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Discoveries

FORUM REVIEW ARTICLE

S-Persulfidation: Chemistry, Chemical Biology, and Significance in Health and Disease

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Abstract

Significance: S-Persulfidation generates persulfide adducts (RSSH) on both small molecules and proteins. This process is believed to be critical in the regulation of biological functions of reactive sulfur species such as H_2S, as well as in signal transduction. S-Persulfidation also plays regulatory roles in human health and diseases.
Recent Advances: Some mechanisms underlying the generation of low-molecular-weight persulfides and protein S-persulfidation in living organisms have been uncovered. Some methods for the specific delivery of persulfides and the detection of persulfides in biological systems have been developed. These advances help to pave the road to better understand the functions of S-persulfidation.
Critical Issues: Persulfides are highly reactive and unstable. Currently, their identification relies on trapping them by S-alkylation, but this is not always reliable due to rapid sulfur exchange reactions. Therefore, the presence, identity, and fates of persulfides in biological environments are sometimes difficult to track.
Future Directions: Further understanding the fundamental chemistry/biochemistry of persulfides and development of more reliable detection methods are needed. S-Persulfidation in specific protein targets is essential in organismal physiological health and human disease states. Besides cardiovascular and neuronal systems, the roles of persulfidation in other systems need to be further explored. Contradictory results of persulfidation in biology, especially in cancer, need to be clarified. *Antioxid. Redox Signal.* 33, 1092–1114.

Keywords: S-persulfidation, persulfides, reactive sulfur species, chemical reaction, chemical probes, detection, diseases

Introduction

S-PERSULFIDATION (also named as S-perthiolation or S-sulfhydration) leads to the formation of persulfides (RSSH) from thiols (RSH), in which R can be both small molecules and proteins. Persulfides belong to the family of reactive sulfur species, which play regulatory roles in biology. In mammals, the amounts of low molecular weight (LMW) persulfides and S-persulfidated proteins are unexpectedly high. For instance, up to 100 μM of LMW persulfides (such as glutathione persulfide [GSSH]) were found in the animal tissues (45). Ten percent to 25% of the proteins in mouse liver lysate, including actin, tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are S-persulfidated under physiological conditions. Protein S-persulfidation is a recently defined oxidative post-translational modification that leads to the alteration of protein structures and functions (32, 48, 128). This concept was formulized along with the identification of H_2S as a NO-like signaling molecule. For example, S-persulfidation augments GAPDH activity and enhances actin polymerization (80). Additionally, S-persulfidated proteins are rich in pancreatic MIN6 cells, especially under the condition of endoplasmic reticulum (ER) stress (36).

Given the importance of S-persulfidation in biology, it is crucial to understand some fundamental knowledge of this process, as well as difficulties associated with persulfidation

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research. This review summaries several key aspects about S-persulfidation, which include (i) pathways accounting for endogenous persulfidation; (ii) *in vitro* methods for protein S-persulfidation; (iii) fundamental chemistry and reactions of persulfides that are important for understanding biological functions of persulfidation; (iv) reagents that can specifically release persulfides; (v) methods for the detection/quantification of persulfidation; and (vi) the links between persulfidation and either organismal physiological health, such as vasorelaxation, learning and memory, or various human disorder states, such as cardiovascular diseases, neurodegenerative diseases, metabolic/immune disorders, and cancer. The readers are also encouraged to read some excellent reviews on this topic published recently (32, 65, 128).

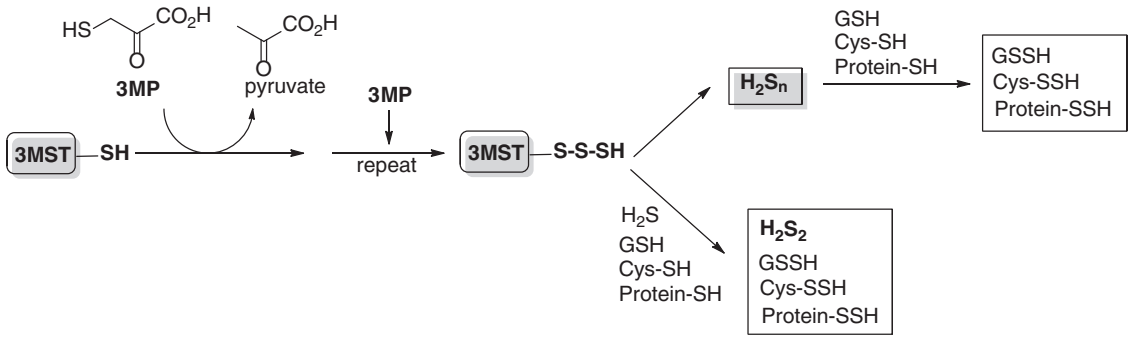
Endogenous S-Persulfidation

S-Persulfidation is believed to be involved in H_2S-based signal transduction. From the chemistry perspective, H_2S cannot directly react with protein cysteine residues (-SH) to form persulfides. However, H_2S could react with the oxidative forms of cysteine, such as disulfides (-S-S-), sulfenic acid (-S-OH), nitrosothiol (-SNO), to form persulfides. It should be noted that in aqueous solutions, the major form of H_2S is its anion (HS^-). The reactions of H_2S are mainly performed through HS^-. The reaction between H_2S and LMW disulfides (such as cystine and GSSG) is a slow and reversible process, usually yielding a mixture of products (35). Such a reaction could be significantly accelerated in protein cases. For instance, the reaction between H_2S and active disulfide of sulfide quinone oxidoreductase (SQR) protein is increased by $\sim 10^6$-fold with respect to free cystine (78). It should be noted that in the cytosol, the concentrations of LMW and protein disulfides are quite low and the formed persulfides should be very limited. Hence, this reaction may be more relevant in ER and under oxidizing conditions (32).

Intracellular persulfide levels were found to increase by the treatment with H_2O_2, while to decrease by inhibiting cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (24). The phenomenon could be explained by two sequential reactions, that is, (H_2O_2 + P-SH → P-SOH + H_2O) and (H_2S + P-SOH → P-SSH + H_2O). Importantly, this process can potentially protect against H_2O_2-mediated oxidative injury in cells. It is known that P-SOH can be further oxidized to sulfinic acids (P-SO_2H) and sulfonic acids (P-SO_3H) (117, 118), which are irreversible oxidized products of the original protein cysteine adducts. However, S-persulfidated proteins (P-SSH) would produce P-SSO_2H and P-SSO_3H under similar oxidative challenges. Both P-SSO_2H and P-SSO_3H can eventually be reduced back to P-SH and recover their normal function in the cells.

S-Nitrosation (RSNO) is another oxidative post-translational modification of protein cysteine residues (15, 69, 94). Similar to S-persulfidation, S-nitrosation is also involved in the regulation of protein functions, although sometimes functions of these two modifications are different or even opposite (113). In one study, 36% overlap of S-persulfidation and S-nitrosation proteomes was noted (36). In theory, RSNO can react with H_2S to form persulfides and HNO. However, this reaction was found to be thermodynamically unfavored (+26 kJ/mol) (63). Nevertheless, this possibility should not be completely ruled out as it may be facilitated by protein microenvironments (32).

Polysulfide species such as H_2S_n or RS_nSH have been suggested by some researchers to be important regulating molecules in sulfur signaling while some other researchers disagree with this assessment (32). Polysulfides belong to the sulfane sulfur family and can transfer sulfur atom to cysteine residues to cause protein S-persulfidation. Such a process has been demonstrated with proteins including transient receptor potential (TRP) A1 and phosphatase and tensin homolog (PTEN) (37, 58). A series of studies by Kimura *et al.* suggested that endogenous H_2S_n are produced mainly from 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (3MST) (59). They also found that H_2S_n could be generated from H_2S by 3MST and rhodanese, and 3MST also produced cysteine- and GSH-persulfides (CysSSH and GSSH) (57). These reactions are described in Scheme 1, with persulfidated 3MST (3MST-SS_nH) as the proposed key intermediate.



