



Detection of sulfane sulfur species in biological systems

Meg Shieh, Shi Xu, Oren L. Lederberg, Ming Xian^{*}

Department of Chemistry, Brown University, Providence, RI, 02912, USA

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ABSTRACT

Sulfane sulfur species such as hydropersulfides (RSSH), polysulfides (RS_nR), and hydrogen polysulfides (H_2S_n) are critically involved in sulfur-mediated redox signaling, but their detailed mechanisms of action need further clarification. Therefore, there is a need to develop selective and sensitive sulfane sulfur detection methods to gauge a better understanding of their functions. This review summarizes current detection methods that include cyanolysis, chemical derivatization and mass spectrometry, proteomic analysis, fluorescent probes, and resonance synchronous/Raman spectroscopic methods. The design principles, advantages, applications, and limitations of each method are discussed, along with suggested directions for future research on these methods. The development of robust detection methods for sulfane sulfur species will help to elucidate their mechanisms and functions in biological systems.

1. Introduction

Reactive sulfur species (RSS) are a series of sulfur-containing molecules found in biological systems. These include thiols (RSH), disulfides (RSSR), hydrogen sulfide (H_2S), hydropersulfides (RSSH), polysulfides (RSS_nSR), hydropolysulfides (RSS_nH), hydrogen polysulfides (H_2S_n , $n \geq 2$), and *S*-modified cysteine adducts (e.g., $-\text{SNO}$, $-\text{SOH}$, $-\text{SO}_2\text{H}$, etc.). Some RSS, such as thiols, have long been known as critical redox regulators. The functions of many others are only recently appreciated. For example, H_2S was recently classified as a nitric oxide (NO)-like signaling molecule in many physiological and/or pathological processes [1]. H_2S functions as an endothelial cell-derived relaxing factor via direct activation of ATP-sensitive potassium (K_{ATP}) channels. The deprivation of endogenous production of H_2S contributes to the development of hypertension. Moreover, H_2S has shown beneficial effects on oxidative stress, inflammation, and fibrosis. While research on H_2S is still actively ongoing, another interesting topic in this field has emerged which focuses on a series of H_2S -related sulfane sulfur species [2]. Sulfane sulfur refers to sulfur atoms with six valence electrons but no charge (represented as S^0). H_2S -related sulfane sulfur compounds include hydropersulfides (RSSH), polysulfides (RSS_nSR), hydropolysulfides (RSS_nH), hydrogen polysulfides (H_2S_n), and protein-bound elemental sulfur (S_8). The most attractive sulfane sulfurs among these are hydropersulfides (RSSH) and hydrogen polysulfides (H_2S_n) because these species are actively involved in redox signaling [2]. Increasing evidence has demonstrated the broad impact of these RSS in health as they are linked

to a number of physiological and pathological processes, such as activating the transient receptor potential ankyrin 1 (TRPA1) channel, regulating the tumor suppressor phosphatase and tensin homolog (PTEN), upregulating the transcription of antioxidant genes, and relaxing vascular smooth muscles. However, due to their inherent instabilities, RSSH and H_2S_n are understudied molecules from a chemistry point-of-view. As a result, the detection of RSSH and H_2S_n in biological samples remains a challenge and major gap in the field, preventing researchers from better understanding their biological behaviors and therapeutic potentials. In this article, we review current methods and techniques that are used for the detection of sulfane sulfurs in biological samples and include discussion on their potential limitations and applications.

2. Cyanolysis

Cyanolysis is one of the very first methods used for the detection of sulfane sulfurs, primarily protein hydropersulfides [3]. This method employs the unique reactivity of the cyanide anion (CN^-) toward sulfane sulfur to form thiocyanate (SCN^-), which can then be determined spectrophotometrically (at 460 nm) upon reacting with ferric nitrate to form a red-colored complex $[\text{Fe}(\text{SCN})_6]^{3-}$ [4]. This method is commonly used for purified proteins or samples in simple matrices. However, cyanolysis displays poor selectivity and specificity for sulfane sulfur species, especially in complex matrices, and cannot be used for the identification of the type of sulfane sulfur species (e.g. persulfides vs

^{*} Corresponding author.

E-mail address: ming_xian@brown.edu (M. Xian).

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polysulfides). Additionally, this method requires post-mortem processing and the destruction of biological samples.

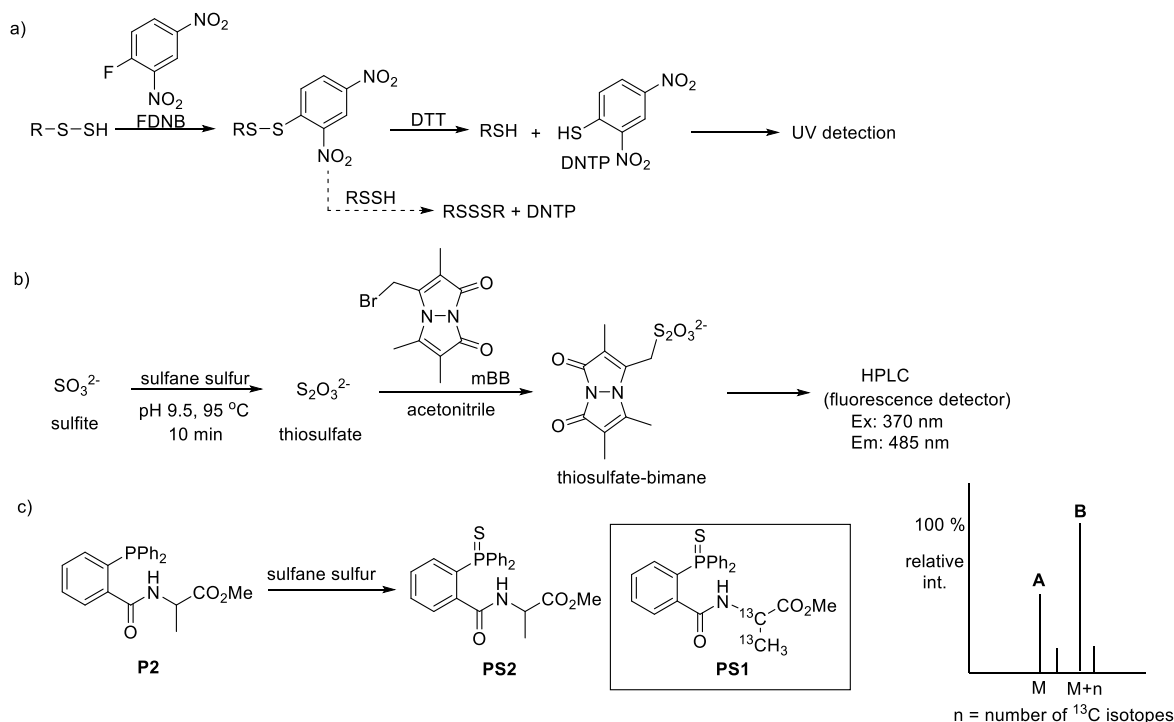
3. Sulfane sulfur/Persulfide derivatization and detection

Since hydropersulfides (RSSH) are unstable species, a common detection method involves chemically converting them to stable derivatives for characterization. 1-Fluoro-2,4-dinitrobenzene (FDNB), a reagent often used for this purpose, can react with both proteins and small molecule hydropersulfides to form dinitrobenzene disulfides (Scheme 1a) [5]. The following reduction of the disulfide by DTT would produce dinitrothiophenol (DNTP), a compound that can be easily measured by spectroscopic methods ($\lambda_{\max} = 408 \text{ nm}$, $\epsilon = 13,800 \text{ M}^{-1} \text{ cm}^{-1}$). As such, the concentration of DNTP reflects the original hydropersulfide concentration. This method is suitable for simple and relatively clean RSSH systems, rather than complex systems such as persulfidated protein mixtures. Still, this method can distinguish RSSH from thiols, disulfides, and polysulfides (RSS_nSR). However, the reaction between FDNB with RSSH, especially small molecule RSSH, is not quantitative as dinitrobenzene disulfides possess high reactivities with RSSH. This side reaction would produce polysulfide as the product and lead to an underestimated persulfide concentration. Additionally, FDNB can react with hydropersulfides (RSS_nSH) and the following reduction will also release DNTP. This will give underestimated sulfane sulfurs as inner S⁰ atoms of RSS_nSH will not generate DNTP. Another commonly used alkylating agent for RSSH detection is monobromobimane (mBB), which can convert unstable hydropersulfides to stable bimane adducts. HPLC-MS/MS analysis of the bimane products with isotope labeled standards can then be used to quantify small molecule hydropersulfides. Using this strategy, Ida et al. was able to measure cysteine- and GSH-derived per- and polysulfides in biological samples [6]. Their results indicated significant levels (50–100 μM) of per- and polysulfides in mammalian cells, plasma, and tissues. Kimura et al. also used mBB to analyze hydrogen polysulfides in rat brains [7]. Homogenates of rat brains and solutions of sodium trisulfide and tetrasulfide were derivatized with mBB in CHES (0.5 M, pH 8.4). The resulting

polysulfide-bimane adducts were then analyzed by HPLC. This method allowed for the quantitative determination of the hydrogen polysulfide concentration in wet brain tissue ($\sim 25 \text{ nmol/g}$), suggesting that hydrogen polysulfides are sufficient to induce Ca^{2+} influx in astrocytes and activate TRPA1 channels.

