. An important aspect of polysulfide induced biological effects is the fact that a common contamination in aged sulfide stock solutions (see Section 6) is expected to be polysulfide species that are generated via metal ion catalyzed oxidation reactions of sulfide by dissolved oxygen. Sulfide is a reductant and polysulfides are expected to behave as oxidant species in biological systems (where the redox potential of the polysulfide largely depends on the number of zerovalent sulfur atoms). Therefore, they are likely to induce vastly different biological processes and hence, some of the controversial biological observation could be due to polysulfide- versus sulfide-mediated actions. In fact, we have observed very efficient inactivation of the tumor suppressor *phosphatase or tensin homolog* (PTEN) protein via sulfhydration of the active site Cys71 and Cys124 residues by sulfide solutions that only contained traces amounts of polysulfide species, which suggests that extreme caution is needed when investigating (or interpreting) sulfide mediated biological events. Therefore, in future studies it is fundamental to 1) separate sulfide and polysulfide mediated events by undertaking adequate control experiments and 2) to understand, whether polysulfide induced biological processes are a result of endogenous or exogenous polysulfide species.

The HSOH intermediate can be further oxidized at larger oxidant concentrations in reactions that favor the formation of oxysulfur species (such as thiosulfate $S_2O_3^{2-}$, tetrathionate $S_4O_6^{2-}$, sulfite SO_3^{2-} or sulfate SO_4^{2-}). The distribution of polythionates will also depend on the pH, on the ratios of sulfide vs. oxidant concentrations as well as on the nature of the oxidant species. Sulfite, thiosulfate and sulfate were reported to be the major sulfide oxidation products i) in mammalian and in vertebrate mitochondria [12,13] and ii) of activated neutrophil white blood cells [67]. In addition, thiosulfate measurement in urine and blood samples has been used as a forensic marker of sulfide poisoning [68,69]. Furthermore, inhaled H_2S leads to sulfite and sulfate formation in the lung [70] and sulfide treatment of liver, plasma, colon or muscle tissue resulted in thiosulfate and sulfate production. Sulfate is relatively inert, but sulfite, thiosulfate and tetrathionate are expected to be reactive under biological conditions and hence they can be viewed as bioactive sulfide metabolites.

The above mentioned biological observations call for a better understanding of sulfide redox biology, for which a systematic elucidation of the kinetics and mechanisms of ROS mediated sulfide oxidation reactions is fundamental.

2.3. Coordination chemistry

Beside the rich redox chemistry of sulfide it also coordinates efficiently to transition metal ions. Due to the soft character of sulfur it binds tightly to copper centers. In fact, this chemical property is utilized in the development of sulfide specific fluorescence probes (see Section 4.3). Cu–S coordination has also been shown to have physiological significance. For example, it has been demonstrated recently that the extensive smell of volatile sulfur compounds even at very low concentrations is due to copper ion-mediated activation of odorant receptors via sulfur–copper coordination chemical interactions [71]. In addition, the toxic nature of sulfide is associated with the inhibition of the mitochondrial respiratory chain protein, *cytochrome c oxidase* (CCO), with a mechanism that involves reduction and tight sulfide coordination to its copper center (reviewed in [72]). Sulfide binding to the Fe^{III} center of the CCO heme prosthetic group and its sulfide-mediated reduction has also been well documented [72]. In fact the interesting property of sulfide to induce a hibernation like state [73] was associated with a reversible inhibition of CCO via fine tuned sulfide coordination chemical mechanisms that involve reactions with both the copper and iron centers [72,74].

Extensive studies on the reactions of sulfide with the heme centers of hemoglobin and myoglobin to form sulfhemoglobin or sulfmyoglobin have revealed a unique structure for these compounds, where the sulfur is incorporated into one of the pyrrole rings of the heme porphyrin [75,76] (see Structure 3 in Fig. 2), but the mechanisms of sulfhemoglobin and sulfmyoglobin formations are not well understood. However, the requirement of oxygen and the possible formation of these species with H_2O_2 suggest the involvement of oxy-ferryl complexes. This highlights the fact that sulfur can interact with different enzyme forms via different chemical mechanisms. In general, the reactivity and mechanism of sulfide–metal interactions depend on: 1) accessibility of the active site, 2) nature and abundance of different enzyme forms (such as the different oxy- forms of the heme iron e.g. Comp. I or Comp. II), 3) total sulfide concentrations (as well as metal to sulfide ratios), and 4) chemical properties of neighboring functional groups (in particular the polarity of the active site environment and whether they are electron withdrawing vs. electron donating, have H-bond donor or acceptor properties). From the perspective of sulfide distribution in biological sulfide pools the interactions of sulfide with metal centers can involve 1) reversible week coordination, 2) redox reactions to give reduced-metal–sulfide complexes and polysulfides or polythionates, and 3) incorporation of sulfide into a heme pyrrol ring via carbon–sulfur covalent bonds. Importantly, very different conditions are required to liberate sulfide from these modifications. For example, the hard nature of Fe^{III} as opposed to the soft sulfide ligand is expected to give rise to labile Fe^{III}–sulfide complexes (see Structure 1 in Fig. 2). On the other hand, Fe^{II} has a so-called "borderline" character (in between soft and hard) and therefore it is expected to bind sulfur more tightly (see Structure 2 in Fig. 2) and liberation of sulfide from covalent C–S interactions (such as sulfhemoglobin or sulfmyoglobin; e.g. Structure 3 in Fig. 2) would require harsh experimental conditions.

3. Most commonly used sulfide detection methods

The above described rich chemistry of sulfide, allowed the development of a plethora of different methods for its detection (also reviewed in [23–25,27,29,77]). However, due to the promiscuous nature of its chemical properties, it is problematic to achieve adequate specificity and selectivity. In this section we briefly describe some of the most commonly used sulfide detection methods and discuss their strengths and limitations based on chemical grounds. We have established some of these methods in our laboratory and therefore we also give a personal perspective with regard to practicality and point out potential pitfalls.

It is important to emphasize that with adequate caution all of these methods can be used to measure sulfide concentrations in simple solutions (e.g. when sulfide is used as a reagent). However, they provide very different sulfide concentrations in biological samples due to the differences i) in the nature of the chemical reactions that are utilized to detect sulfide and ii) in the applied experimental conditions. It is now well accepted that beside free sulfide a large pool of biomolecule-bound sulfide is present in biological systems (for potential biomolecule–sulfide interactions see Section 5.1) that can be liberated by for example acidification, alkalization or under reducing conditions. These modifications from normal physiological conditions perturb biomolecule–sulfide binding (and sulfide release) equilibria in different ways (for more details see Section 5.2 and the chemical background is discussed in Section 2), which is most likely the reason of the large discrepancy in physiological sulfide levels measured by the following techniques. The methylene blue method (MB; Section 3.1) is conducted under strongly acidic conditions and measures relatively large sulfide concentrations in biological samples by liberating acid labile sulfide. The monobromobimane (MBB; Section 3.2) and gas chromatography (GC; Section 3.4) based measurements can be performed at physiological pH and hence provide smaller numbers. However, due to irreversible sulfide binding or shifts in phase transition equilibria, respectively, these methods also potentially liberate loosely-bound sulfide. Ion selective electrodes (ISE; Section 3.3) have the advantage of specifically measuring the levels of free sulfide due to the fact that they utilize only a small fraction of the non-bound physiological sulfide pools. In general, this section highlights the chemical features of these methods and in Section 5 we further elaborate on the possible reasons for the huge deviations of measured sulfide concentrations in biological specimens.