Scheme 1

Besides 3MST, other sulfur transfer enzymes may also be responsible for the formation of endogenous persulfide species (both LMW and protein substrates). These include rhodanese, SQR, CSE, CBS, cysteine desulfurase, and so on. Ida *et al.* found high levels (up to 100 μM) of LMW persulfides (CysSSH/GSSH) exist in cells and tissues, and these molecules were suggested to be the main biologically relevant persulfidation reagents (45, 88). A very recent work by Akaike *et al.* found that prokaryotic and mammalian cysteinyl-tRNA synthetases (CARSs) could effectively catalyze the production of cysteine persulfide (CysSSH) and polysulfides ($CysS_nSH$) from cysteine (1). CARS was also found to integrate cysteine polysulfides into proteins during translational

process. These LMW or protein-bound polysulfides can act as the precursors of H_2S_n, and they may work collectively to induce endogenous protein S-persulfidation.

A recent work by Vitvitsky *et al.* found that H_2S could reduce cytochrome c (Cyt C) *via* electron transfer process (114). This Cyt C-dependent H_2S oxidation leads to HS radical formation. The resultant reactive sulfur species can further react with intracellular protein thiols to form persulfides, therefore enhancing persulfidation. This process is speculated to be particularly important in ischemia/reperfusion (I/R)-related injury as it protects proteins from reactive oxygen species (ROS)-induced hyperoxidation during the reperfusion phase.

In Vitro Methods for Protein S-Persulfidation

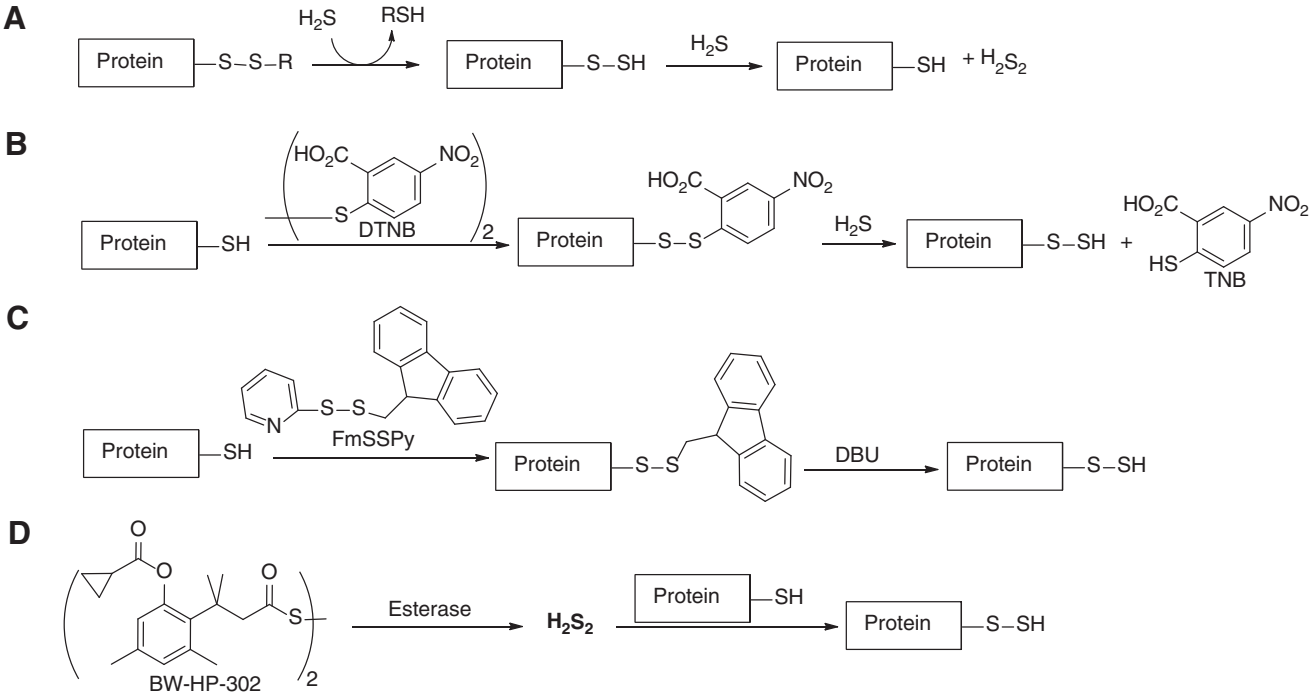
Protein persulfides are a class of important biological persulfides. While they are endogenously generated, relatively pure forms of protein persulfides are sometime needed for understanding their functions. To this end, several methods for the preparation of protein persulfides have been developed (Scheme 2):

(i) Using papain as the model protein, Fukuto and colleagues showed that protein disulfides (P-SSR) could react with H_2S to form persulfides (P-SSH) (35). Interestingly, excess H_2S could further react protein persulfides to resume the original protein (P-SH), presumably with hydrogen persulfide (H_2S_2) as the byproduct.

(ii) 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent) was used to react with protein-SH to form mixed disulfides of the protein, which could then react with H_2S to form protein persulfides. In the latter step, a highly colored species, thionitrobenzoate ($\lambda_{max} = 412$ nm), was formed and could be easily detected. As such, it provided a way to quantify protein persulfides. This method was validated on papain (35) and glutathione peroxidase (GPx) 3 (89).

(iii) A H_2S-free method to prepare protein-SSH utilized 9-fluorenylmethyl pyridinyl disulfide (FmSSPy) to selectively block protein-SH (92). Then, the mixed protein disulfide was treated with DBU to release the 9-fluorenylmethyl protecting group and form protein persulfide.

(iv) Wang and colleagues developed a donor of H_2S_2 (BW-HP-302) that was activated by esterases (124). The liberated H_2S_2 could act as an electrophile to transfer the sulfur atom to protein-SH and furnish the persulfide. The authors tested GAPDH, and the persulfidated GADPH was analyzed by the tag-switch assay. All these methods provide indisputable syntheses of protein-SSH and open access to study the roles of protein persulfidation.

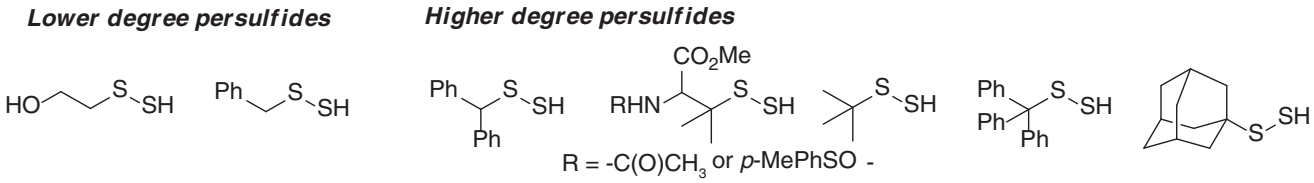


Scheme 2

Fundamental Chemistry of Persulfides

The chemistry and reactivity profiles of persulfides (R-SSH) are best studied using small-molecule substrates. Presently, a limited number of model persulfide substrates have been prepared, isolated, and fully characterized (5, 6, 40). These comprise a small fraction of lower degree (primary) persulfides such as 2-hydroxyethane persulfide [$HO(CH_2)_2SSH$] and benzyl persulfide (Bn-SSH), and the major pool of known persulfide substrates that include higher degree (secondary and tertiary) persulfides such as diphenylmethane-, *tert*-butyl- (*t*Bu-), trityl- (Trt-), adamantyl (Ad-) persulfides, and derivatives of

penicillamine persulfides (Scheme 3). Persulfides are inheritably unstable species, and trends of their stabilities are similar to those observed for *S*-nitrosothiols and sulfenic acids (4, 28, 127); where higher degree substituted persulfides are less prone to decomposition than lower degree substituted persulfides (5, 40). In most cases, the products of persulfide decomposition are polysulfides and elemental sulfur, that is, S_8 (5).



Scheme 3

Persulfides are the sulfur equivalents of organic hydroperoxides with opposite reduction/oxidation (redox) properties. However, they share similar polarization across the O-O and S-S bond and exhibit electrophilic properties. Both sulfurs in the persulfide moiety are reactive electrophilic sites. Density functional theory and experimental data suggest difference in the regioselectivity of the addition of nucleophiles. The regioselectivity of the reaction of thiols and persulfides is dictated by the steric hindrance of the substituents. When the substituent on the persulfide is not sterically constrained, for instance a methyl group, nucleophiles preferentially react with the internal sulfur of persulfides (R-*S*SH), whereas sterically hindered persulfides such as t-BuSSH and TrtSSH are prone to be attacked by nucleophiles at the external sulfur (R-S*S*H) (5, 131).