Another method for sulfane sulfur detection exploited the known ability of sulfite to react with sulfane sulfurs to form thiosulfate, leading Xun et al. to develop a HPLC-based method for sulfane sulfur detection in biosystems [8]. They found that the reaction could complete quantitatively at 95 °C in a solution at pH 9.5 for 10 min. The resultant thiosulfate was then derivatized by mBB in acetonitrile to form thiosulfate-bimane, which could be analyzed by HPLC with a fluorescence detector (Scheme 1b). This method was used to quantify total sulfane sulfur concentrations in biological samples including bacteria, yeast, mammalian cells, and animal organs. The sulfane sulfur levels in different mouse organs were in the range from 1 to 10 nmol/(mg of protein). Interestingly, it was found that the sulfane sulfur contents of microorganisms varied at different growth stages; the content increased in the early growth phase, was steady in the middle to late of growth, and then sharply decreased in the late stage of growth. Meanwhile in zebrafish, sulfane sulfur levels peaked on the 6th day before egg hatching and declined after several weeks of growth as adult fish. A drawback of this method is that it uses harsh reaction conditions for S⁰-thiosulfate conversion. As high pHs and temperatures can promote polysulfide degradation through hydrolysis, some sulfane sulfurs can be lost as bimane-S-OH.

An additional detection technique that utilized the electrophilicity of sulfane sulfurs, such as reacting with phosphines (R_3P) to form phosphine sulfide ($\text{R}_3\text{P}=\text{S}$) under mild conditions, was our lab's isotope dilution mass spectrometry (IDMS) method for sulfane sulfur (e.g., persulfides and polysulfides) quantification [9]. The mechanism of IDMS is described in Scheme 1c, which relies on the quantitative reaction between triaryl substituted phosphine P2 with sulfane sulfurs to form phosphine sulfide PS2. The concentration of PS2 can then be measured by IDMS using a synthetic $^{13}\text{C}_3$ -labeled phosphine sulfide PS1 as the internal standard. This method was applied to measure the levels



Scheme 1. Selected derivatization reactions of hydropersulfides and sulfane sulfurs. a) FDNB-based persulfide derivatization. b) Sulfite-mBB derivatization. c) Phosphine-based isotope dilution mass spectrometry method.

of sulfane sulfurs in mouse tissues including brain, kidney, lung, liver, heart, spleen, and blood. The average concentrations were found to be in the nmol/(g of tissue) scale.

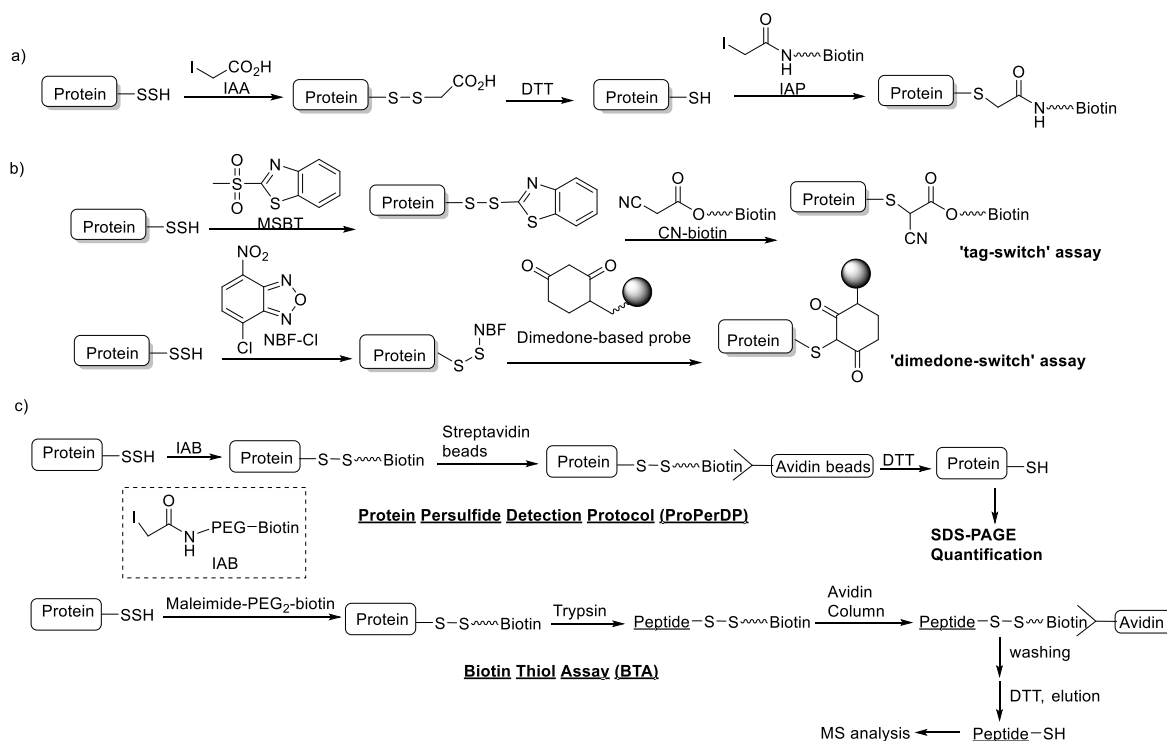
4. Methods for proteomic studies of persulfidation

RSSH derivatization has also been used in proteomic studies of protein persulfidation (protein-SSH). Considering hydropersulfides (-SSH) and thiols (-SH) are nucleophilic species, they both should react with common -SH blocking reagents such as MMTS, NEM, and IAA. Thus, the detection of protein-SSH mainly relies on multi-step reactions that first block the -SSH/-SH residues and then manipulate the disulfide adducts generated from -SSH to differentiate -SSH from -SH. Currently, the following methods have been developed:

- 1) Tonks et al. reported a 3-step strategy to label protein hydropersulfides (Scheme 2a) [10]. The sample was firstly treated with iodoacetic acid (IAA) to block -SH and -SSH. Then, the disulfides formed from -SSH were reduced by dithiothreitol (DTT) to give free -SH. Finally, the -SH residues were treated with iodoacetamide-linked biotin (IAP) to form biotin-conjugates. This method was used to identify H₂S-mediated inactivation of protein tyrosine phosphatase 1B (PTP1B) under endoplasmic reticulum stress. It should be noted that DTT treatment may also reduce natural -S-S- bonds in proteins and cause false positives.
- 2) Our lab developed a 'tag-switch' assay for protein hydropersulfide labeling [11]. This method utilizes two step reactions to selectively label P-SSH (Scheme 2b). In the first step, methylsulfonyl benzothiazole (MSBT) is used to block both -SH and -SSH. Thioether (P-S-BT) and disulfide (P-S-S-BT) are formed as the products. Since benzothiazole-containing disulfides (P-S-S-BT) display high reactivity to certain carbon-based nucleophiles, the subsequent 'tag-switch' step utilizing cyanoacetate-biotin conjugate can convert P-S-S-BT to P-S-biotin adducts. This step does not affect P-S-BT. Using this method, protein hydropersulfides can be selectively

labeled by biotin or other reporting molecules, such as fluorophores, for further analysis and characterization. The concept of the tag-switch assay can also be found in the recently developed dimedone switch method [12]. First, proteins are reacted with 4-chloro-7-nitrobenzofurazan (NBF-Cl) to label hydropersulfides while thiols, sulfenic acids and amino groups on the proteins are also labeled. Next, commercially available dimedone-based probes are used to selectively label the protein hydropersulfides by switching with the NBF tag. NBF-adducts from other residues are not affected. This method was applied to proteins from a wide range of sources (i. e. cells, worms, rats, lysates) and demonstrated that protein persulfidation occurs in multiple cells to protect proteins against over-oxidation. It was also found that persulfidation extends lifespan, decreases with aging, and increases in events of caloric restriction.