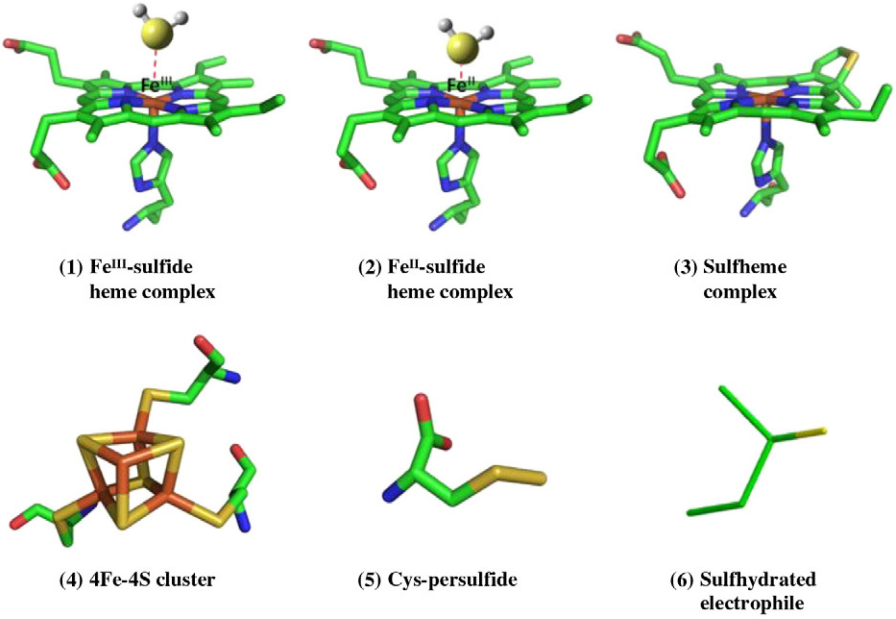
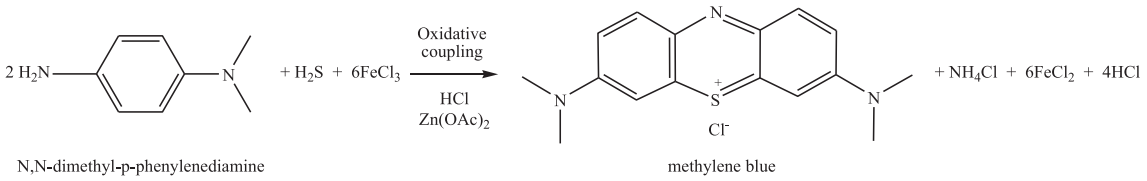


Fig. 2. Some proposed sulfide–biomolecule interactions. (1) Coordination of sulfide to an Fe^{III} heme center. (2) Coordination of sulfide to an Fe^{II} heme center. (3) Incorporation of sulfide into a pyrrole ring of a heme prosthetic group. (4) A 4Fe–4S cluster from an activated aconitase. (5) Crystal structure of a stabilized Cys-persulfide at the active site of cysteine C–S lyase from *Synechocystis*. (6) Proposed structure of a sulfhydrated electrophile. Structures were generated by using PDB files 1MBO [157] for (1) and (2), 1YMC [158] for (3), 6ACN [159] for (4) and 1ELU [160] for (5).



Scheme 3. Detection of sulfide by the methylene blue method. After $Zn(OAc)_2$ precipitation of sulfide ZnS is redissolved under acidic conditions, where N,N-dimethyl-p-phenylenediamine reacts in an oxidative coupling reaction with sulfide to give methylene blue. The oxidizing equivalents are provided by Fe^{3+}.

3.1. The methylene blue method

This is probably the most commonly used method for sulfide measurement both in stock solutions and biological samples. The method is based on the reaction of sulfide with *N,N-dimethyl-p-phenylenediamine*, in a ferric chloride catalyzed reaction with a 1:2 stoichiometric ratio to give the methylene blue dye, which is detected spectrophotometrically (see Scheme 3). A weakness of the method (and all other methods that rely on colorimetric detection) is interference of other chromophores in the sample. Chromatographic separation was proposed to be a possible solution to overcome this problem and increase the level of sensitivity, (see e.g. [78–81]), which can be further improved by replacing the spectrophotometer detector with a mass spectrometer (see e.g. [82]). Another possibility to separate sulfide from other chromophores is via precipitation by Zn-acetate, which is currently the most widely used technique. The ZnS precipitate is than redissolved under highly acidic conditions in the presence of *N,N-dimethyl-p-phenylenediamine*, and methylene blue is detected spectrophotometrically [83]. The molar extinction coefficient of methylene blue at 667 nm in water solution is 71,000 M^{-1} cm^{-1} [24,84], but it drops under acidic conditions (see later). Therefore, the lower limit of detection by this method is around 1 μM (in a 1 cm cell), but at higher sulfide concentrations deviations from linearity was observed. This was suggested to be the result of methylene blue dimer and trimer formation [24]. Although the metachromatic nature of methylene blue due to formation of water assisted aggregates has been extensively studied, these investigations were carried out in pure water solutions [84,85]. A highly cited paper in the oceanography literature demonstrated that the different slopes of the calibration curves that were obtained for sulfide measurements are apparently due to the fact that at higher sulfide concentration a dilution of the final methylene blue solution is required in order to keep the absorbance values below 1 (which is a requirement to obey the Lambert–Beer law). This dilution is often made by water, which shifts the final pH and causes a significant change in the molar extinction coefficient of the dye in the 0–2 pH region[3] [86]. Because acidification of the samples is required to redissolve the ZnS precipitate, this is the exact pH range, where the measurements are carried out for sulfide detection. They also showed that by correcting for the shift in the pH (or adjusting the molar extinction coefficient of methylene blue) the method measures sulfide levels with less than 3% deviation in a wide concentration range (1–1000 μM) [86]. Another important aspect of this fact is that the final pH upon acidification will also depend on the buffer (because it consumes protons). Therefore it is important to develop the calibration curves for sulfide detection under exactly the same conditions with the sample solutions.

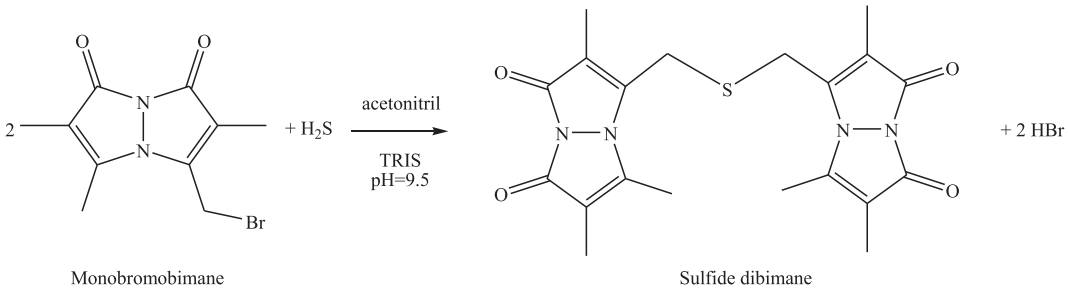
3.2. The monobromobimane method

Following up on Fahey's methodology to detect biological thiols, a fluorescent detection of sulfide after derivatization with monobromobimane was reported [87]. This method is based on consecutive nucleophilic substitution reactions of sulfide with two monobromobimane molecules to give the stable and highly fluorescent sulfide dibimane (SDB) product (see Scheme 4). SDB can be separated from monobromobimane by reverse phase chromatography, because it contains 2 hydrophobic bimane residues. Detection of SDB is achieved by a fluorescence detector with excitation and emission wavelengths of 390 and 475 nm, respectively. The method was further improved for optimal sulfide detection in biological samples [26,28,88,89], and the limit of detection was suggested to be down to the low nM region [26]. However, the calibration curves were developed by a serial dilution of solutions that were made from authentic SDB in the absence of substantial monobromobimane. Notwithstanding, we have observed that in real samples, where a large concentration of monobromobimane is present (5–10 mM reagent is required to achieve adequate derivatization within 30 min), quantification of sulfide can interfere with the monobromobimane peak front below 1 μM. However, the sensitivity can be improved by extraction of SDB into ethyl acetate [26,89]. The method has been used to detect "persulfide" and "acid labile" sulfide based on reduction by tris(2-carboxyethyl)phosphine (TCEP) or sample acidification, respectively [28]. We have used this method to measure the amounts of polysulfides that form during the reactions of sulfide with different oxidants. This was achieved by measuring the increase in sulfide concentrations in the reaction mixtures upon the reduction of polysulfide products with dithiotreitol (DTT; unpublished results). For example, using this method we corroborated our previous observation [52] that a close to complete conversion of sulfide into polysulfides can be achieved upon reaction with HOCl at a 4–5 molar excess of sulfide [52].