Persulfides are weak acids (p*K*a $\sim$6.2), and the acid dissociation of -SSH is favored under normal physiological conditions, establishing the perthiolate anion (-SS^-) as the major form in the biological medium. Therefore, persulfides are potential biological nucleophiles and prone to nucleophilic additions with biological electrophiles. Notably, the chemical reactions that perthiolate anions undergo with electrophiles are similar to those observed for R-SH. However, products derived from these reactions differ. For instance, in biological systems, persulfides can react with sulfur-containing electrophiles such as RSSR, RSOH, and H_2O_2 to yield trisulfides, polysulfides, and perthiosulfinate, respectively. Reactions with carbon-based biological electrophiles such as 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) yield RSS-cGMP products, which in turn regulate signal transduction (45).

For decades, significant interest has been paid to the single electron chemistry of reactive sulfur species, especially hydrogen and electron transfer reactions of RSH to produce thiyl radicals ($RS^\bullet$) (27, 100). The formation of thiyl radicals can arise from exogenous stimuli, such as photolysis and radiolysis, and also by the action of oxygenated radicals, such as hydroxyl radical or superoxide, and electron transfer reactions with Fe-containing proteins. Implications of these sulfur radicals, for example, in proteins, are detrimental as they can lead to protein damage or altered function (16, 100). Compared with thiols (RSH), persulfides (RSSH) are more effective hydrogen donors as well as electron donors, making RSSH efficient radical scavengers. Persulfides are known to produce perthiyl radicals (20). In contrast to RSH, the weak bond dissociation enthalpy of the S-H bond in RSSH and stability of the perthiyl radical ($RSS^\bullet$) favor the formation of perthiyl radicals from the reaction with Fe(III) or the transfer of a hydrogen atom with other radical species, including peroxyl ($ROO^\bullet$), thiyl ($RS^\bullet$), alkoxyl ($RO^\bullet$), and alkyl radicals ($R^\bullet$) (8, 20). Compared with thiyl radical ($RS^\bullet$), $RSS^\bullet$ are more stable and in consequence, less reactive and less toxic, due to the α-effect. Tertiary substituted substrates have mainly been utilized to study the chemistry of perthiyl radicals. Nonetheless, the formation of these radicals in proteins has been suggested. For instance, perthiyl radicals in *Escherichia coli* ribonucleotide reductase and pyruvate formate lyase were detected by electron spin resonance and proven to be vital for the inactivation of these two enzymes (23, 91). The major role elicited by perthiyl radicals is their ability to scavenge oxidants, suggesting their pivotal roles in the cell antioxidant defense (31).

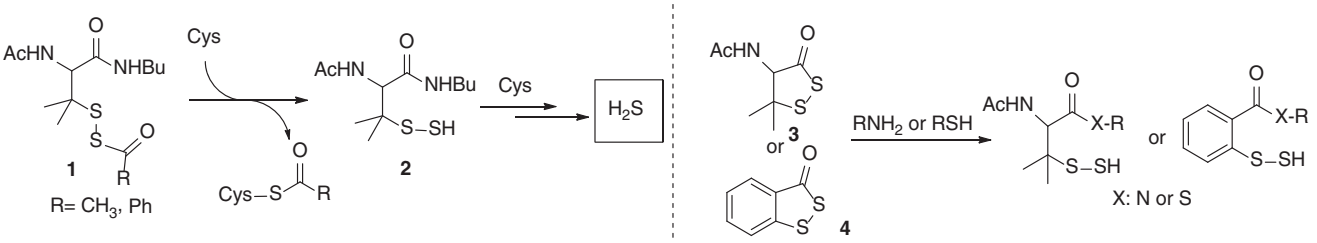
Biocompatible Persulfide Donors

Persulfide donors are reagents that are capable of delivering RSSH payloads upon stimulation. The mechanism to trigger the release of persulfide should be unique and compatible to biological systems. Several strategies have been developed in this regard and can be classified in the following four categories.

Thiol-activated RSSH donors

Diallyl disulfide (DADS) and dially trisulfide (DATS) have been considered as H_2S donors activated by cellular thiols (Cys and glutathione [GSH]). The mechanism of H_2S release from DATS and DADS was recently revisited, and it was found that DATS is a rapid H_2S donor *via* the intermediacy of S-allyl persulfide (68). The authors confirmed the generation of S-allyl persulfide and other polypersulfides (RSS_nSH) derived from the reaction between GSH and DATS by intercepting the intermediates with an alkylating agent 9-bromobimane and liquid chromatography–mass spectrometry analysis. The products allyl-SS-bimane, allyl-SSS-bimane, and allyl-SSSS-bimane were confirmed. In contrast, similar experiments performed with DADS only produced trace amount of allyl-SS-bimane, and no detectable signal for allyl-SSS-bimane and allyl-SSSS-bimane. As such, DADS is not a useful H_2S donor under the treatment of GSH. Previous discovery of DADS as a H_2S donor is likely due to other polysulfide impurities in DADS samples. Notably, the

formation of allyl-SS-bimane demonstrates that DATS is a potential persulfide donor. According to DFT calculations, the nucleophilic reaction of biological thiols to DATS is kinetically and thermodynamically driven toward the peripheral sulfur atom, which in turn produces the S-allyl perthiolate anion (18) rather than previously described S_N2 reaction on the α-carbon. In addition, the calculations confirmed a significantly slower reaction for DADS when compared with DATS. These combined data provided direct evidence about the pre-

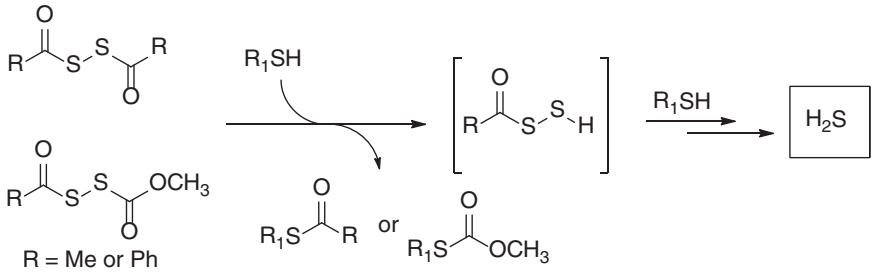


Scheme 4

dominant persulfide donors in garlic, and new insights into the mechanism of H_2S generation from garlic.

Our group reported a series of S-acyl penicillamine substrates **1** (S-acyl disulfides) as donors of H_2S (134). These are also activated by nucleophilic thiols, such as Cys, through an S-acyl transfer reaction, which in turn reveals the penicillamine persulfide (Scheme 4). In this study, the penicillamine persulfide was not isolated. Instead, excess Cys triggered the decomposition of the persulfide and emanated H_2S. Although this class of persulfide/H_2S donors was found effective to repair myocardial I/R injury, it still

Another class of S-acylated disulfide are those derived from dithioperoxyanhydrides (97) (Scheme 5). These compounds can release persulfide intermediates and subsequently H_2S by the action of Cys or GSH. Mechanistically, an S-acyl transfer drives the formation of the persulfides. The profile of H_2S release was illustrated in various aqueous medium including Dulbecco's phosphate-buffered saline buffer and cell lysate. An *ex vivo* vasorelaxation in rat aorta model demonstrated the efficacy of this class of donors, especially methyldithioperoxy anhydride [$CH_3(C=O)SS(C=O)CH_3$], to relax aortic rings in a dose-dependent manner.