- 3) In 2016, Nagy et al. reported a method named ProPerDP for the identification of S-persulfidated proteins in intact cells and tissues [13]. This method utilizes iodoacetyl-PEG₂-biotin (IAB) to link protein-SSH as biotin conjugates, which are then pulled down from the sample mixtures using streptavidin-coated magnetic beads (Scheme 2c). Persulfide (-SSH)-derived disulfide adducts can be reduced by DTT and released from the beads while thiol (-SH)-derived thioether adducts cannot be reduced by DTT and should stay attached to the beads. The released proteins can then be identified and quantified on SDS-PAGE. The authors acknowledged that ProPerDP is unable to distinguish between protein hydropersulfides and polysulfides, and proteins containing both sulfhydryl groups of the -SSH group and other surface-exposed -SH groups may yield false negatives because the presence of a non-persulfidated -SH residue could cause the protein to be immobilized on the streptavidin. While there is also potential for false positives from disulfide-linked proteins if additional non-persulfidated free -SH residues exposed on the surface are alkylated, analyses using two-dimensional diagonal gel electrophoresis, A549 and HEK293 cells, and CBS- or CSE-deleted yeast cells determined that this potential is likely minor. Methodological improvements to overcome



Scheme 2. Representative methods for proteomic studies of persulfidation. a) IAA-IAP based persulfide labeling. b) Tag-switch and Dimedone-switch methods. c) ProPerDP and BTA methods.

the aforementioned limitations using mass spectrometry and tryptic digestion before affinity purification were proposed in the original paper. Hatzoglou et al. reported another method, the biotin-thiol assay (BTA), based on the same concept [14]. A maleimide-linked biotin reagent is first used to label protein-SSH residues (Scheme 2c). Then, samples are trypsin-digested, and biotin-conjugated peptides are enriched by streptavidin beads. Finally, the RSSH-derived disulfide-based peptides are reduced by DTT and eluted from the beads for MS analysis. Another similar persulfide labeling approach, qPerS-SID, was combined with the stable isotope labeling with amino acid in culture (SILAC) method and reported by Longen et al. [15]. Modifications to qPerS-SID were later made to obtain selectivity by precipitating samples with trichloroacetic acid, adding dimedone, and using maleimide-PEG2-biotin [16]. These prevented modification of reactive thiols, blocked sulfenylated thiols, and alkylated free thiols and *S*-sulfhydrated cysteines, respectively. Overall, these methods allow for quantitative RSSH proteomics analyses.

Another important development in this field is a method named low-pH quantitative thiol reactivity profiling (Low-pH QTRP) reported by Yang et al. in 2020 [17]. This method utilized a thiol-reactive probe, iodo-N-(prop-2-yn-1-yl) acetamide (IPM), to specifically conjugate RSSH at pH 5.0. It was believed that RSSH should react with the probe under this acidic pH while RSH should not, because of the much lower *pK_a* of RSSH compared to that of RSH. The probe-labeled proteome was then digested into tryptic peptides. The probe-labeled peptides were next conjugated with both light and heavy azido-UV-cleavable-biotin reagents via click chemistry. Finally, the biotinylated peptides were captured with streptavidin and photo-released for MS-based shotgun proteomic analysis. This method allows for the unbiased and site-specific mapping of cysteine persulfidation in complex proteomes. It provided a remarkably higher number of persulfide identifications than that generated from other methods. It was also observed that up to half of the measured hydropersulfides showed at least twofold loss of reactivity at decreased pH (5.0 vs 7.4), indicating that some RSSH are likely to be stabilized and deactivated by the low pH of the protein microenvironment. The authors also pointed out several limitations of the method: 1) it can only be used for proteomic studies; 2) it cannot identify IPM-labeled protein hydropersulfides (P-SS_nH), likely due to the low stoichiometry of the species or loss from sample preparation; 3) it is still difficult to effectively analyze endogenous persulfidation as the method relies on the negative selection of highly abundant probe-labeled thioether peptides for enrichment.

5. Possible artifacts in persulfide/polysulfide labeling and detection

As described above, thiol-blocking reagents (NEM, IAA, etc.) are often used in the detection of cysteine modifications such as persulfidation. Recently, it was found that the use of these reagents could breakdown polysulfides [18]. As shown in Scheme 3a, the sulfur-sulfur

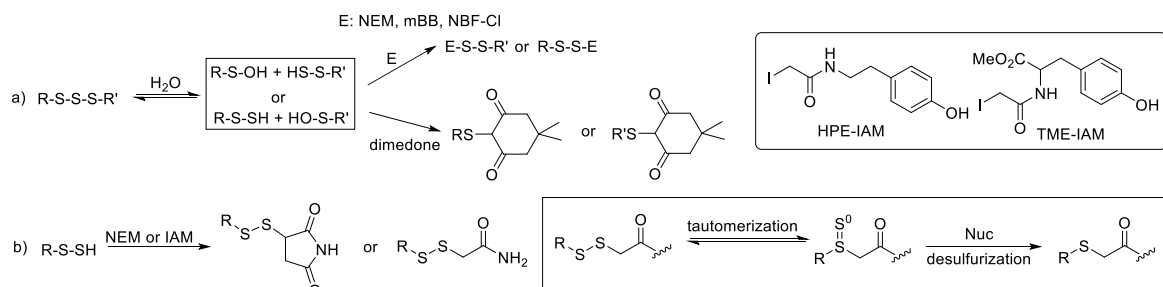
bonds in polysulfides (with trisulfide as an example to illustrate the reaction) can be sensitive to hydrolysis under weak alkaline and physiological conditions. The resulting products are nucleophilic hydropersulfides (-SSH) and electrophilic sulfenic acids (-SOH). Normally, there should be an equilibrium which favors the more thermodynamically stable polysulfides. The use of thiol-blocking reagents, however, could trap the hydropersulfide product and break the equilibrium. Dimedone, an -SOH labeling reagent, could cause similar effects. This property may lead to false positive results in the detection of cysteine modifications as polysulfides exist ubiquitously. Interestingly, Akaike et al. found that hydroxyphenyl-containing compounds could markedly suppress the hydrolysis of polysulfides, though the detailed mechanism behind it was still unclear [19]. Nevertheless, two hydroxyphenyl-containing thiol-labeling reagents, HPE-IAM and TME-IAM, were designed and tested [19]. These two compounds retained good -SH blocking ability but minimized polysulfide hydrolysis. Thus, they may be more suited as reagents in cysteine assays. In another recent study, Dick et al. found that the selection of alkylation reagents was critical [20a]. mBB was the most efficient reagent for capturing persulfides while IAM and NEM were less effective. The problem was due to the carbonyl group in the β -position of the disulfide formed from NEM or IAM, which could promote tautomerization to form thiosulfoxide (Scheme 3b). The presence of ambient nucleophiles would rapidly abstract the sulfur atom from thiosulfoxide and lead to false negative results in the MS analysis. The use of mBB or bulky IAM derivatives, such as *N*-*t*-butyl-IAM, could attenuate this side reaction. While their findings may seem controversial due to a claim in an earlier report by Bogdandi et al. [20b] that mBB and maleimide-based alkylating agents promote cleavage of polysulfide chains, resulting in a lower detected per/polysulfide levels while IAM could capture more polysulfides in the sample, these two papers discuss different experiments. Dick et al. focused on the persulfide modification of a model protein, while Bogdandi et al. built upon the polysulfide speciation of low molecular weight thiols. These works [20] indicate that even very similar experiments can lead to different conclusions depending on what is monitored and how experiments are performed. It was also clear that maleimide-based alkylating agents are not suitable for per/polysulfide detection, even though these agents are often used in studies.