3.3. Sulfide selective electrodes

Sulfide-ion selective electrodes have been extensively used for measuring sulfide concentrations in biological systems (e.g. [19,90–96]). Measurements are usually performed against a glass pH electrode and hence the measured electromotive force will not only depend on sulfide concentrations but also on the pH of the solution [97]. Therefore, it is important to keep the pH constant during the measurements and develop the calibration curves under strictly similar conditions. A major disadvantage of the method was suggested to be the requirement of reducing alkaline conditions (by an often called "antioxidant buffer" e.g. [92,94,95]), which was explained by the fact that the electrode measures S^{2-} ions, which only has reasonable abundance at high pH [25,27]. However, it has been shown that the electrode gives a linear response down to 10^{-15} M total sulfide at pH 7 (which in fact does not necessarily mean that it measures HS^- and not S^{2-} ions, but reflects extraordinary sensitivity) [98]. Such low sulfide concentrations cannot be obtained by a serial dilution of standard solutions, because trace metal ion contaminations consume sulfide in the reaction vessel. Although metal chelators and ascorbate decrease the extent of sulfide loss, even in their presence sulfide titration curves deviate from linearity at <1 μM concentrations [97]. We have observed a similar phenomenon in phosphate buffered saline (PBS), but linearity was maintained down to 0.3 μM sulfide levels in tris(hydroxymethyl)aminomethane (TRIS) buffer, suggesting lower levels of metal ion contamination in TRIS compared to PBS (see Fig. 3). Searcy and Peterson could obtain their previously mentioned linear response in the very low 1 fM–

[3] The change in the molar extinction coefficient of methylene blue with the pH can either be a result of shifting protonation equilibria or a pH dependent formation of aggregates.



Scheme 4. Detection of sulfide by the monobromobimane method. 2 Monobromobimane molecules react with sulfide in consecutive nucleophilic substitution reactions to give the fluorescent sulfide dibimane.

1 μM concentration range with an insightful method of using saturated solutions of sparingly soluble metal salts, which was a clear demonstration of the extraordinary sensitivity of this method [98]. Further advantages of the method are 1) that it does not require sulfide derivatization and therefore it measures free sulfide concentrations (for further explanation see Section 5.2) and 2) that due to the fast electrode response (often less than a minute) it allows dynamic measurements of sulfide concentrations. However, measurements are perturbed in the presence of biomolecules, such as thiols (a phenomenon that we confirmed in our laboratory), most likely due to interference with the Ag^+/Ag_2S system on the electrode surface, representing a major disadvantage for its application in biological systems. Another disadvantage of the method is the fact that due to formation of Ag_2S on the electrode surface, it needs to be reconditioned (typically for 1 h in 5 mM Na_2S) and calibrated daily.

To overcome the interference with biomolecules and problems associated with frequent reconditioning, a polarographic sulfide sensor was developed, which has a sulfide selective membrane to protect the cathode, anode and electrolyte from solution constituents [99,100]. This technology conserved all of the above mentioned advantages of sulfide selective electrodes and it allows detection of free sulfide levels in a dynamic fashion even in in vivo models (see e.g. [25,27,35,89,99]).

3.4. Gas chromatography

Initially gas chromatographic analysis of sulfide was performed after derivatization to bis(pentafluorobenzyl)sulfide [101]. The product was extracted into organic solvents, followed by GC analysis of the organic phase. This technology was used to study blood sulfide concentrations after alkalization [102] and in forensic studies for sulfide poisoning post mortem [102,103]. Further development of the method allowed detection of protein bound persulfide moieties or polysulfides after reduction at pH 5, alkylation and extraction into hexane [104]. Although this latter method responds to albumin bound sulfide down to 0.3 μM, it could not detect measurable persulfide concentrations in rat or human plasma. However, significant amount of protein sulfhydration was detected in rat tissue samples (see Table S2) [104]. Measurement of tissue sulfide concentrations was also investigated by direct analysis of the headspace H_2S gas (without derivatization) after tissue homogenization and acidification with a detection limit of 6 pmol total H_2S [105]. On the other hand, Levitt's research group measured much lower mouse tissue sulfide levels at pH 5.7 by headspace GC analysis albeit using different experimental conditions and sample preparation method (see Table S2) [106]. It was pointed out recently that in tissue samples continuous sulfide producing and consuming reactions operate (with different relative rates) and therefore these measurements most likely correspond to steady state sulfide concentrations [107]. The same study demonstrated an improved procedure for dynamic sulfide measurement in murine tissue samples by GC. However, a disadvantage of measuring headspace sulfide concentrations was proposed to be the relatively long incubation times that are needed for gas evolution in the headspace, which could make real-time measurements problematic [25]. More specifically, it is important to separate the kinetics of sulfide metabolism from H_2S volatilization. This can be challenging, because the equilibrium between solvated and gas phase H_2S largely depends on the experimental conditions (i.e., temperature, pressure, pH, chemical composition of the gas phase, nature and relative rates of sulfide consuming and producing reactions, concentration of biomolecules that reversibly bind sulfide and the equilibrium constants of these reactions in the liquid phase, etc.).

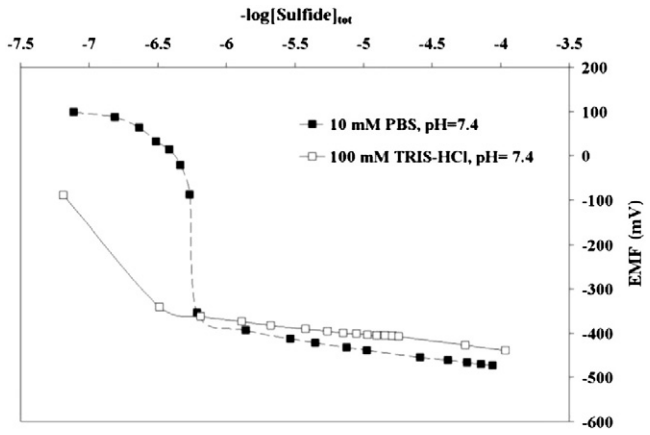


Fig. 3. Calibration curves obtained for a sulfide selective electrode in different buffers. Calibration of a Radelkis OP-S-O711P sulfide selective electrode in the 100 nM–100 μM concentration range, at room temperature against a Methrom 6.0106.100 glass electrode (used as a reference electrode).

4. Fluorescent probes

The above mentioned technologies mostly require the extraction of sulfide from cells or tissues, during which the biological and chemical environment changes significantly. In order to better understand the biological roles of sulfide it is instrumental to build robust imaging technologies that allow dynamic detection of endogenous sulfide in intact, live biological systems without significant perturbation of normal functions. As a result of this recognition a significant effort was devoted to build specific and sensitive fluorescent sulfide probes in the last couple of years. The major requirements for a probe to give the most realistic picture of endogenous sulfide actions are to 1) be highly specific, 2) have adequate sensitivity with a linear response, 3) be membrane permeable, 4) respond quickly, 5) not be photosensitive, 6) give a stable florescence signal in time, 7) be inert towards other biological molecules and 8) do not affect biological functions. Due to the previously discussed promiscuous chemical properties of sulfide a number of strategies are available to visualize this reactive molecule. However, for the same reason it is a difficult task (if not impossible) to fulfill all the above criteria. The most widely applied strategies to capture sulfide is by utilization of its redox, coordination