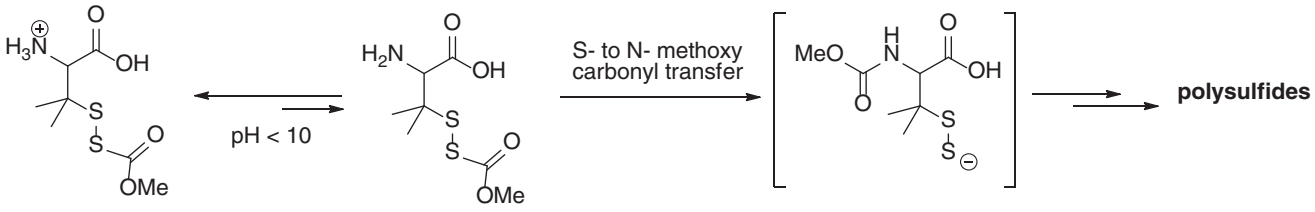
Scheme 5

remains an open question of whether the observed activity is attributed to the direct action of H_2S or through some regulatory mechanisms associated with persulfides. More recently, we extended this class of molecules to a cyclic framework (51). From a chemistry standpoint, the cyclic framework was sought to exert different reactivity profiles when compared with acyclic structures as the tertiary α-carbon of the disulfide bond can induce some ring strain in compound **3**. Biological reactive nucleophiles such as amines and thiols were tested, and both liberated the persulfide intermediate. Other persulfidated molecules such as benzodithiolane **4**, and its selenyl surrogate yielded persulfide and selenylsulfide, respectively. Interestingly, the reactivity profiles of **3** and **4** in the presence of Cys and GSH significantly differed as H_2S generation from **3** was ~8-fold higher than **4** after 1 h. These results suggested a distinct reactivity and stability between the persulfidated species generated from **3** and **4** (*e.g.*, tertiary-SSH *vs.* aryl-SSH). Such diverse reactivity widens the scope of the current library of S-acyl disulfide-based persulfide donors.

pH-activated RSSH donors

The ideal platform to release an active molecule is directly by aqueous environments and the variations in pH. The work by Galardon and colleagues demonstrated a slow and sustained production of H_2S from an S-acylated disulfide scaffold (dithioperoxyanhydrides derivatives) at physiological pH 7.4 (97). Additional studies by this group led to a refined structure, which is a protected disulfide penicillamine derivative (Scheme 6) (3). In aqueous buffers, the $-NH_2$ group triggered an *S*- to *N*-methoxy carbonyl transfer (analogous to

native chemical ligation) to free up a perthiolate anion intermediate. Not surprisingly, a rapid release was attained at higher pH values (8.5) in contrast to lower pH values (6.5) as dissociation of the α-amino group increases as function of pH. It is worth noting that this persulfide donor was unable to produce H_2S. Instead, a mixture polysulfides were identified as the end products. This persulfide donor may be suitable for studies centered in disseminating regulation of protein S-persulfidation in a H_2S-free environment.



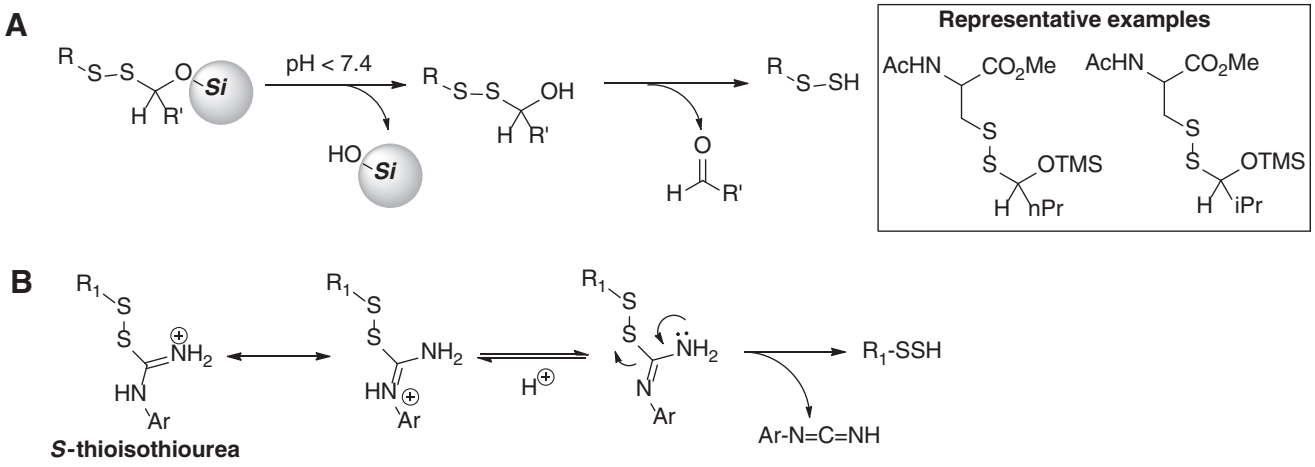
Scheme 6

In this category, our group recently developed a general scaffold for the release of persulfides (52). The scaffold involves *O*-silyl protected unsymmetrical disulfides (Scheme 7A). This design combines the acid labile nature of the *O*-silyl group and the instability of hemithioacetals toward hydrolysis to unmask the persulfide under aqueous conditions. Several substrates were studied to explore the effects of the R-group and the lability of the *O*-silyl group. Substrates containing *O*-trimethylsilyl (-OTMS) were able to produce the corresponding persulfides at a wide range of pH (5.0, 6.0, and 7.4). For substrates containing *O*-triethylsilyl group (-OTES), the persulfide formation was much slower. These donors may serve as a framework to develop tools to deliver persulfides in cell organelles where pH is slightly acidic such as the lysosome.

formation. In addition, RSSH release of these donors was pH dependent as the release was slower at lower pH.

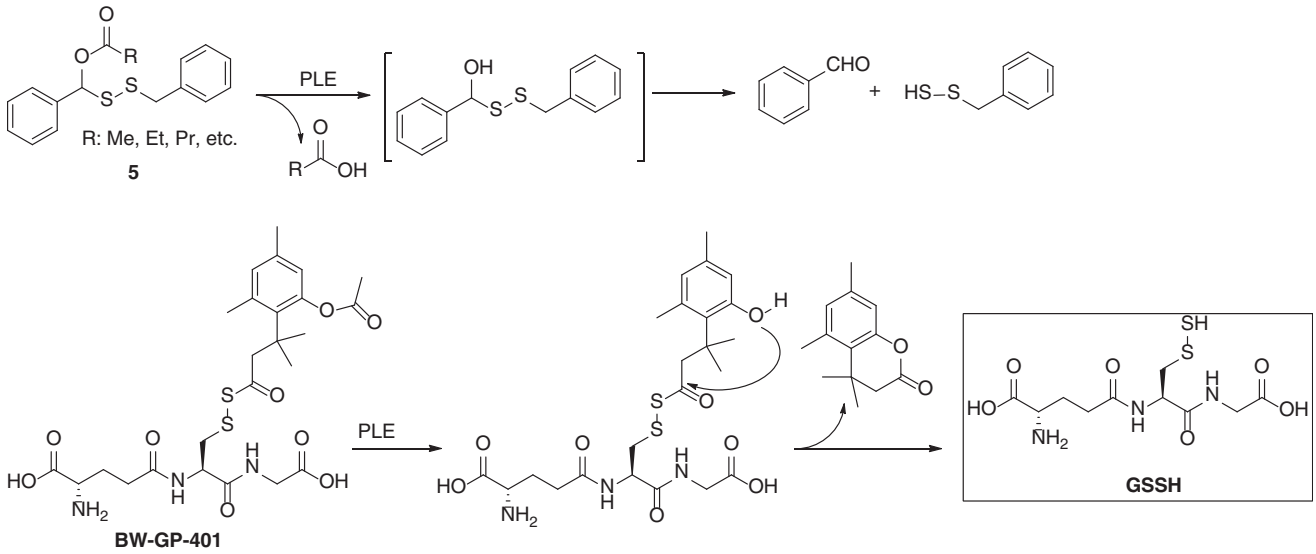
Enzyme-activated RSSH donors

Enzyme-catalyzed release of bioactive payloads is paramount for prodrug design, and this strategy has been applied in the design of persulfide donors. In 2017, Wang and colleagues reported the first esterase-triggered RSSH donors **5** (Scheme 8) (137). Porcine liver esterase (PLE) could promote deacylation of **5** and led to the formation of benzyl persulfide. Later, the same group extended this strategy for the development of a GSSH donor BW-GP-401 (126). Upon PLE-catalyzed hydrolysis, an intramolecular lactonation produced a lactone and released