6. Fluorescent probes for the detection of sulfane sulfurs

Another popular method for monitoring sulfane sulfur level changes in biological samples is fluorescent imaging. So far, many reaction-based fluorescent probes have been developed. These probes can recognize either all sulfane sulfurs or specific individual species (e.g., persulfides or hydrogen polysulfides).

6.1. Fluorescent probes for general sulfane sulfur detection

In 2013, our lab reported the first fluorescent probes, SSP1 and SSP2, for sulfane sulfurs and have since developed additional probes in the

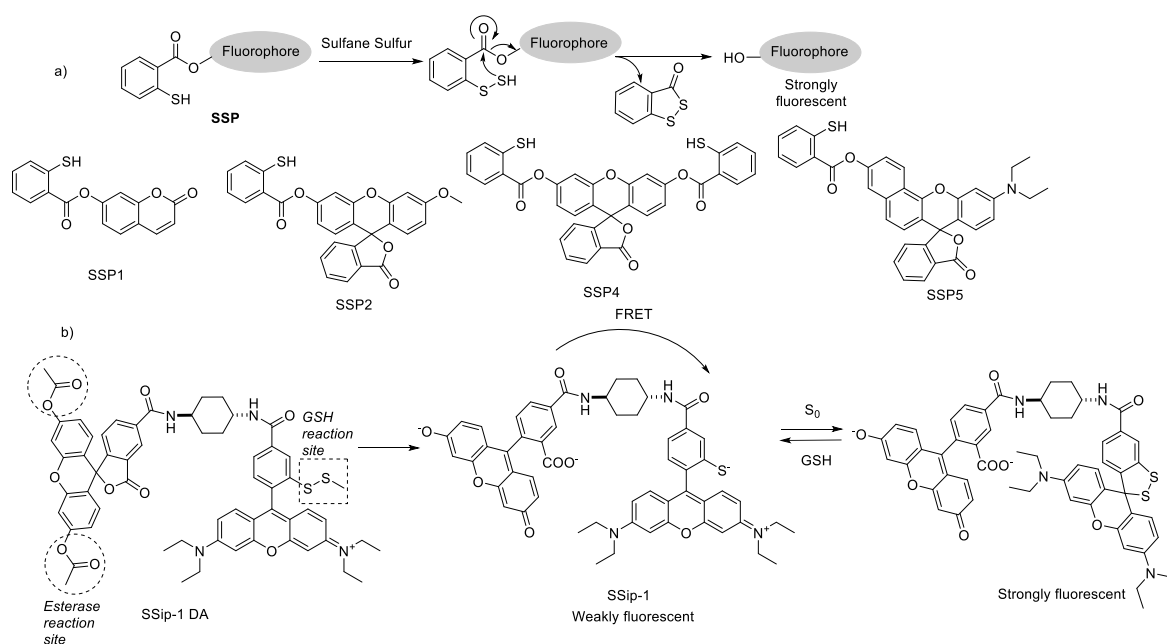


Scheme 3. Possible side reactions in hydropersulfide/polysulfide detection. a) Hydrolysis of polysulfides. b) Tautomerization and desulfurization of NEM/IAM-captured persulfide derivatives.

sulfane sulfur probe (SSP) series [21,22]. Our design exploits the electrophilic nature of sulfane sulfurs and their special affinity to thiol-based nucleophiles. All SSPs have a thiophenol moiety, an ester linker, and a hydroxyl-containing fluorophore. The nucleophilic thiophenol will attack the electrophilic sulfane sulfur to form an –SSH intermediate, which will then quickly attack the pendant ester moiety and release the fluorophore (Scheme 4a). This process is highly selective to sulfane sulfurs. Other cellular species, such as biothiols, ROS, and amino acids, cannot take part. Thus, SSPs are only responsive to sulfane sulfurs. It should be noted that the reaction between SSP and RSSH is complicated as both sulfur atoms of RSSH can potentially react with the thiophenol residue of SSP. The reaction with the external S (e.g. sulfane sulfur) will give the desired cyclization, while the reaction with internal S (the non-sulfane sulfur) will not, but such a reaction is reversible. Therefore, SSP eventually can pick up sulfane sulfurs. In addition, the external S of RSSH is more accessible, especially when RSSH are on proteins. That warrants the reactivity of SSP toward protein-based RSSH. SSP2 was first used to investigate the activity of CBS and CSE in live A549 cells. By employing SSP2 to evaluate the sulfane sulfur levels in cells over-expressing CBS and CSE and using LC-MS/MS to identify distinct sulfane sulfur species in the cells, Ida et al. found that overexpression of these enzymes dramatically increased the levels of Cys-SSH and Cys-SSSH [6]. Knockdown of CBS led to a decrease of Cys-SSH. Results of the study identified Cys-polythiolated proteins, demonstrated that overexpression of CBS and CSE in cells resulted in an increase of protein polysulfide residues, and suggested that reported H₂S-associated biological activities could potentially be from persulfides instead. To improve upon the moderate sensitivity of SSP2, SSP4 was created in 2014 using fluorescein as the fluorophore and demonstrated a much higher fluorescence enhancement toward sulfane sulfurs as compared to SSP2 (>900-fold vs 50-fold) [21b]. Ichinose et al. first used SSP4 in human umbilical vein endothelial (HUVEC) cells to identify sulfide metabolites increased by sodium thiosulfate in cells treated by lipopolysaccharide and in SH-SY5Y cells to determine that increased intracellular sulfane sulfur levels may have protective effects against neurodegenerative diseases [23,24]. SSP4 has also been reported to detect sulfane sulfur levels to the nanomolar range in HUVECs, mouse lung ECs, mouse tissues, and human platelets [25]. Since then, SSP4 has become the most widely used probe for its high photoactivity, selectivity, and sensitivity and has been commercialized by Dojindo Laboratories. This probe has also been

applied in a variety of systems including plants, bacterial, mammalian cells, etc. [26,27]. Kimura et al. utilized a combination of SSP4, LC-FL and LC-MS/MS to identify the number of sulfur atoms in H₂S_n found in the brain and determine the producing enzyme [27]. By examining H₂S production with lysates of COS cells expressing 3MST, they found that 3MST produced H₂S₃ and 3-MP, a substrate of 3MST, produced H₂S. SSP4 was later used to examine the cellular localization of H₂S₃ in COS cells expressing 3MST and in primary neuronal cultures. Results demonstrated that H₂S₃ was localized in the cytosol in both cases. Their study offered new insights into H₂S_n and H₂S biology and into potential novel therapeutic approaches for diseases involving these molecules. SSP4 has also been applied to quantitatively determine the levels of endogenously generated polysulfides in various biological samples by Bibli et al. [28]. They developed an H₂S_n assay based on the selective reaction of SSP4 with cell- and tissue-generated H₂S_n capable of transverse cell membranes. H₂S_n in cultured HUVECs and murine lung endothelial cells, in different cellular subcompartments of human endothelial cells, in frozen tissues of different mouse organs, and in isolated human platelets were studied using 10 μM SSP4. Supernatants were collected for LC-MS/MS and fluorescence measurements. Standard curves of fluorescein, the fluorophore found in the probe, and SSP4 were generated to allow for the quantification of H₂S_n production. The SSP4 and LC-MS/MS-based method allowed for the quantitative detection of polysulfides in different biological samples.

A non-traditional probe, SSP5, has also been developed and takes advantage of the rhodol fluorophore's long excitation wavelength (582 nm), far-red emission (>600 nm), and excellent photostability for use as a smartphone-based point-of-care (POC) device [22]. Combined with the POC device, SSP5 enabled rapid, low-cost detection of sulfane sulfur in synthetic urine systems. Other SSP-based probes have since been synthesized as well. SSNIP, a near-infrared (NIR) probe, shares a thiophenol moiety and an ester linker with the SSP series and was used to determine levels of sulfane sulfur in plant roots [29]. Results suggested that sulfane sulfurs may act as signaling molecules in plant physiology. Probes in the SSP series can detect a wide range of sulfane sulfur species with high specificity and selectivity, are well-established for bioimaging in various models, and have allowed for many discoveries. However, similar to many fluorescent probes, a limitation is that they are unable to distinguish between members of the sulfane sulfur family, such as persulfides and polysulfides, or identify specific locations of persulfidation



Scheme 4. Fluorescent probes for general sulfane sulfurs. a) The reaction and structures of SSP probes. b) The reaction of SSip probes.

site(s) in proteins.