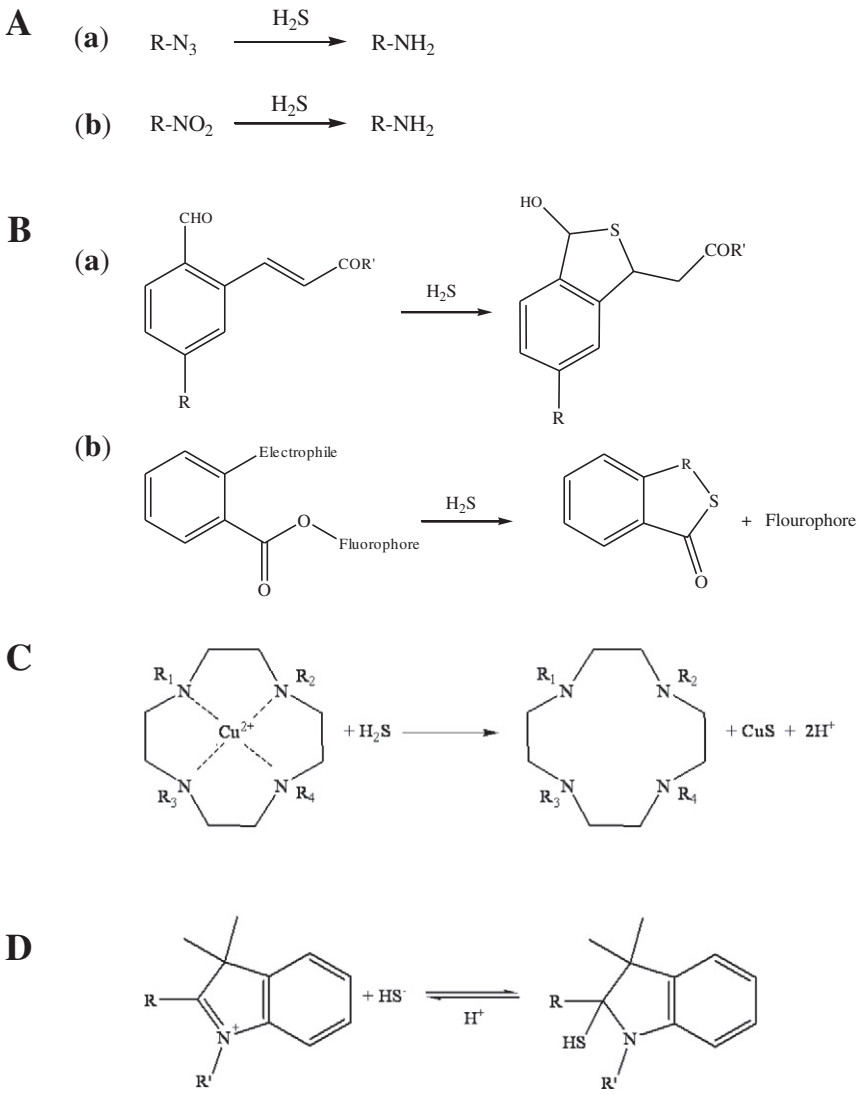
chemical or nucleophilic properties. The major challenge to overcome is to eliminate the interference of other thiol species such as glutathione or protein bound cysteine derivatives that are highly abundant in most cellular compartments and biological fluids and have similar chemical properties to sulfide [63]. In 2011 five research groups independently reported developments of novel sulfide specific fluorescent probes that have potential for detecting endogenous sulfide using different chemical strategies [108–113], which triggered an extensive effort to further develop and optimize fluorescent sulfide detection in biological systems.

4.1. Reduction of azides or nitrocompounds (Scheme 5A)

Chris Chang's group is using a reaction based approach, where they search for chemoselective reactions for sulfide that are bioorthogonal to other cellular events. They utilized the azide reducing property of sulfide [114,115] to form amines (Scheme 5A(a); azides are widely used molecules in biological systems, with adequate inertness [116]). They built two azide-caged rhodamine analogues (SF1 and SF2), which give fluorescent rhodamine products upon reduction by sulfide [108]. They showed that their probe functions in aqueous solution at physiological pH with relatively good selectivity over a range of ROS, reactive nitrogen species (RNS) and reactive sulfur species (RSS). However, they conducted their measurements in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, in which the lifetime of some of the tested oxidant species (such as HOCl) is expected to be very short, due to fast reaction with HEPES. On the other hand the selectivity of SF1 and SF2 over physiological concentrations of Cys, GSH and other reducing sulfur species is convincing. Despite of the relatively slow response (on an hour timescale) of this probe it was demonstrated to be able to measure sulfide in intact cells.

Wang's group also utilized the azide reduction by sulfide reaction but they attached it to Dansyl (a different fluorophore) to give the fluorescent DNS-Az probe [110]. A major advantage of this probe is its fast response rate, which was achieved by attaching the strong electron withdrawing sulfonyl group to the azide moiety, making it more electrophilic.

DNS-Az appears to give a linear response to sulfide concentrations (in Tween buffer and in bovine serum as well), with a detection limit of a few μM and with significantly increased reactivity (with half life on the second time scale at 10–100 μM concentrations). The selectivity of the probe against 18 different anions was convincingly demonstrated, but relatively low concentrations of different thiol species induced



Scheme 5. Chemical basis for fluorescent sulfide detection. **A** Reduction of (a) azides or (b) nitrocompounds. **B** Bi-nucleophilic cyclization to (a) generate a fluorescent product or (b) release a fluorophore tag. **C** Precipitation of the Cu^{2+} center from macrocyclic copper complexes. **D** Reversible single nucleophilic addition to a quaternary ammonium compound, where the decomposition of the product is acid catalyzed.

considerable signal intensities. The observed background signal in bovine serum is also suggestive of some interference with biological thiols.

Also using the azide reduction procedure, Das et al. recently developed a two-photon probe (FS1), which has the advantage of sulfide imaging in tissue owing to increased penetration depth, localized excitation and prolonged observation times [117]. This probe is sparingly soluble in aqueous media, but it was able to detect endogenous sulfide in HeLa cells. It has relatively good selectivity (although interference with ROS was studied in HEPES, a buffer that reacts with some oxidants), it can detect sulfide down to low μM levels, but it requires relatively long reaction times (hours).

Recently a number of new probes were developed based on the reduction of an azide tag that is attached to different fluorophores [118–122] and a slightly different strategy based on sulfide mediated reduction of nitrocompounds (Scheme 5A(b)) is also emerging [120, 123,124].

4.2. Bi-nucleophilic reactions (Scheme 5B)

Xian and coworkers [109] utilized the unique property of sulfide (over aliphatic thiols and other biological nucleophiles) to be able to engage in two consecutive nucleophilic substitution reactions (the same chemical feature is responsible for the selectivity of the MBB method, see Section 3.2). They developed a sensor (probe 1), which captures sulfide in a nucleophilic substitution reaction followed by a cyclization process via a second nucleophilic displacement, which results in the release of a flourophore tag (see Scheme 5B(b)). The specificity of this probe is clearly a great advantage over the azide containing ones. However, the fact that this probe also reacts with other nucleophiles (albeit in a single nucleophilic substitution reaction, which does not result in fluorescence signal) introduces the requirement that it always needs to be in access not only over sulfide but also over other nucleophiles, which is hard to achieve in biological systems. The probe is capable of quantitatively detecting very low concentrations of sulfide with a linear response, but it requires relatively long reaction times (in the order of hours). It was shown to be able to detect sulfide in plasma as well as in cells.

The work by Quian et al. also utilizes the ability of sulfide to engage in bi-nucleophilic reactions (Scheme 5B(a)[111]). They developed two probes (SFP-1 and SFP-2) in which an initial addition of sulfide to an aldehyde moiety is followed by cyclization via a proximal acrylate to form a thioacetal. These probes also react with other thiols, albeit in a reversible reaction to give an intermediate species that decomposes to restitute the parent aldehyde derivatives. Hence, it is only expected to result in a decreased reactivity of the probe with sulfide in the presence of other nucleophiles by decreasing the available active probe concentrations and possible rate determining dissociation of other probe–nucleophile complexes. They exhibit a relatively slow (hour timescale) but linear response at pH 7.7 and 37 °C in PBS with sensitivity at the low μM region. Although a sizable interference was observed in the presence of 1 mM concentrations of thiol species the excitation spectra suggested that choosing the right wavelength could provide adequate selectivity for SFP-1 and SFP-2, but only on the expense of the sensitivity. Both probes could detect intracellular sulfide, but both generated a measurable background signal, which is most likely not solely due to endogenous sulfide but also a result of thiol interference. These probes were further developed to give a faster response (SFP-3) by replacing the acrylate ester functional group with an α,β-unsaturated phenyl ketone moiety [125]. The reaction time dropped to a few minutes even in blood plasma, but the reported high plasma sulfide concentrations (56 μM) reflects derivatization-mediated irreversible sulfide quenching (for details see next Section 5.2).