Scheme 7

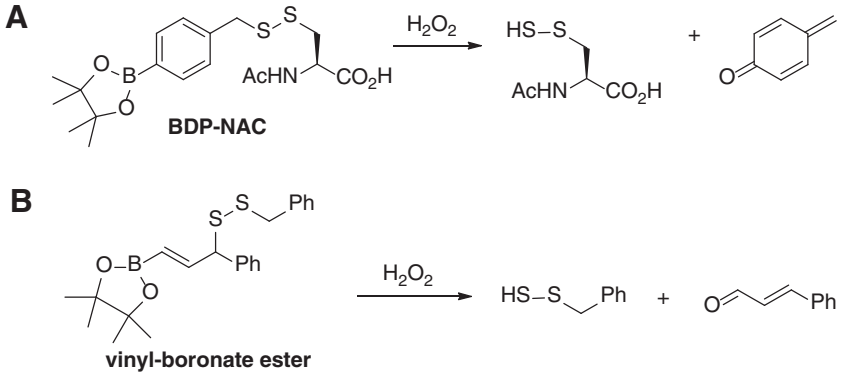
Another class of pH-activated RSSH donors was reported by Khodade and Toscano (56). The design of this new class of donors employed a urea moiety as part of unsymmetric disulfides to yield the *S*-thioisothiourea functionality (Scheme 7B). At pH 7.4, these donors produced persulfides as demonstrated in GSSH. Direct evidence of the formation of GSSH was confirmed by capturing GSSH with DNFB. However, it is unclear how specific this donor is toward esterases, as similar acyl disulfides have been demonstrated to react with thiols *via* S-acyl transfer (3, 97, 134). Such a reaction may also lead to GSSH formation.



Scheme 8

ROS-activated RSSH donors

One possible way of generating protein-SSH is the reaction of H_2S with protein sulfenic acids (P-SOH). Since protein-SOH are produced *via* the action of cysteine thiols and H_2O_2, it suggests that persulfides may coexist with oxidants like H_2O_2. Recently, Matson and colleagues reported a disulfide scaffold bpin-disulfide prodrug-N-acetyl cysteine (BDP-NAC) (Scheme 9A) that released cysteine persulfide in response to H_2O_2 (93). The scaffold contains a boronic ester, which is proven to selectively react with H_2O_2. The following self-degradation releases persulfide. Another work by Chakrapani and colleagues showed that vinyl-boronate ester-based disulfides could act as H_2O_2-triggered RSSH donors too (Scheme 9B) (13). It should be noted that persulfides are known to be highly reactive toward H_2O_2. Therefore, whether the released persulfides can be useful for the following applications is uncertain.

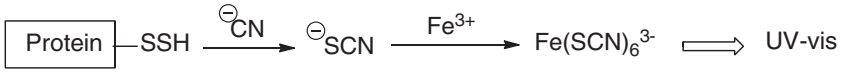


Scheme 9

Detection of S-Persulfidation

Cyanolysis

The first method that was used in the identification of protein persulfides is cyanolysis. As shown in Scheme 10, cyanide anion (CN^-) can react with persulfides to form thiocyanate (SCN^-), which can then be determined spectroscopically following the reaction with a ferric reagent.



Scheme 10

CN^- could also react with other sulfane sulfur species like polysulfides and therefore causing false positives. This method is usually used for purified proteins. It cannot be used for identifying persulfide-containing proteins in biological samples such as cell lysates.

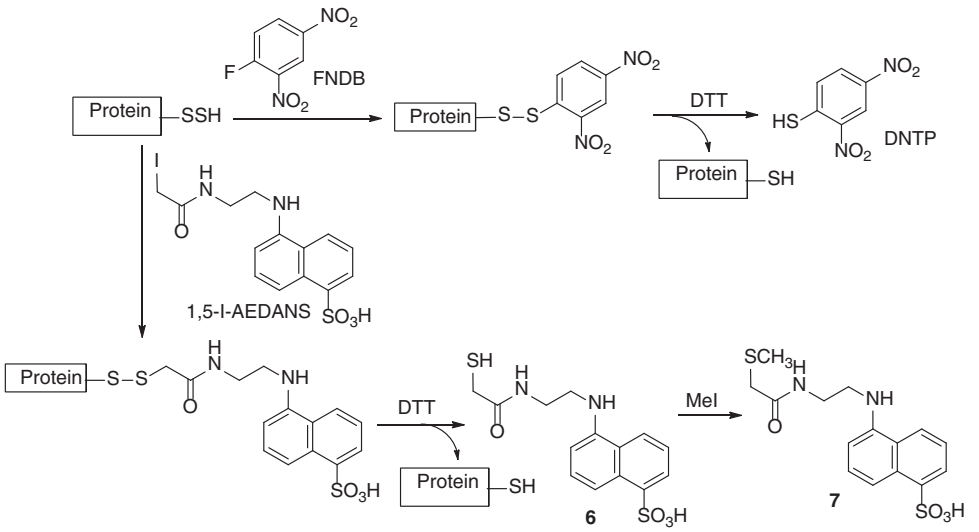
Persulfide-derivatization-based methods

Due to the strong nucleophilicity of persulfides, certain electrophiles can be used to derivertize persulfides and applied for their detection. In one example, 1-fluoro-2,4-dinitrobenzene (FDNB) reacted with protein-SSH to form dinitrobenzene disulfide, which was next reduced by dithiothreitol (DTT) to release DNTP (Scheme 11) (99). The original concentration of persulfide could be determined by measurement of DNTP *via* spectroscopic methods. In another example, *N*-(iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid (1,5-I-AEDANS) was used to identify NifS-bound persulfide (135). The resulted NIfS-bound disulfide was cleaved by DTT to form NifS-SH and thiol **6**. Methylation of **6** gave a stable thioether **7**, which could be easily quantified. Again, these persulfide derivatization methods are limited to individual and purified proteins.

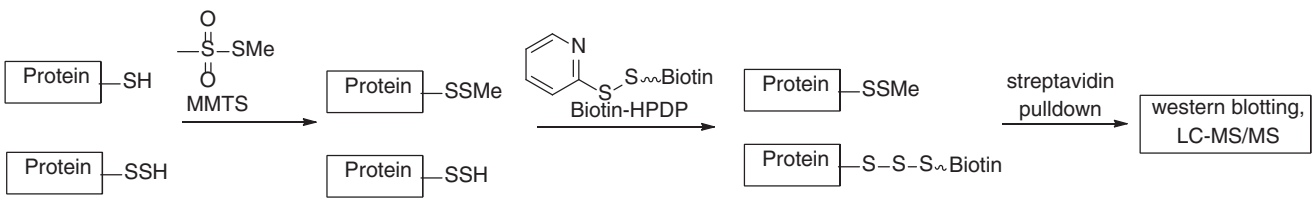
Modified biotin-switch methods

The first method that was used to identify persulfidated proteins in cell lysates is a modified biotin-switch method reported by Snyder and colleagues in 2009 (80). The rationale of this method is illustrated in Scheme 12. It was believed that methyl methanethiosulfonate (MMTS), a thiol-blocking reagent, could specifically block protein thiols (–SH) while leaves persulfides (–SSH) untouched. Then, the persulfide residues were tagged by a biotinylating agent, biotin-HPDP (*N*-[(6-biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide). With this method, it was found that 25%–50% hepatic proteins in mouse liver were persulfidated. However, the specificity of this method has been questioned as the selectivity of MMTS for –SH *versus* –SSH is hard to verify. Normally, –SSH are believed to be stronger nucleophiles than –SH. Thiol-blocking reagents such as iodoacetamide (IAM), NEM, MMTS, DTNB, and *N*-acetylcysteine pyridyldisulfide have been found to effectively block persulfides (89).