Takano et al. reported two reversible FRET-based fluorescent probes, SSip-1 and SSip1-DA, for the detection of intracellular sulfane sulfur species in 2017 [30]. Their design exploited the formation of a spirocyclization adduct after the reaction of sulfane sulfur with the -SH group on the 2-thiorhodamine B (RB) moiety of the probe (Scheme 4b). Spirocyclization disrupts the π conjugation between the two aromatic rings of rhodamine, leading to a loss of absorption in the visible region. Interestingly, sulfane sulfur treatment in the presence of GSH led to the initial decrease and gradual recovery of absorbance, suggesting that the disulfide bond of the spirocyclization adduct was reduced by GSH, and 2-thio RB was regenerated. This allows the probe to be 'reversible'. 2-Thio RB was thus coupled to fluorescein with a cyclohexyl linker to construct the FRET-based on/off probe. Upon the addition of sulfane sulfur species, spirocyclization would occur and inhibit FRET, yielding a strong fluorescence (from fluorescein). To improve membrane permeability, SSip1-DA was developed. The diacylation and thiol protection on SSip1-DA enhanced the molecule's lipophilicity, making it more cell-permeable. Upon entry into the cytosol, SSip1-DA readily converts to SSip-1 via hydrolysis by esterase and reduction by GSH. Indeed, SSip1-DA showed fluorescence in Na_2S_4 -treated A549 cells. The fluorescence decreased with further incubation and recovered upon the second addition of Na_2S_4 . This phenomenon was attributed to the reducing environment in the cytosol, indicating that SSip1-DA could reversibly detect sulfane sulfur species in cells. SSip1-DA has been used in a variety of biological studies. For example, Miyamoto et al. used SSip-1 to monitor H_2S_n production from the reaction of H_2S and NO in dorsal root ganglion neurons [31]. They found that H_2S_n could activate transient receptor potential ankyrin (TRPA1) channels. More recently, Marutani et al. used SSip-1 DA and SSP4 to estimate persulfide levels in mice brain tissues and SH-SY5Y cells, respectively [32]. Results demonstrated that sulfide:quinone oxidoreductase (SQOR) expression protected against hypoxia-induced sulfide accumulation, bioenergetic failure, and ischemic brain injury.

6.2. Protein-based probes for sulfane sulfurs

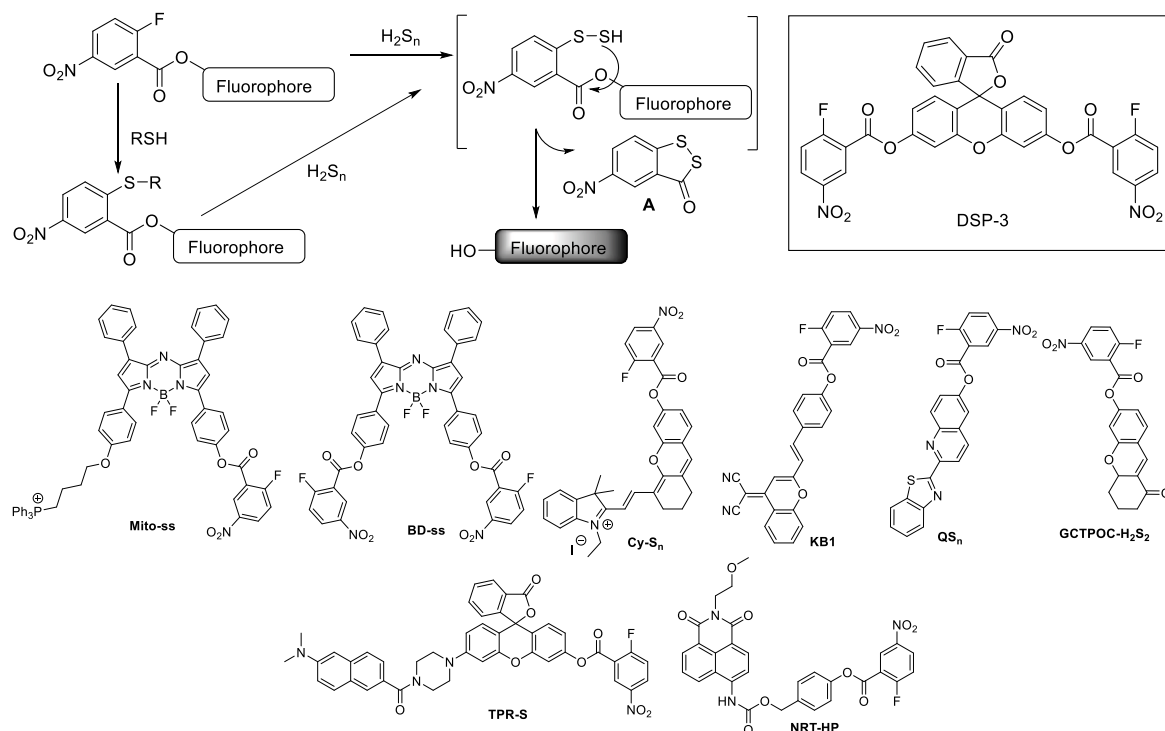
In 2019, Liu et al. reported two protein-based fluorescent probes for sulfane sulfurs: one based on green fluorescent protein (GFP) and the other based on red fluorescent protein (mCherry) [33]. They first carried out 3D structure analyses on the proteins and identified possible sites for mutation. Then, two amino acid residues near the protein chromophore were mutated to cysteine residues. The selection of sites was critical as the distance between the two cysteines needed to be long enough to react with sulfane sulfurs to form a trisulfide (-S-S-S-) bond but not a disulfide bond (2.05 Å). After reacting with sulfane sulfurs, the trisulfide bond formation near the chromophore altered the photophysical properties of the proteins and led to fluorescence signal changes. In the case of GFP, the best probe, psGFP1.1, had two Cys mutations at 147 and 202. When reacted with sulfane sulfur, an increase in the 400 nm excitation peaks was noted together with a decrease in the 475–490 nm region. The excitation spectra (408/488 nm ratio) were used to evaluate psGFP1.1's response to sulfane sulfur. In the case of mCherry, A150 and S151 were mutated to Cys. The trisulfide bond formation on this mutant, psRFP, decreased the intensity of the 610 nm emission (excitation at 587 nm) and slightly increased the intensity of the 466 nm emission (excitation at 406 nm). The 466/610 nm emission ratio was used to measure the levels of sulfane sulfur. These protein-based probes have the potential to detect sulfane sulfur in subcellular organelles as they can be expressed in locations such as the cytoplasm and mitochondria. psRFP and psGFP1.1 had a similar detection range and sensitivity, though their sensitivity was generally lower than that of small molecule probes. The reaction time of psRFP with sulfane sulfur was also longer (~50 min) than that of psGFP1.1 (~15 min).

6.3. Fluorescent probes for hydrogen polysulfides (H_2S_n)

H_2S_n belong to the sulfane sulfur family but exhibit unique reactivity and biological functions. Several fluorescent probes have been reported specifically for H_2S_n . These are summarized below:

2-Fluoro-5-nitrobenzoic ester-based probes: Similar to H_2S , H_2S_n can be considered nucleophiles that can undergo nucleophilic reactions twice. Due to this property, compounds containing two electrophilic groups may selectively capture H_2S_n . In 2014, we reported the first H_2S_n fluorescent probes, DSP1-3, based on this strategy [34a]. The employment of 2-fluoro-5-nitrobenzoic ester as the recognition group allowed the DSP probes to sense H_2S_n based on a two-step mechanism: an $\text{S}_\text{N}2$ reaction, and a subsequent intramolecular cyclization by the -SSH intermediate to release the fluorophore. Although thiols can react with DSP, the corresponding adducts will not lead to a fluorophore release. On the other hand, such adducts can be readily reactivated by H_2S_n (Scheme 5). This design ensures excellent selectivity for H_2S_n . It is important to note that H_2S_n are unstable in aqueous solutions and likely to be in a combination of multiple polysulfide anions (whether $n = 2, 3, 4$, etc.) [34b]. DSP probes should react with H_2S_2 rapidly to form the desired cyclized product A (shown in Scheme 5) while the cyclization with other species (H_2S_3 , H_2S_4 , etc.) should be much slower. So, eventually all H_2S_n are likely to produce the most stable cyclized product A. In our studies, A was the only observed product, and we did not see other cyclized products (with more than 2 sulfur atoms). Among the DSP probes, DSP-3 showed the best fluorescence property, with a 137-fold fluorescence increase and a detection limit of 71 nM. The DSP template has also been adopted by other researchers for the development of H_2S_n probes with a range of fluorescent properties. For instance, Chen et al. incorporated this strategy with BODIPY to make NIR H_2S_n probes Mito-ss and BD-ss [35]. The fluorescence of Mito-ss was initially quenched by a donor-excited photo-electron transfer (d-PET) due to the 2-fluoro-5-nitrobenzoic ester. Upon treatment with H_2S_n , the fluorophore was unmasked and a fluorescence enhancement at 730 nm could be observed. Additionally, Mito-ss included a triphenylphosphonium cation for mitochondria localization. This probe was used to investigate the mechanism for endogenous H_2S_n generation, as well as for the qualitative evaluation of H_2S_n in mice organs. Similarly, BD-ss responded to H_2S_n at 737 nm and was used for the quantification of H_2S_n levels in mice serum and imaging endogenous H_2S_n generation in RAW 264.7 cells. Both Mito-ss and BD-ss were used to image H_2S_n in vivo with mice models. Xu et al. reported NIR-PHS1, another H_2S_n probe that utilized the BODIPY fluorophore and featured a detection limit of 12 nM [36a]. Other NIR fluorophores were also used in the construction of H_2S_n probes. Peng et al. developed Cy- S_n by employing a classic NIR scaffold, semiheptamethine [36b]. Cy- S_n showed strong fluorescence enhancement at 720 nm when treated with Na_2S_4 and was successfully applied in the sensing of endogenous H_2S_n in RAW 264.7 cells and mice. Additionally, KB1, a probe with the dicyanomethylene-benzopyran fluorophore, could sense H_2S_n in both colorimetric and NIR regions and showed good sensitivity for endogenous H_2S_n in MCF-7 cells [36c].

2-Fluoro-5-nitrobenzoic ester with two-photon fluorophores could give the probes a number of advantages, including weak self-absorption, low autofluorescence, better tissue penetration, and lower photobleaching. Liu et al. reported a two-photon H_2S_n probe, QS_n , using 2-benzothiazol-2-yl-quinoline-6-ol as the fluorophore [37]. QS_n demonstrated a 24-fold fluorescence 'turn on' in the presence of Na_2S_4 , with one and two photon excitations at 368 and 730 nm, respectively. QS_n 's two-photon action cross-section was 50 GM at 730 nm and was able to image H_2S_n in zebrafish embryos. Another two-photon H_2S_n probe was GCTPOC- H_2S_2 , reported by Lin et al. [38]. The GCTPOC dye released by the probe exhibited a large two-photon cross-section (500 GM at 780 nm). GCTPOC- H_2S_2 could detect H_2S_n in mice liver tissue to a depth of 180 μm . Tan et al. reported a FRET-based ratiometric two-photon H_2S_n probe TPR-S [39]. Consisting of a rhodol and a naphthalene, the inactive probe was in the spiro ring-closing form and only showed emission at

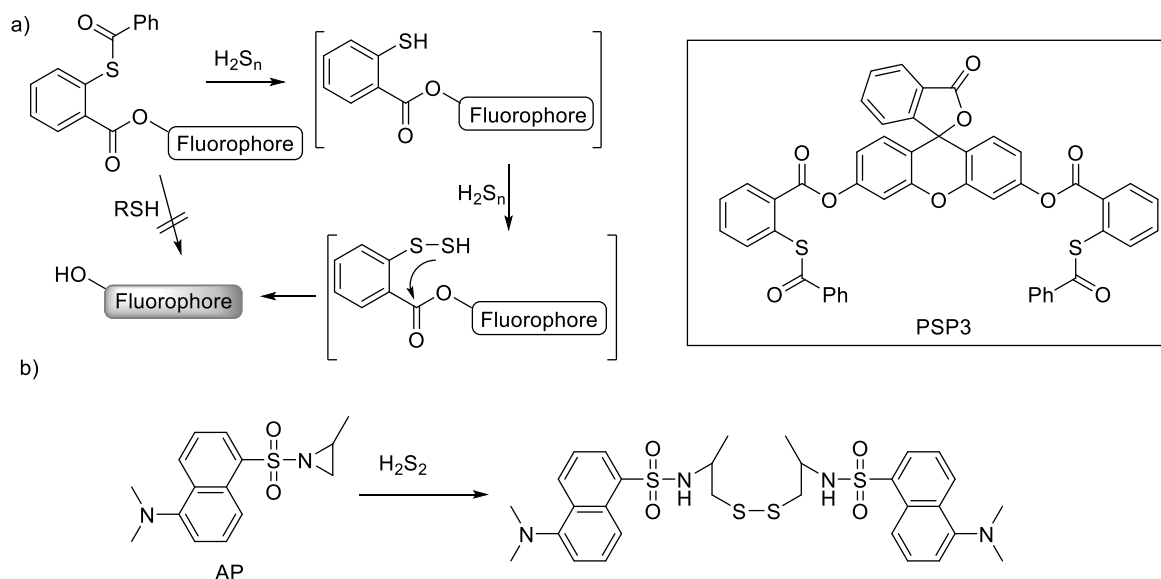


Scheme 5. The reaction and structures of 2-fluoro-5-nitrobenzoic ester-based fluorescent probes.

440 nm. After H_2S_n treatment, the FRET process was on, and the probe exhibited a new emission at 541 nm. Apart from visualizing H_2S_n in HeLa cells, TPR-S was also used for the detection of H_2S_n in rat liver tissues and organs of LPS-induced acute injuries. NRT-HP, another two-photon ratiometric H_2S_n probe, was developed by Liu et al. [40]. This molecule used a 1,8-naphthalimide for efficient ICT effect. Upon reacting with H_2S_n , the original 460 nm emission of the probe was decreased, along with a new emission of 542 nm. NRT-HP was successfully applied in tissue imaging and could detect H_2S_n at a tissue depth of up to 300 μm at a 800 nm excitation. Recently, a few more ratiometric two-photon H_2S_n probes employing 1,8-naphthalimide fluorophore were reported. These include mitochondria- and

lysosome-targeting probes [41] and a FRET-based ratiometric probe [42].

2-(Acylthio)benzoate-based probes: While 2-fluoro-5-nitrobenzoate-based probes showed good selectivity for H_2S_n , the probes could be consumed by biothiols due to the high reactivity of the fluorobenzene structure. To address this limitation, we explored more H_2S_n -specific reactions and developed another series of probes (PSP) based on a 2-(acylthio)benzoate template [43]. H_2S_n can serve as both nucleophiles and electrophiles, so they should readily undergo thioester exchange reactions with the template to form a thiophenol intermediate. This should then abstract sulfane sulfur from H_2S_n and turn on the pendant fluorophore (Scheme 6a). While biothiols could also theoretically react



Scheme 6. a) The reaction of 2-(acylthio)benzoate-based fluorescent probes. b) The reaction of aziridine-based fluorescent probes.