Based on similar chemical grounds Xu et al. developed a different ratiomeric probe (E1) with a similarly fast response time as SFP-3 (few minutes) but with improved sensitivity (down to the sub μM region) [126]. E1 gave a linear response and showed adequate selectivity against thiol species, but just as the SFP probes or probe 1 it is not capable of detecting sulfide in a dynamic fashion.

It would be interesting to see whether the probes that are based on incorporation of sulfur in a heterocyclic ring (probe 1, SFP-1/2 and E1) would exhibit interference with polysulfides, because they could potentially form polysulfide containing heterocycles too.

4.3. Coordination to Cu^{II} (Scheme 5C)

Following up on the observation of Choi et al. that the a dipicolylamine–fluorescin complex of Cu^{2+} is capable of detecting sulfide [127], Sasakura et al. developed a novel probe that operates on similar chemical basis, but shows great selectivity to sulfide [112]. They used a series of azomacrocyclic ring complexes of Cu^{2+}, which release their Cu^{2+} center upon reaction with sulfide (Scheme 5C), but not with GSH (even at 10 mM concentration). Detection is based on the loss of the fluorescence quenching effect of the paramagnetic Cu^{2+} center upon its reaction with sulfide (and precipitation of CuS), which lights up the molecules. The HSip-1 compound appeared to be highly sensitive (low μM), gave an immediate response, showed selectivity for sulfide over a range of inorganic and organic sulfur species (at adequate concentrations), ascorbate and a variety of ROS and RNS (at physiologically relevant concentrations). Although the probe appeared to be membrane impermeable its diacetylated derivative entered the cell and proposed to hydrolyze to HSip-1 by intracellular esters. Although it did not exhibit any toxicity up to 100 μM concentrations, the authors report intracellular sulfide detection only upon the addition of a large concentration (500 μM) of exogenous sulfide. A similar approach was used by Hou et al. where they used an 8-hydroxyquinoline-appended fluorescein derivative to complex Cu^{2+} [128,129]. The chemistry and the characteristics of this probe are similar to Hsip-1.

4.4. Single, reversible nucleophilic addition (Scheme 5D)

Recently Chen et al. introduced a merocyanin derivative as a ratiomeric fluorescent sulfide probe (CouMC) [130]. CouMC binds sulfide via a nucleophilic addition reaction (Scheme 5D). The probe responds to sulfide concentrations in a linear fashion with adequate sensitivity (down to 1 μM). Although the selectivity was demonstrated by various thiol species, due to the fact that detection is based on a single nucleophilic addition, other much more nucleophilic protein thiols such as peroxiredoxins, thioredoxin or protein tyrosine phosphatases [61] could pose interference problems. A potential advantage of this probe is the reversible nature of its reaction with sulfide (see Section 5.2). However, reversibility was demonstrated by strong acidification and at high temperature (indirectly in the source of a mass spectrometer). Therefore, in order to assess the potential of CouMC for dynamic sulfide detection it would be very interesting to see how quickly the signal is quenched when sulfide is taken away from the reaction mixture and to know the stability constant of the sulfide adduct. Another promising feature of this probe is that it is not only permeable to cell membranes but it seems to accumulate in the mitochondria and therefore, it could be used to obtain information on the function of mitochondrial sulfide.

5. Kinetic and thermodynamic aspects of sulfide binding and release by biomolecules in relation with sulfide detection in biological samples

It is now well appreciated that in biological systems the majority of sulfide is bound to biomolecules via different chemical interactions. Based on the applied conditions (by the different detection methods) that were thought to be responsible for triggering bound sulfide release (i.e. acidification, alkalization or reduction), the so-called “acid labile”, “alkaline labile” and “persulfide” pools are the most frequently quoted sulfide reserves in the literature. In this section we discuss

some thermodynamic and kinetic aspects of sulfide consumption and release by these pools from the perspective of sulfide detection by the above discussed methods.

5.1. Some proposed sulfide–biomolecule interactions that could liberate sulfide

5.1.1. Heme protein complexes

As discussed in the coordination chemistry section (Section 2.3), there are a variety of heme–sulfide interactions with different chemical properties. The Fe^{III}–sulfide complex (Structure 1 on Fig. 2) is expected to be labile, but higher sulfide to heme concentration ratios can trigger the formation of Fe^{II}–sulfide complexes (Structure 2 on Fig. 2), and sulfheme derivatives (Structure 3 on Fig. 2). Fe^{II}-complexes are generally more stable than Fe^{III}-complexes and liberation of sulfide from Fe^{II}-complexes is expected to be slower too (because $K = k_{on} / k_{off}$ and k_{on} is not expected to be faster for Fe^{II}-complexes than for Fe^{III}-complexes, where K is the stability constant of the complex and k_{on} and k_{off} are the rate constants of complex formation and dissociation, respectively). Release of sulfide from sulfheme derivatives most likely requires harsh experimental conditions. In addition, sulfide should also coordinate to other protein bound transition metal centers, such as copper, zinc or manganese via reducing or non-reducing chemical interactions.

5.1.2. Iron–sulfur clusters

Acidification was shown to result in disassembly of Fe–S clusters (Structure 4 on Fig. 2) [131] and acid labile sulfide formation was associated with mitochondrial ferredoxin proteins (such as aconitase) in the brain [132].

5.1.3. Persulfides

Persulfides (Structure 5 on Fig. 2) can release sulfide upon reduction by for example DTT, TCEP or another thiol derivative or via alkaline hydrolysis [28,31,56,133].

5.1.4. Sulfhydrated electrophiles

Michael addition of sulfide to electrophiles generates aliphatic thiols (Structure 6 on Fig. 2) that were proposed to be stable (just like the most well known aliphatic thiol, cysteine) [33]. On the other hand if the Michael addition reaction destroys a conjugated double bond, the reaction could acquire a reversible nature with reasonable decomposition rates of the adduct (as we observed for the glutathionylation of superoxide modified tyrosine residues [134]).

5.1.5. Disulfides

Alkaline hydrolysis of disulfides was proposed to occur via two major pathways (see Scheme 6) [135–138]. We recently confirmed that 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) undergoes rapid base hydrolysis via mechanism 1 (Scheme 6), and the rate increases with the pH [139,140]. At a first glance, mechanism 1 is not a major concern in relation with measuring sulfide concentrations at high pH, but with the highly reactive intermediate sulfenic acids (RSOH; discussed in [61,63,141,142]) a series of reactions could be devised that could eventually either consume or liberate sulfide. For example, sulfenic acids could react with persulfide species to give thiosulfinate ester derivatives and liberate sulfide (reaction (11)).

$$RSOH + R'SSH \rightleftharpoons RS(=O)SR' + H_2S \quad (11)$$

Alternatively, RSOH species could consume free sulfide to give persulfides (reaction (12)).

$$RSOH + H_2S \rightleftharpoons RSSH + H_2O \quad (12)$$

These are only theoretical examples, because it is difficult to predict, which reactions are most likely to occur in complex biological systems and the partitioning of different reaction pathways will largely depend on the experimental conditions. Furthermore, in mechanism 2 (Scheme 6) a β-elimination step results in persulfide formation from disulfides, which could liberate sulfide via many different reactions, e.g. base hydrolysis (reaction (13)) or reaction with a thiol (reaction (14)):

$$RSSH + OH^- \rightleftharpoons RSOH + HS^- \quad (13)$$

$$RSSH + R'SH \rightleftharpoons RSSR' + H_2S. \quad (14)$$

Significant disulfide hydrolysis via mechanism 2 was observed for insulin at pH 12–13 on a reasonable timescale [143] and persulfide formation was recently confirmed to occur by mass spectrometry even under normal tryptic digest conditions (at pH 8–9 and 37 °C; albeit quite slowly) [144]. For practical reasons it is also worth mentioning that mechanism 2 is facilitated at higher temperatures [145]. In addition, a significant amount of sulfide release was observed from bovine serum albumin (BSA) at high pH as measured by a sulfide ion selective electrode [146].