In another work by Tonks and colleagues, a different biotin-switch method was used in determining S-persulfidated proteins (Scheme 13A) (64): Both P–SH and P–SSH were first blocked by iodoacetic acid (IAA). Then, persulfide-derived disulfides were reduced by DTT to give free –SH. Subsequently, the –SH was labeled by iodoacetamide-linked biotin



Scheme 11

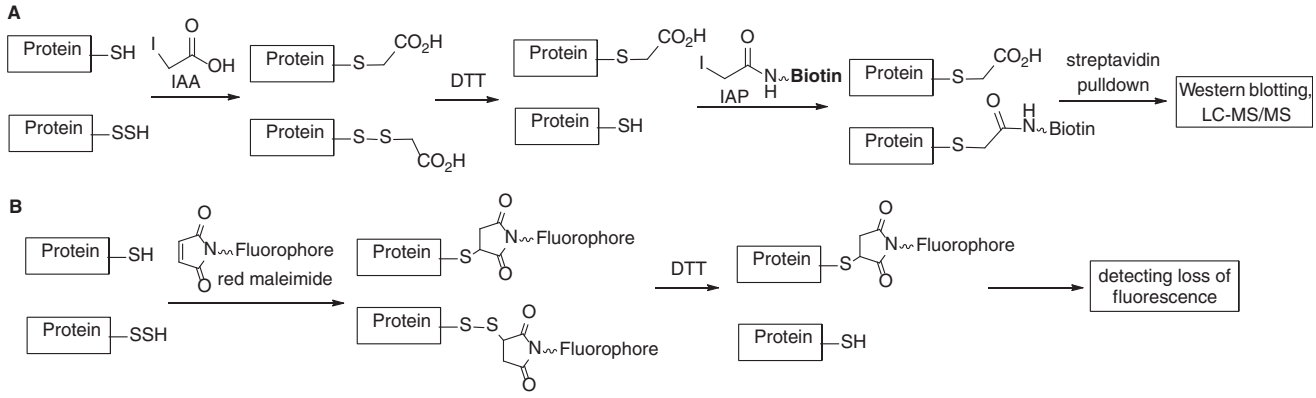


Scheme 12

(IAP) for further isolation and analysis. Using this method, it was found that protein tyrosine phosphatase 1B could be reversibly inactivated by H_2S and led to ER stress response. A similar method employing fluorophore-linked maleimide to detect protein persulfides was reported by Snyder and colleagues (Scheme 13B) (101). The protein mixtures were treated with fluorescent dye-linked maleimide to block both –SH and –SSH. DDT reduction should remove the fluorescent payload from –SSH-derived conjugates. The decrease of fluorescence could be quantified on a polyacrylamide gel and used to identify the original persulfides.

Other biotin-labeling-based persulfide detections

In 2016, Nagy and colleagues reported an easy-to-use and highly specific method for the detection/identification of protein persulfides, named ProPerDP (29). This method can be used in intact cells and tissue samples. With this method, the authors were able to quantify 1.52 and 11.6 μg/mg protein steady-state protein persulfide concentrations in HEK293 cells and mouse liver, respectively. The chemistry mechanism of the protocol is shown in Scheme 15: in the first step, protein-SH and –SSH groups are alkylated by iodoacetyl-



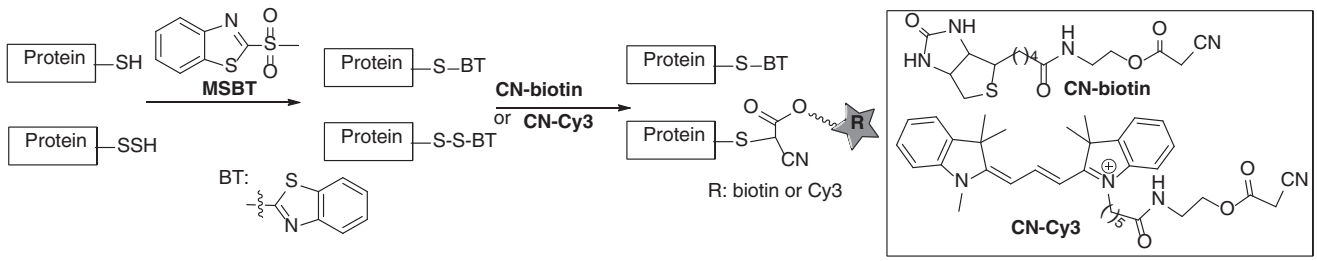
Scheme 13

Tag-switch method

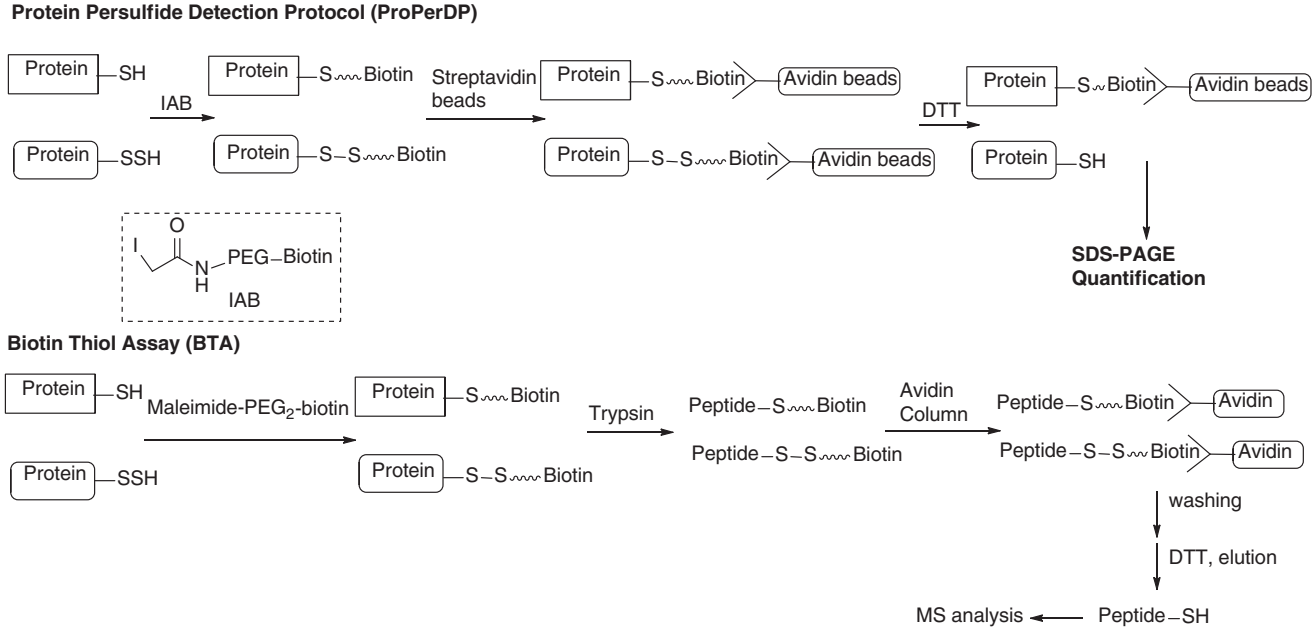
In 2014, our laboratory developed a tag-switch assay, which could be used for proteomic study of protein persulfidation (45, 129). This method employs two reagents to selectively label protein persulfides in a stepwise ''tag-switch'' process (Scheme 14). In step 1, both P–SH and P–SSH are blocked by methylsulfonyl benzothiazole (MSBT) to give the corresponding thioether (P-S-BT) and disulfide (P-SS–BT) adducts. Since benzothiazole-containing disulfides (P-S-SBT) are reactive toward certain carbon-based nucleophiles such as cyanoacetate, in step 2 reporting molecules can be introduced to P–S-SBT to form stable conjugates, indicating the original sites of protein persulfides. This process is selective for P–SSH as MSBT-blocked adducts from thiols (P-SH) do not react with carbon nucleophiles. Several cyanoacetate derivatives such as CN-biotin and CN-Cy3 have been developed as ''tag-switch'' labeling reagents. S-Persulfidated proteins identified by this method include protein disulfide isomerase, heat shock proteins, aldo-keto reductase, GAPDH, enolase, and phosphoglycerate kinase.

PEG_2-biotin (IAB). The resultant biotin-linked proteins are then pulled down from the protein mixture using streptavidin-coated magnetic beads. Persulfide-derived proteins can be further reduced by DTT to form protein-SH and released from the beads, whereas thiol (-SH)-derived proteins will remain bound to the beads. The released proteins can be quantified or visualized on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which should reveal the original identity of persulfidated proteins.