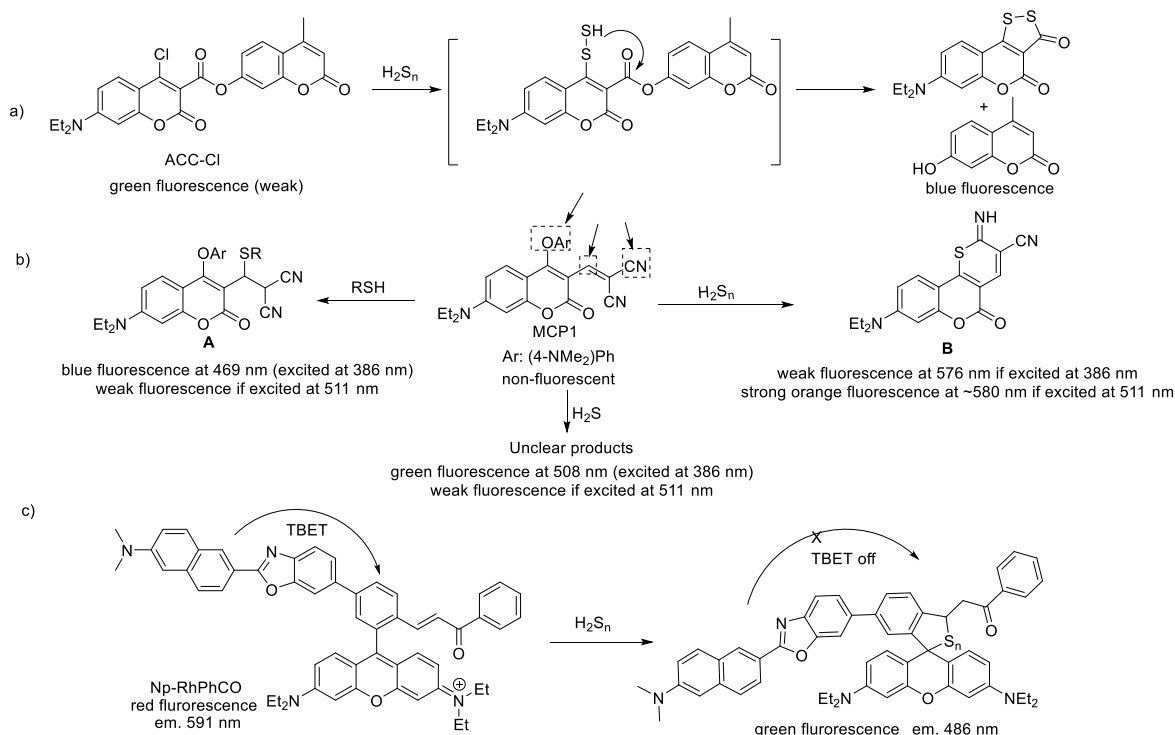
with the thioester, such reactions are unfavored under physiological concentrations and pHs. Thus, PSP probes demonstrated excellent selectivity and sensitivity to $\text{H}_2\text{S}_\text{n}$. PSP3 was also applied for the imaging of endogenous $\text{H}_2\text{S}_\text{n}$ in COS-7 and Vero cells. This template has since been used to conjugate to other NIR and two-photon fluorophores [44, 45], as well as a probe that could detect $\text{H}_2\text{S}_\text{n}$ and H_2S with different fluorescence signals [46].

Aziridine-based probes: We also found that aziridine was a unique recognition group for $\text{H}_2\text{S}_\text{n}$ due to its strong nucleophilicity, and this led to the development of the fluorescent probe AP [47]. The probe itself only exhibited weak fluorescence due to a twisted intramolecular charge transfer (TICT) effect on the dansyl fluorophore. Upon treatment of H_2S_2 , AP's emission at 530 nm increased. This was attributed to the H_2S_2 -mediated aziridine ring opening and the suppression of TICT (Scheme 6b). AP showed a detection limit of 0.3 μM and good two-photon photophysical properties. It also demonstrated a high solid state luminescent efficiency, suggesting that it could be a potential candidate for an organic emitter. However, the application of AP in cell imaging was unsuccessful [47].

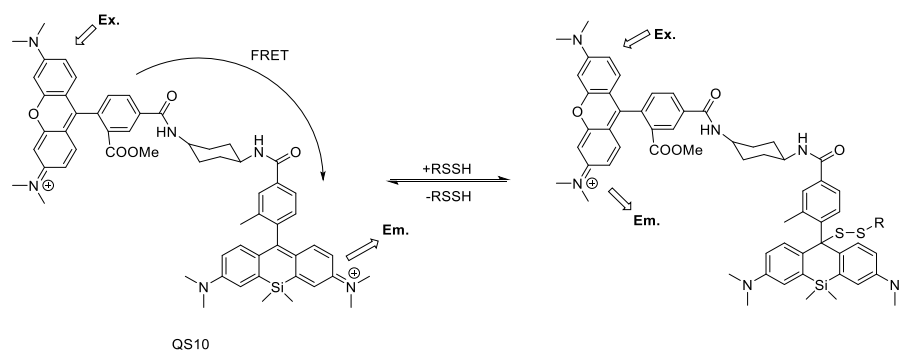
Other $\text{H}_2\text{S}_\text{n}$ probes: Several other probes for $\text{H}_2\text{S}_\text{n}$ have also been developed utilizing the strong nucleophilicity of $\text{H}_2\text{S}_\text{n}$. For example, Chen et al. reported a ratiometric probe, ACC-Cl, which employed 4-chloro-7-diethylamino coumarin ester (CDCE) to trap $\text{H}_2\text{S}_\text{n}$ and promote the intramolecular cyclization to release the pendant fluorophore (7-hydroxy-coumarin) [48]. When excited at 385 nm, ACC-Cl showed a weak green fluorescence at 510 nm (from CDCE). Upon treatment of $\text{H}_2\text{S}_\text{n}$, this emission decreased, accompanied by a gradual increase of blue fluorescence at 461 nm (from 7-hydroxy coumarin) over 120 min (Scheme 7a). Additionally, the same group developed MCP1, an interesting probe that could differentiate between $\text{H}_2\text{S}_\text{n}$, H_2S , and biothiols [49]. MCP1 was based on the diethylaminocoumarin template. Three possible reaction sites were introduced on the template: 1) a substituted phenoxy group, 2) an activated α,β -unsaturated bond, and 3) the cyano group. As such, the reactions between MCP1 and biothiols, H_2S , or $\text{H}_2\text{S}_\text{n}$ could produce different products and lead to different fluorescence outcomes. The authors were able to confirm the formation of **A** (from

thiols) and **B** (from $\text{H}_2\text{S}_\text{n}$) (Scheme 7b). The reaction with H_2S was found to be complicated and unclear. Nevertheless, MCP1 displayed distinct fluorescence toward these sulfur species while MCP1 itself was nearly non-fluorescent. With GSH, MCP1 gave a strong blue fluorescence at 469 nm (excited at 386 nm). With H_2S , it demonstrated green fluorescence at 508 nm (excited at 386 nm) and only yielded a slight fluorescence at 576 nm when excited at 386 nm with $\text{H}_2\text{S}_\text{n}$. However, when excited at 511 nm, the probe yielded a strong orange fluorescence (at ~ 580 nm) with $\text{H}_2\text{S}_\text{n}$ and only a weak fluorescence with thiols and H_2S . Yuan et al. developed a ratiometric probe, Np-RhPhCO, for $\text{H}_2\text{S}_\text{n}$ [50]. The probe contains three parts: an α,β -unsaturated ketone as the recognition unit with rhodamine and naphthalene as fluorescent moieties. When excited at 420 nm, the probe mainly exhibited rhodamine emission at 591 nm due to a Through-Bond Energy Transfer (TBET) effect. The nucleophilic $\text{H}_2\text{S}_\text{n}$ would attack the enone component to produce a persulfide intermediate, which should rapidly form the spirocyclic product and turn off the TBET effect. As such, it would show naphthalene emission at 486 nm (Scheme 7c). Np-RhPhCO was incorporated into self-assembled nanoparticles based on an amphiphilic copolymer to construct PPG-Np-RhPhCO. This material showed good photostability, biocompatibility, and selectivity to $\text{H}_2\text{S}_\text{n}$ over other RSS. It was applied to study $\text{H}_2\text{S}_\text{n}$ in a nonalcoholic fatty liver disease (NAFLD) model. For example, it was used to monitor endogenous $\text{H}_2\text{S}_\text{n}$ generation regulated by 3-MST and CSE. The authors also used PPG-NP-RhPhCO to study the ROS/ H_2S / $\text{H}_2\text{S}_\text{n}$ crosstalk in the NAFLD model and found that increased ROS stimulated H_2S generation, which in turn enhanced $\text{H}_2\text{S}_\text{n}$ levels.