5.2. Problems associated with irreversible free sulfide consumption during detection

Sulfide can be seen as the smallest thiol and hence it exhibits similar reaction properties to cysteine residues. Therefore, one of the major problems that is associated with the specificity of sulfide detection in biological systems is to overcome the interference of the large thiol pool that mainly consists of GSH and protein-cys derivatives. A unique feature of sulfide over other (aliphatic) thiols is that it can engage in two nucleophilic reactions at a time. This property is often utilized in sulfide derivatization methods to obtain adequate selectivity for its detection (see e.g. the MBB method or some of the fluorescence probes in Sections 3.2 and 4, respectively). However, these derivatization reactions are practically irreversible and therefore take free sulfide out of the system. As a result of free sulfide consumption, some of the reversibly bound sulfide complexes will start liberating sulfide to attain equilibrium. As discussed in Section 5.1, different sulfide–biomolecule complexes liberate sulfide with different rates, which largely depend on the applied experimental conditions. Therefore, methods that rely on derivatization of sulfide are likely to measure different sulfide concentrations in the same sample, because they often use different conditions and incubation times. Furthermore, methods that contain a sulfide precipitation step or volatilization of H_2S are also likely to result in a shift in the free vs. bound sulfide equilibria and overestimate free sulfide levels. For example, sulfide measurement by GC via headspace sampling is expected to show a dependency on the applied experimental conditions, because sulfide protonation and phase transition equilibria will depend on the pressure, temperature, pH, etc. Therefore, a major advantage of sulfide selective electrodes is that they do not consume considerable amounts of the free sulfide pool (and despite previous proposals they do not require alkaline conditions either).

The above mentioned limitations that arise from free sulfide consumption are also associated with most of the fluorescent probes, because they bind (or consume) sulfide in an irreversible manner. For example, DNS-Az was used to measure sulfide concentrations in whole mouse blood, but the obtained relatively high 32 μM value suggests that this method does not measure the concentration of free sulfide (see Section 4.1), but rather a segment of the biomolecule bound sulfide reserve. Detection with probes that contain a Cu^{II}-center also rely on irreversible heterogeneous reactions, which could not only shift chemical equilibria in biological systems, but CuS precipitation or the potential release of highly toxic free Cu^{I} cations could lead to perturbations of normal biological functions. In addition, derivatization of

Mechanism 1. Alkaline hydrolysis of disulfides via disproportionation of intermediate sulfenic acids

$$2RSSR' + 2OH^- \rightleftharpoons 2RSOH + 2R'S^-$$
$$2\ RSOH \rightarrow RSO_2H + RSH$$

net reaction: $\mathbf{2RSSR' + 2OH^- \rightarrow RSO_2H + RSH + 2R'S^-}$

Mechanism 2. Alkaline hydrolysis of disulfides via β-elimination

$$\mathbf{RSSR' \xrightarrow{OH^-} R"CH{=}CH_2 + R'SSH}$$

Scheme 6. Proposed models for base hydrolysis of disulfides.

sulfide with fluorescent probes does not allow dynamic sulfide monitoring, which would be instrumental in better understanding its biological actions. Therefore, a selective fast responding fluorescent probe with high sensitivity that uses only a fraction of sulfide (in order not to liberate H_2S from biological sulfide pools) is desired.

5.3. Blood sulfide pools

Relatively high blood sulfide concentrations (ten to hundreds of μM) were measured by a number of investigators using the MB or the ISE methods (see Table S1), which today is accepted to be mostly sulfide that is bound to different biomolecules (see Section 5.1) and liberated under acidic or alkaline conditions, respectively. Free blood sulfide levels are much lower as reported by Olson's and Kraus's research groups, who found no detectable free sulfide in a series of vertebrate blood by a polarographic electrode [99,146]. In addition, Kevil's group also measured sub-μM amounts of free and low μM concentrations of "persulfide" or "acid labile" sulfide in human plasma [26,28]. Whitfield et al. have shown that BSA, bovine plasma or whole blood consumes added sulfide in rapid reactions (few minutes) [146]. However, 10 μM sulfide spikes could be recovered with the MB method from different vertebrate plasma, suggesting that added sulfide was incorporated into the acid labile pool. Notwithstanding, >80% of the added sulfide was lost from the acid labile pool of whole blood and they suggested that it was not incorporated into the alkaline labile pool either (measured in trout blood) [27,147]. In these experiments the samples were not aerated and hence oxidation-mediated sulfide loss was proposed instead of H_2S volatilization.

Wintner et al. also detected low free sulfide levels in animal blood (in the range of 0.3–1.2 μM) using the MBB method. They measured a similar rate of added sulfide loss in human plasma samples with the MBB method and with a polarographic sensor. However, in contrast to the polarographic sensor, which could not detect sulfide in spiked rat blood, a slow loss of sulfide spikes was measured with the MBB method, suggesting reversible coordination to blood cell biomolecules in a loosely bound manner. They have confirmed these findings in in vivo measurements too (in rats). Furthermore, Wintner et al. also shown by administering radio-labeled sulfide that this loosely bound sulfide sink is not in rapid exchange with endogenous sulfide pools and they proposed that it represents persulfide species. However, we could detect BSA or PTEN bound persulfides with the MBB method under similar conditions, only after reduction by DTT [31] (Shen et al. had similar observations [28]). It has to be noted though that theoretically other labile persulfides could liberate sulfide during the MBB derivatization step. In addition, because Whitfield et al. could not recover sulfide from whole blood with the MB method, the experiments with MBB suggest that blood cell bound sulfide pools are catalytically consumed (oxidized or more tightly bound) under acidic conditions. Based on the above arguments we hypothesize that this loosely bound sulfide pool may represent hemoglobin bound Fe^{III}–sulfide complexes (see Structure 1 in Fig. 2). A healthy human should have approximately 15 g/dl hemoglobin in their blood with normally 1–2% of it being methemoglobin that would correspond to about 100 μM of Fe^{III}–heme. Corroborating our hypothesis and the observation by Whitfield et al., Van de Louw showed methemoglobin induced rapid H_2S loss in vitro that could not be recovered by the MB method [148]. Furthermore, consistent with this loosely bound sulfide pool, Table 2 in Pietri et al. [72] shows rapid formation reactions of different hemoglobin Fe^{III}–sulfide complexes with reverse (sulfide liberation) rates on the minutes timescale at pH 7. An important role of this sulfide pool is highlighted by the fact that intravenously administered sulfide appears rapidly in exhaled air [149,150].

In summary, free blood sulfide levels are in the sub- to low μM level, but ten to hundreds of μM sulfide reserves are available in blood that can be liberated under different conditions. At physiological pH this sulfide pool most likely serves as a buffer to maintain free sulfide concentrations constantly below toxic levels but at the same time provide feedback upon sulfide consumption. Sulfide liberation reactions from different sulfide–biomolecule complexes (upon free sulfide consumption) occur with different rates, which is likely to serve as a modulator of sulfide-mediated signaling events. In addition, blood cells are capable of rapidly binding administered sulfide in loose interactions and to keep this added sulfide pool separate from endogenous sulfide reserves. This latter mechanism could play an important role in detoxification of exogenous sulfide.