In almost the same time ProPerDP was developed, Hatzoglou and colleagues reported a biotin-thiol assay that was able to identify persulfide proteins based on a very similar mechanism (36). As shown in Scheme 15, maleimide-linked biotin is first used to label both protein –SH and –SSH residues. Then, proteins are trypsinized. Biotin-conjugated peptides (*via* thioether linkage or disulfide linkage) are immobilized by streptavidin beads. The disulfide-based peptides can be reduced by DTT and eluted from the beads for subsequent mass spectrometry analysis. These peptides are originally derived from protein persulfides so their identity/quantification can be used to deduce the information of persulfides.



Scheme 14

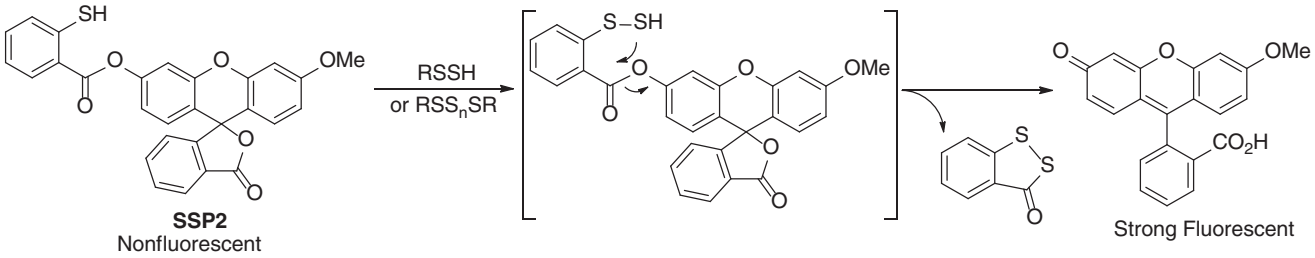


Scheme 15

Another similar persulfide-labeling approach (qPerS-SID) was combined with the stable isotope labeling with amino acid in culture method (73). This method allowed for quantitative persulfide proteomics analysis.

Fluorescent sensors for persulfides

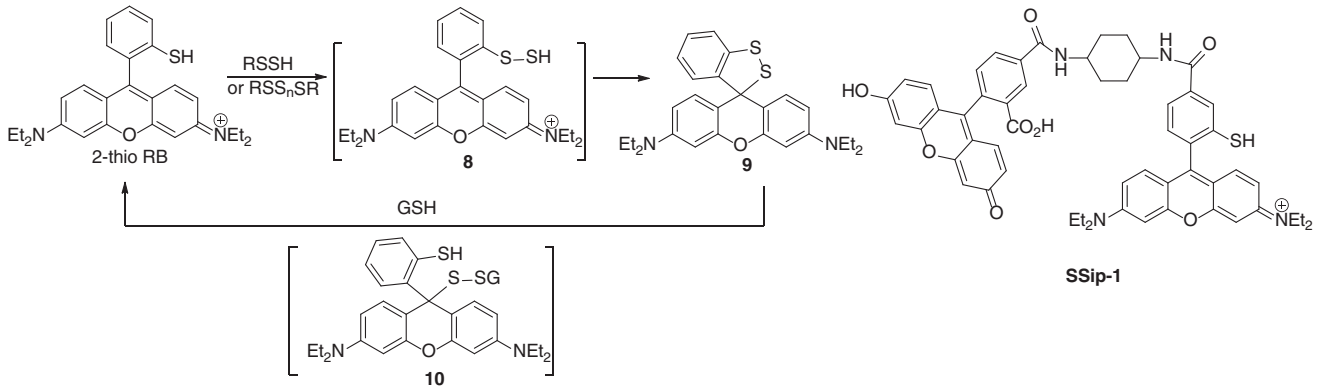
Fluorescence-based methods have been widely used in the detection of biomolecules due to their rapid sensitive responses and spatiotemporal resolution capability. Up to date, fluorescent sensors that can specifically imagine protein persulfides have not been reported. However, several sensors have been developed for the detection of small-molecule persulfides. These works are summarized below.



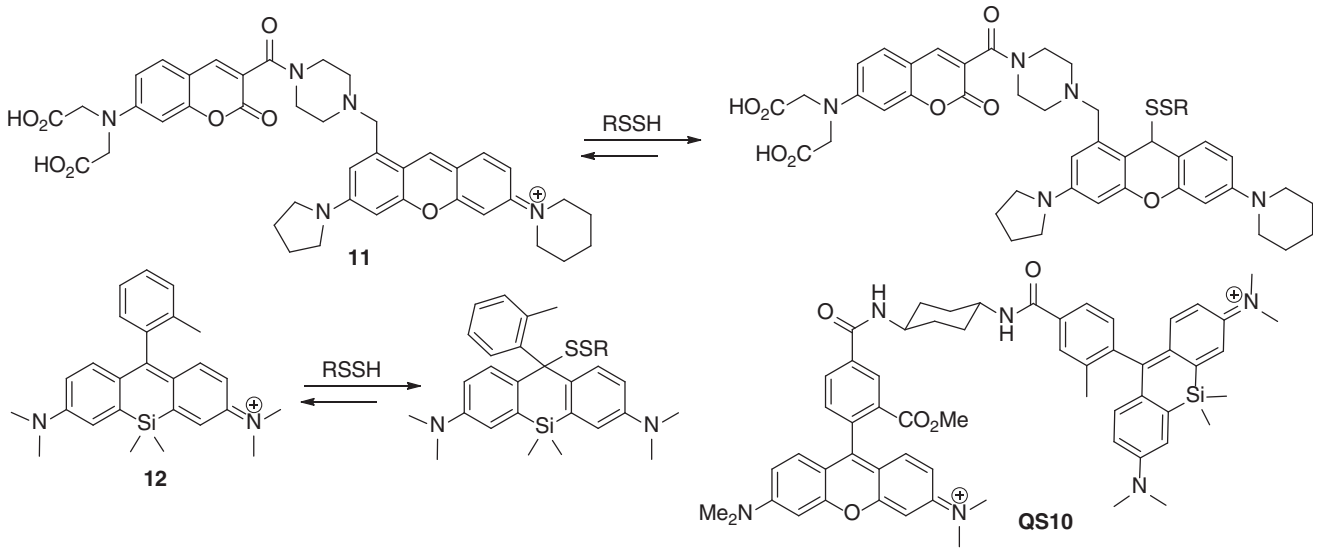
Scheme 16

SSP probes. Persulfides belong to the sulfane sulfur family, in which molecules contain a sulfur atom with six valence electrons but no charge (known as S^0). A unique reactivity of sulfane sulfurs is that they can react with certain nucleophiles, for instance, reacting with cyanide ion (CN^-) to form thiocyanate (SCN^-). Based on this reactivity, sulfane sulfur probes (such as SSP2) have been developed and were used to detect persulfides (Scheme 16). These probes employ a thiophenol to react with sulfane sulfurs to form an Ar-S-SH intermediate, which subsequently undergoes an intramolecular cyclization to release the pendant fluorophore. While these probes are selective for sulfane sulfurs including persulfides, they cannot differentiate each member in the sulfane sulfur family, for example, persulfides *versus* polysulfides.

SSip-1. The reaction between persulfides and thiolate has also been used to develop reversible fluorescent sensors for persulfides. Urano and colleagues found that 2-thio rhodamine B (2-thio RB, Scheme 17) could react with persulfides or polysulfides to form **8**, which then formed spirocycle **9**, leading to fluorescence decrease at 560 nm (108). Interestingly, GSH could restore the fluorescence, *via* the reduction of the disulfide bond to form **10** and then elimination of GSSH to reform 2-thio RB. Based on this reactivity, a fluorescence resonance energy transfer (FRET)-based sensor SSip-1 was developed. SSip-1 could reversibly visualize sulfane sulfurs including persulfides in living cells.