Reversible fluorescent probes for RSSH and $\text{H}_2\text{S}_\text{n}$: Umezawa et al. developed a reversible probe for the detection of hydropersulfide and $\text{H}_2\text{S}_\text{n}$ in 2018 [51]. The design was based on an intermolecular nucleophilic equilibrium between silicon-derived rhodamine (SiR) and thiol-containing nucleophiles such as GSH. As RSSH and $\text{H}_2\text{S}_\text{n}$ are much more nucleophilic, the authors envisioned GSH-insensitive SiRs could be useful for RSSH/ $\text{H}_2\text{S}_\text{n}$ detection. Indeed, compound 2' Me SiR650 exhibited a 10,000-fold selectivity for H_2S_2 over GSH. Taking advantage of this finding, the authors designed a ratiometric probe, QS10 (Scheme



Scheme 7. Sensing mechanisms of several other $\text{H}_2\text{S}_\text{n}$ probes. a) ACC-Cl. b) MCP1. c) Np-RhPhCO.



Scheme 8. The reversible reactions of QS10 with RSSH.

8). When excited at 550 nm, QS10 itself showed the SiR-based emission at 670 nm through the FRET effect. Upon treatment with Na_2S_2 , a new emission at 595 nm appeared with the decrease of emission at 670 nm. QS10 was able to respond to both Na_2S_2 and 4-trimethylsilyl benzyl-persulfide (BnSSH, in-situ generated), while remaining inert to GSH. Additionally, the ratiometric fluorescent change was reversible. Due to these advantages, QS10 was applied in various biological settings. For instance, it was used to visualize the cellular GSSH-GSSSG redox cycle in real time. The authors also used it to quantify persulfide levels in live cells (12–25 μM), depending on the cell lines.

6.4. Other detection methods

Ikeda et al. reported a combined assay, “Elimination Method of Sulfide from Polysulfide” (EMSP) with the methylene blue (MB) sulfide detection assay, to quantitatively determine polysulfides in human albumin and plasma, saliva, and tear samples [52]. Sulfide Antioxidant Buffer (SAOB) is a strong redox buffer containing 0.5 M of sodium salicylate, 0.12 M ascorbic acid, and 2.2 M NaOH, which is used to prevent sulfide oxidation in samples. Olson et al. noticed that millimolar levels of sulfides were produced in plasma after SAOB treatment and attributed this to the NaOH-promoted elimination of HS^- from thiols [53]. However, the studies from Ikeda et al. showed that SAOB was unable to produce sulfide (S^{2-} or HS^-) from thiols, disulfides, or thioethers. Only polysulfides, such as GSSSG and diallyl trisulfide, could produce sulfide by SAOB. Components of SAOB were then modified, and EMSP was developed (e.g. incubation with ascorbic acid (0.3 M) and KOH (1 M) for 3 h at 37 °C). This process led to an optimized production of sulfides from polysulfides. The liberated sulfide could then be quantified by the standard MB method, and Na_2S was used to construct a standard curve. Further EMSP-MB assay applications determined approximately 8000, 130 and 1000 μM of endogenous polysulfides in human plasma, saliva, and tear samples, respectively. Overall, results suggested that polysulfides are derived from cysteine residues in proteins, HSA is important for maintaining a pool of polysulfide in human blood circulation, and the assay’s quantitative capabilities may allow it to elucidate functional consequences of protein-bound cysteine polysulfides. It must be noted that the sulfane sulfur measurements were not obtained from thiosulfate, so it is unknown if this may be a significant source of sulfane sulfur in the blood. This is an important area for investigation in the future.

In 2019, Xun et al. reported a reaction-free and nonintrusive method for sulfane sulfur detection [54]. This method utilizes resonance synchronous spectroscopy (RS_2) with a conventional spectrofluorometer to simultaneously scan the excitation and emission ($\Delta\lambda = \lambda_{\text{em}} - \lambda_{\text{ex}}$) of the analytes. Sulfane sulfurs, including H_2S_n , GSSH, GSSSG, and protein-bound persulfides, gave obvious RS_2 signals in the range of 400–600 nm. It was interesting to find that the protonated form of hydropersulfides (RSSH) showed strong RS_2 signals while the anionic form (RSS^-) showed no RS_2 signal. This method was utilized to measure the pK_a value of hydropersulfides (GSSH), reaction kinetics of sulfane

sulfurs (e.g., the reactions of $\text{H}_2\text{S}_n + \text{GSH}$; $\text{H}_2\text{O}_2 + \text{H}_2\text{S}$; $\text{H}_2\text{O}_2 + \text{GSSH}$), and disproportionation reactions of GSSH.

The S–S stretch of polysulfides exhibits peaks at around 480 cm^{-1} by conventional Raman spectrometry. This property was utilized by Sue-matsu et al. to develop a surface-enhanced Raman spectroscopy (SERS)-based method for the non-invasive detection of sulfane sulfurs in biological samples [55]. In this method, gold was deposited at a diagonal angle to boehmite-based nanostructures to create random arrays of horse-bean-shaped nanostructures named gold-nanofeve (GNF). This generated many electromagnetic hotspots as SERS excitation sources and enabled the large-area visualization of molecular vibration fingerprints of metabolites in tissue samples. With SERS, Raman signals of polysulfides are enhanced because the excitation of surface-plasmon resonance amplifies the local electromagnetic field. Using this technique, they visualized endogenous polysulfides via the peak at 485 cm^{-1} in the tumor-bearing brain tissues and the peak at 480 cm^{-1} in liver xenografts. Very recently, the same group used this method to identify sulfur metabolites associated with chemoresistance mechanisms of certain cancer patients [56]. It was found that clear cell carcinoma (CCC) patients with greater SERS signals at 480 cm^{-1} showed significantly shorter survival times than those with lower SERS signals after the surgery, indicating CSE-derived polysulfides could be a predictive marker for the overall survival of CCC patients. The potential limitation of SERS is that this technology can hardly capture more sterically hindered protein-bound sulfane sulfurs as the distance between adjacent Au nanoparticles is too small to allow proteins to access hot spots of the near field light. Also, glutathione-derived polysulfides give weak SERS signals suggesting SERS mainly detects signals from inorganic or low-molecular weight organic polysulfides (such as cysteine-derived polysulfides).

The structural confirmation of inorganic molecules relies heavily upon mass spectrometry due to their minimal responses towards other analysis techniques. Oligosulfurs, such as S_8 and other allotropic forms, have proved to be challenging to analyze via mass spectrometry due to rapid fragmentation during MS analysis. Matsuno et al. reported a novel polyaromatic capsular matrix that allows for accurate MS analysis and stabilization of otherwise labile S_6 and S_{12} clusters [57]. Electrospray ionization time-of-flight (ESI-TOF) MS analysis of S_8 revealed a 1:2 host-structure that was confirmed by NMR, UV–Vis, and X-ray crystallography. Even in water under highly diluted conditions and under ambient aqueous conditions, the host-guest matrix remained intact. Similarly, the less stable allotropes S_6 and S_{12} were stabilized once encapsulated by the matrix, likely as a result of the polyaromatic shell of the matrix. This could be further analyzed by ESI-TOF. The capsular matrix stabilized S_8 and unstable oligosulfurs, allowing for the otherwise unobtainable MS analysis and leading to a potential analytical tool for understanding the functions of inorganic sulfane sulfurs in biological settings.

7. Conclusions

The emerging importance of sulfane sulfur species in redox biology has spurred the rising need to explore their origins, functions, and mechanisms of action. This has led to the rapid advance of sulfane sulfur detection methods in recent years. Due to the unstable nature of many sulfane sulfurs, such as RSSH and H₂S_n, many methods rely on chemical reactions to convert labile sulfane sulfurs to stable adducts for quantification. However, other sulfur species normally co-exist with sulfane sulfurs, and they tend to share similar reactivities (e.g., RSSH vs RSH, H₂S_n vs H₂S). Additionally, it is expected that sulfane sulfurs and other sulfur species form a complex but balanced equilibrium among these inter-convertible species in biological systems. Any chemistry to react with one or a few species could shift the equilibrium and lead to inaccurate results. These factors contribute to the challenge of sulfane sulfur detection. Recent discoveries of alkylating reagent-triggered polysulfide hydrolysis and disulfide arrangement further complicate data interpretation [18–20]. Therefore, the selection of appropriate reagents is critical, and exploring more effective and selective reagents for each sulfane sulfur category is still needed. On the other hand, while fluorescent probes have been widely used in this field, few can differentiate hydropersulfides from polysulfides or be used for sulfane sulfur quantification. These technical challenges are expected to be addressed in future research, with the aid of new chemistry developments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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