5.4. Tissue sulfide measurements

Sulfide measurements in tissue samples are even more problematic than in physiological fluids, because in tissue there are a plethora of different enzymatic pathways that constantly produce and consume sulfide. The actual rates of these enzymatic sulfide production and consumption processes largely rely on experimental conditions and even a small perturbation of production or consumption rates can result in several folds of differences in measured sulfide levels [107]. For example, significantly lower sulfide production rates were observed at pH 5.8 vs. at 7.4, because some enzymatic pathways are more affected by the change in the pH than others [107]. In addition, the presence of oxygen markedly accelerates sulfide clearance in a concentration dependent manner, most likely via oxidative pathways [151]. Furthermore, unrealistically high added cysteine was suggested to underestimate sulfide production rates in a tissue specific manner, because the kinetic properties of different sulfide producing enzymes will show different Cys concentration dependencies (e.g. in contrast with CSE, the preferred catalytic route of CBS to produce sulfide not only depends on cysteine, but also on homocysteine concentration, see Scheme 1 [152,153]). However, large amounts of added Cys could also liberate sulfide by reducing persulfide species via reaction (14).

An insightful study by DeLeon et al. has pointed out a potential pitfall in sulfide measurements using three different biological apparatuses that require constant gas delivery (i.e. tissue culture plates,

Langendorff apparatus and muscle myograph bath) [154]. They have observed significant and rapid sulfide loss via H_2S volatilization as a result of continuous sample bubbling. Corroborating this observation we show in Section 6 that sulfide loss from stock solutions via H_2S volatilization is fast even in the absence of bubbling. Therefore, tissue sulfide measurements should always be carried out with extreme care, preferably in steady state and as close as possible to physiological conditions.

In addition, sample maceration or cell lysis results in unrealistic conditions and destroys among others enzyme, metal ion and oxidant/reductant compartmentalization, which most likely will have a profound effect on sulfide producing and consuming reactions. Although freezing production and consumption pathways via lowering the temperature to 4 °C is an alternative way of estimating steady-state concentrations [106], a dynamic measurement of sulfide production and consumption is desired to better understand biological functions [107]. The most realistic picture is expected to come from in vivo studies. Both the polarographic and MBB methods were shown to be able to measure sulfide levels in vivo [99,146] and a preferably two photon fluorescent probe that is capable of sulfide detection in larger tissue depth in a dynamic fashion is desired.

In summary, due to the dynamic nature of sulfide production and consumption reactions (that are largely tissue specific and very sensitive to reaction conditions), tissue sulfide measurements should be carried out in a dynamic manner with the least perturbation of normal physiological functions.

6. Practical advices on using sulfide as a reagent and handling stock solutions

The high reactivity of sulfide together with the volatile nature of H_2S, makes it important to handle sulfide reagent solutions with care in order to avoid artifactual observations. In this section we discuss important chemical aspects of handling sulfide as a reagent based on our own experience and a literature review. To begin with, it is important to know the composition (including trace contaminations) of the chemicals (H_2S gas, sulfide salt, buffer, other salts, etc.) that are used to make sulfide solutions. For example, Table 1 shows that stock solutions that were prepared from different freshly opened commercial sulfide salts based on molecular weights (provided by the vendor) show large variations in their sulfide content. Reliable sulfide concentrations were measured by the MB (Sulfide content 2) and the DTNB methods (Sulfide content 3; based on reduction of DTNB and quantification of the highly absorbing 5-thio-2-nitrobenzoic acid at 412 nm [155]). However, for practical reasons Table 1 shows that measurements of sulfide concentrations at the absorbance of HS^- (230 nm; Sulfide content 1) gave different results. Sulfide content 1 measurements were made after a dilution of the stock solutions with water to ~50 μM (calculated based on the molecular weights assuming 100% purity). The larger values that were obtained by measurements on the absorbance of HS^- for the cleaner Na_2S solutions are most likely due to interfering absorbances of sulfide oxidation products at 230 nm. However, the lower concentrations that were obtained for the NaHS and lower purity samples are most likely due to the fact that dilution of these solutions resulted in pH < 8 values (as opposed to the purer Na_2S samples where the final pH was always > 9) and therefore a shift in a speciation to give more of the less absorbing H_2S form (see Fig. 1). In addition, substantial polysulfide contaminations were detected in some cases, immediately upon crystal dissolution in double distilled water (Table 1). In fact, some of the crystals were yellow indicating heterogeneous air oxidation on the surface. We have confirmed that washing the surface of sulfide crystals a few times can efficiently eliminate the majority of polysulfide and other oxysulfur compound contaminations [24]. Polysulfides also form readily in sulfide stock solutions via transition metal ion catalyzed air oxidation. Because most of the metal ion contamination is expected to come from buffer salts, the nature and concentration of the buffer that is used to make sulfide stock solutions will make a difference in the rate of air oxidation of sulfide. For example, Fig. 3 shows that sulfide selective electrode calibration curves start deviating from linearity at the >1 μM concentration range. This was previously shown to be due to sulfide consumption via contaminating trace metal ion-mediated coordination and redox chemical reactions [97]. Fig. 3 also shows that in TRIS buffer substantial sulfide

Table 1
Comparison of sulfide and polysulfide contents of some commercially available reagents.

Compound	Purity as provided by vendor	Polysulfide content 290 nm	Sulfide content 1 230 nm	Sulfide content 2 412 nm DTNB	Sulfide content 3 667 nm MB
$Na_2S·9H_2O$ Sigma Aldrich	ACS reagent ≥98.0%	(138 ± 33) μM	(156 ± 56) mM	(112 ± 8) mM	(117 ± 23) mM
$Na_2S·9H_2O$ Sigma Aldrich	≥99.99%	(332 ± 26) μM	(138 ± 33) mM	(117 ± 5) mM	(110 ± 16) mM
Na_2S Sigma Aldrich	97–103%	(154 ± 18) μM	(139 ± 24) mM	(117 ± 11) mM	(114 ± 11) mM
Na_2S Alfa Aesar	96.12%	(403 ± 118) μM	(126 ± 38) mM	(112 ± 14) mM	(94 ± 8) mM
$Na_2S·9H_2O$ Sigma Aldrich	≥98.0%	(103 ± 10) μM	(169 ± 55) mM	(125 ± 15) mM	(120 ± 12) mM
NaHS XH_2O Sigma Aldrich	≥60%	(952 ± 51) μM	(82 ± 11) mM	(105 ± 20) mM	(97 ± 14) mM
$Na_2S·XH_2O$ Sigma Aldrich	≥60%	(593 ± 28) μM	(90 ± 25) mM	(79 ± 7) mM	(72 ± 8) mM
$Na_2S·XH_2O$ Sigma Aldrich	59–65%	(537 ± 31) μM	(71 ± 11) mM	(77 ± 8) mM	(75 ± 7) mM

Experimental conditions: Sulfide stock solutions (150 mM) were prepared by dissolving the calculated amount of the corresponding sulfide salt in double distilled water. The solutions were kept in the dark on ice. Polysulfide contents were determined by measuring the absorbance of water stock solutions at 290 nm using the previously measured extinction coefficient of 811 M^{-1} cm^{-1} for total polysulfide under similar conditions (see supporting information in Ref. [52]). Stock solutions were diluted to 50 μM with double distilled water (for Sulfide content 1) or with 100 mM phosphate buffer (for Sulfide contents 2 and 3) before sulfide concentration measurements. Sulfide content 1: Based on the absorbance at 230 nm using $\varepsilon_{230}^{HS^-} = 7700\ M^{-1}\ cm^{-1}$. Sulfide content 2: Based on the absorbance at 412 nm, after reaction with 5 mM DTNB (3,3′,5,5′dithio-nitrobenzoic acid), which leads to the formation of 2 M equivalents of a strongly absorbing yellow product, 5-thio-2-nitrobenzoic acid (TNB), using $\varepsilon_{412}^{TNB} = 14{,}100\ M^{-1}\ cm^{-1}$ (for details see Ref. [155]). Sulfide content 2: Based on the absorbance measured at 667 nm, using the MB method, $\varepsilon_{667}^{MB} = 30{,}200\ M^{-1}\ cm^{-1}$ at pH = 0.45 (determined in our laboratory under these conditions). Data are means and standard deviations of three independent experiments. In summary, the table shows that sulfide chemicals should be used with caution and washing the surface of the crystals to get rid of air oxidation generated polysulfides is always recommended. For the concentration measurement of sulfide stock solutions we recommend to use one of the above mentioned reaction-based colorimetric methods in comparison with the direct measurement at 230 nm at pH > 9 in water (where a large deviation between the two methods is indicative of substantial contamination). For more detailed discussion see the main text.

loss starts to occur at lower concentrations compared to PBS, reflecting the differences in the purity of the buffer salts. Avoiding sulfide oxidation in aqueous solutions (especially at extended incubation times) is not an easy task and sometimes practically impossible. Therefore, appropriate controls are necessary in biological studies to assess, whether the actual active species is sulfide or one of its oxidation products. As a note of caution, our recent report pointed out that the presence of even traces amounts of polysulfide in working sulfide solutions is enough to trigger polysulfide-mediated biological processes [31].

Although transition metal ion catalyzed sulfide oxidation can give rise to substantial amounts of polysulfide and/or other oxysulfur derivatives, in contrast to previous results [24] we observed only minor inhibition of sulfide loss by diethylenetriamine-pentaacetic-acid (DTPA; a metal chelator) at pH 7.4 in PBS (see Fig. 4). In addition, in our hands argon saturation or protection from light at pH 7.4 did not have a marked effect on sulfide loss either (see Fig. 4). Furthermore, despite the fact that sulfide oxidation is expected to be faster at higher pH (due to the increased nucleophilicity of HS^- compared to H_2S; see Section 2.2 and [39]) we have measured an opposite pH dependence of sulfide loss in buffered sulfide solutions (see Fig. 5). The above observations suggest that the majority of the observed relatively rapid sulfide loss from open stock solutions at physiological pH (see Fig. 4) is mainly due to H_2S volatilization and not to oxidation (which is in agreement with observations by DeLeon et al. [154]).

As a practical advice we have found that the most stable and pure sulfide stock solutions (100 mM) are made by dissolving selected $Na_2S \times 9H_2O$ crystals after washing their surface in double distilled argon saturated water. This results in an alkaline water solution, in which volatilization is slow (due to the high pH) and oxidation is retarded (due to the absence of O_2). The concentration of this stock solution should be checked with the MB or DNTB methods. We suggest to also measure the concentrations at the absorbance maximum of HS^- ($\varepsilon_{230}^{HS^-} = 7700\ M^{-1}\ cm^{-1}$; after a dilution to ~50 μM with H_2O to ensure pH > 9) because significant deviation between the two measurements is suggestive of inadequate purity, in which case the stock solution should be discarded. Stock solutions should be stored on ice away from light (because $1e^-$ sulfide oxidation is expected to be UV-light catalyzed). Working solutions are made by dilution with the desired buffer (having enough buffer capacity to overwhelm the base that is introduced by the stock sulfide solution). Buffers should contain 100 μM DTPA to chelate trace metal ion contaminations and working solutions should be used immediately after dilution.

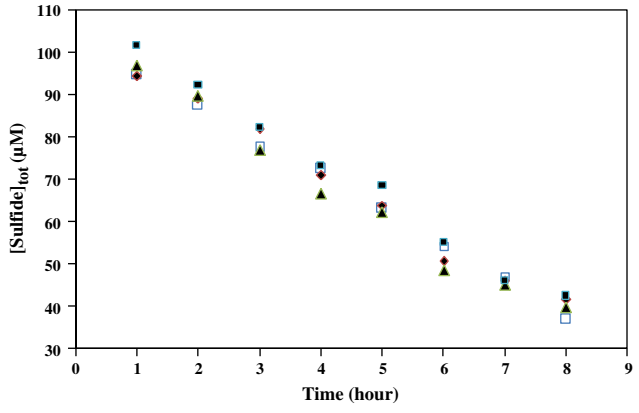


Fig. 4. Stability of sodium sulfide stock solutions in different media. Loss of total sulfide as a function of time from 100 μM stock solutions in 10 mM PBS (pH = 7.4) using different conditions: (□) exposed to laboratory light, (♦) in the dark, (▲) containing 100 μM DTPA in the dark, (■) containing 100 μM DTPA under argon in the dark. Sulfide concentrations were determined by the MB method immediately after a 7 fold dilution with 10 mM PBS (pH = 7.4). Calibration curves for the MB measurements were developed for 10 mM PBS buffer at pH = 7.4.

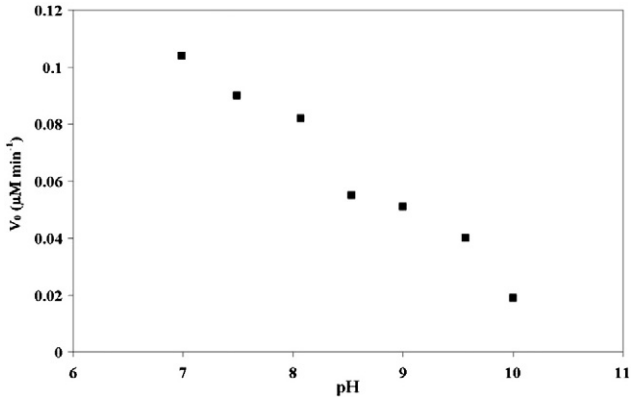


Fig. 5. pH dependency of the kinetics of sulfide loss from aqueous solutions. The initial rates of sulfide loss are shown as a function of pH. Conditions: 300 μM sulfide stock solutions were incubated at room temperature, in the dark in a mixture of 10 mM PBS and 10 mM TRIS buffers containing 100 μM DTPA in the 7 < pH < 10 pH range. Sulfide concentrations at different time points were assayed by the MB method after a 7 fold dilution (with 100 mM phosphate buffer pH = 7.4) followed by an immediate quench with the MB reagent solution. Calibration curves for the MB measurements were developed for 100 mM phosphate buffer at pH = 7.4. The data is representative of three independent experiments, where similar trends were observed.

7. Conclusions

Owing to its versatile chemical properties, it is not surprising that endogenous sulfide has been demonstrated to have an orchestrating role in a plethora of different biological processes. However, a better understanding of sulfide chemistry is essential in order to reconcile the large number of controversial observations around its biological functions. The different sulfide detection methods that are being used in the literature resulted in orders of magnitude differences in measured biological sulfide levels. Investigations that were aimed to find explanations for these inconsistencies lead to the discovery that although free sulfide levels are relatively low, there are huge reserves in most biological systems that can release sulfide via different chemical reactions. Although the messenger function of sulfide was questioned after the recognition that most of the previously detected sulfide was bound in different sulfide pools [27,156], we believe that this kind of sulfide buffering is in fact an important feature to control sulfide mediated biological actions. Due to the diversity of biomolecule–sulfide interactions, sulfide release rates are expected to vary in a wide range. This dynamic system needs to be tightly controlled, because sulfide exhibits toxic properties even at low μM concentrations. In addition, the apparent regulatory roles of sulfide in cellular signaling events also predict the presence of a dynamic control mechanism for its steady-state concentrations. Based on these arguments the following obvious questions can be raised: What are the physiologically relevant concentrations of sulfide that should be used in model in vitro studies? Is it more realistic to use a bolus of sulfide or rather one of the slow releasing sulfide donors? At this point it is difficult to answer these questions, because it should be system specific in a way that it could either be free sulfide, the fast releasing pool or slow sulfide liberation that triggers the corresponding biological function. Therefore, future chemical kinetic and mechanistic studies of sulfide reactions and dynamic biological investigations are fundamental to get a deeper insight into sulfide biology. In addition, some coordination and redox reactions of sulfide can result in bioactive metabolites that are becoming more appreciated to play important roles in sulfide-mediated biological actions. This calls for extreme caution when making and handling reagent sulfide solutions and underpins the importance of adequate control experiments to avoid controversial or artifactual observations.

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Appendix A. Supplementary data

A comprehensive summary of reported blood and tissue sulfide measurements can be found in the online supplementary material to this article at http://dx.doi.org/10.1016/j.bbagen.2013.05.037.

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