Scheme 17



Scheme 18

In another work by Ojida and colleagues, the strong nucleophilicity of persulfides was employed in the design of reversible ratiometric fluorescent sensors (54). For instance, sensor **11** contained a coumarin as the FRET donor, which is conjugated to a pyronine unit as the FRET acceptor. It showed two distinct emissions at 479 and 584 nm (excited at 410 nm) due to the coumarin and pyronine units. Upon addition of persulfides (using Na_2S_2 as the model), the dual-emission spectrum changed dramatically in a seesaw manner, with a large decrease at 584 nm and the concomitant increase at 479 nm. The ratio value R (F_{479nm}/F_{584nm}) could be used to determine the concentrations of persulfides. The sensing mechanism involves a nucleophilic addition of the persulfides with the pyronine unit, which modulates the intramolecular FRET efficiency. In addition, the nucleophilic addition was found to be reversible, and therefore, the sensor could also detect the decreases of persulfide levels. Very recently, Urano and colleagues found silicon-derived rhodamines could also undergo reversible nucleophilic addition with persulfides (110). For example, compound **12** showed high sensitivity and selectivity for persulfides (>10,000-fold over GSH). This template was used to develop QS10, a ratiometric sensor for persulfides.

S-Persulfidation and Diseases

S-Persulfidation in cardiovascular and related health and diseases

Vasorelaxation is one of the most important functions of H_2S, and the underlying mechanisms have been investigated since the report of Kimura and colleagues (41). Several related proteins, such as ion channels and second messengers, are involved in H_2S-induced vasorelaxation (Fig. 1) (7, 60, 81, 105, 109, 133). Among ion channels, ATP-sensitive potassium channel (K_{ATP}) is well documented (21, 47, 81). Classical K_{ATP} channels are composed of Kir6.x and SURx subunits, alongside additional components (102). Physiologically, K_{ATP} channels are controlled by intracellular ATP, as well as ADP and nucleotides. Many reports have shown that K_{ATP} channels can be regulated by H_2S (47, 116, 133). For example, in aorta and mesenteric artery, H_2S-induced vasorelaxation is mainly mediated by K_{ATP} channels (21, 133).

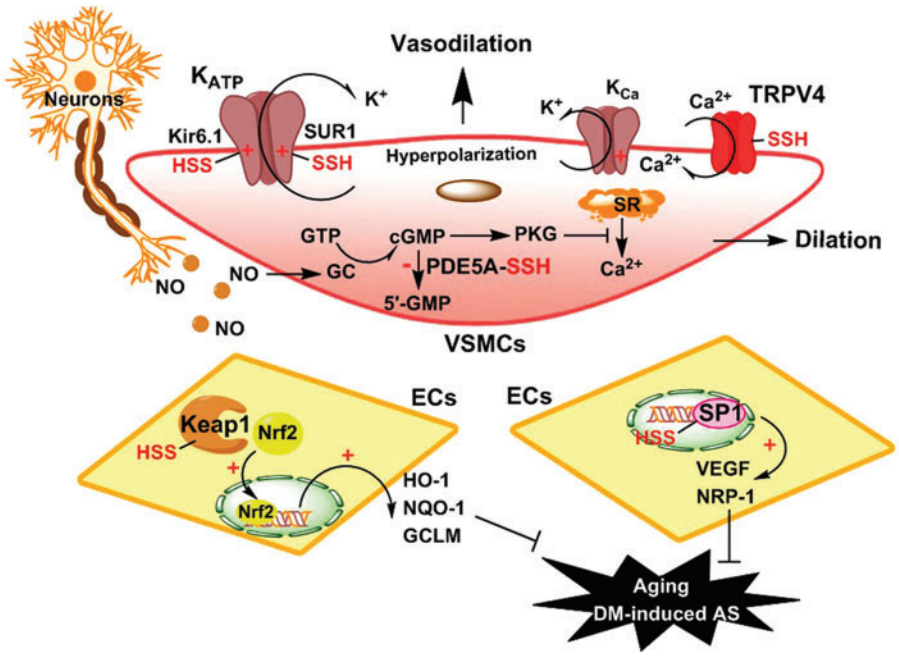


FIG. 1. Possible signaling roles of S-persulfidation in vasorelaxation. Neurons and ECs-released NO dilates VSMCs. In VSMCs, enhancement of K^+ efflux through K_{ATP} or K_{Ca} channel hyperpolarizes cell membrane and induces vasodilation. The *red* P-SSH in K_{ATP} channel Kir6.1 and SUR1 subunits and in TRPV4 channel represents S-persulfidation of these channel proteins. The *red cross* (+) represents opening of the channels. The activation of GC/cGMP/PKC pathway inhibits SRs Ca^{2+} release and induces vasodilation. In ECs, S-persulfidation-induced Keap1 activation releases Nrf2, which undergoes nuclear translocation and induces the expression of HO-1, NQO-1, and GCLM. S-Persulfidation-induced SP1 activation promotes VEGF and NRP-1 expression, thus eliminating aging and DM-induced AS. AS, atherosclerosis; ECs, endothelial cells; GCLM, glutamate-cysteine ligase modifier; HO, heme oxygenase; K_{ATP}, ATP-sensitive potassium channel; K_{ca}, calcium-activated potassium channel; Keap1, Kelch-like ECH-associated protein 1; NQO, NADPH quinone oxidoreductase; Nrf2, nuclear factor-like 2; NRP, neuropilin; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; SP1, specificity protein 1; SR, sarcoplasmic reticulum; TRP, transient receptor potential; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells. Color images are available online.

Jiang *et al.* found that H_2S-induced activation of K_{ATP} channels depends on SUR1 subunit's S-persulfidation at Cys6 and Cys26 residues (47). However, Mustafa *et al.* considered that S-persulfidation of Kir6.1 subunit at Cys43 site might be crucial to H_2S-induced hyperpolarization and vasorelaxation (81).

Besides K_{ATP} channels, another kind of K^+ channel can be controlled by intracellular Ca^{2+} ion content, also known as Ca^{2+} ion-activated K^+ (K_{Ca}) channels. It has been shown that Ca^{2+} ion influx mediated by TRPV4 channels can activate K_{Ca} channels, thereby resulting in endothelial hyperpolarization and subsequent vasodilation (33). It is worth noting that H_2S can S-persulfidate TRPV4 channel proteins at specific cysteine residues, as supported by a DTT assay (82). Apart from activating K^+ channels, normal Ca^{2+} ion influx can cause contraction of muscle cells, including vascular smooth muscle cells. Therefore, lowering intracellular Ca^{2+} content will consequently lead to vasorelaxation. An important example is the signaling of cGMP, a second messenger, which has been demonstrated to reduce cytoplasmic Ca^{2+} content (10). Endogenous cGMP usually exerts its vasodilator action through the activation of cGMP-dependent protein kinase (PKG) (34). In cells, PKG has a wide range of functions to dilate blood vessels. For example, PKG can induce sarcoplasmic reticulum (SR) to store Ca^{2+}, while it inhibits the release of Ca^{2+} by SR, thereby lowering cytoplasmic Ca^{2+} content and dilating smooth muscle cells. It is known that endogenous phosphodiesterase type 5 (PDE5) can hydrolyze intracellular cGMP, thereby balancing the physiological content of cGMP. However, if intracellular cGMP is deficient, low Ca^{2+}-induced vasodilation and penile cavernous relaxation will be impaired, which may result in hypertension and erectile dysfunction. Therefore, inhibition of PDE5 is an important strategy of these dysfunctions through raising intracellular cGMP content. Interestingly, the increase in endogenous H_2S amount or its exogenous application can inhibit PDE5A dimerization and resultant activation *via* S-persulfidation (105), thereby inducing vasodilation and penile cavernous relaxation. The effect mediated by S-persulfidation is similar to the effect of Sildenafil, a clinically used PDE5 inhibitor (12).

Besides vasorelaxation, H_2S maintains vascular function and health through upregulation of vascular endothelial growth factor receptor 2 and neuropilin-1 (Fig. 1). The regulatory effects of H_2S on these two proteins mainly attribute to S-persulfidation of a transcription factor, specificity protein 1 (SP1). It has been shown that S-persulfidation of Cys68 and Cys755 can enhance SP1 transcriptional activity (98).

Under conditions of oxidative lesion, S-persulfidation of redox-related proteins is distinctively involved in blood vessel protection. One of the most important examples is Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor-like (Nrf)2 signaling pathway. The combination of Keap1 with Nrf2 usually activates the ubiquitin-proteasome pathway-mediated degradation, leading to a decrease in

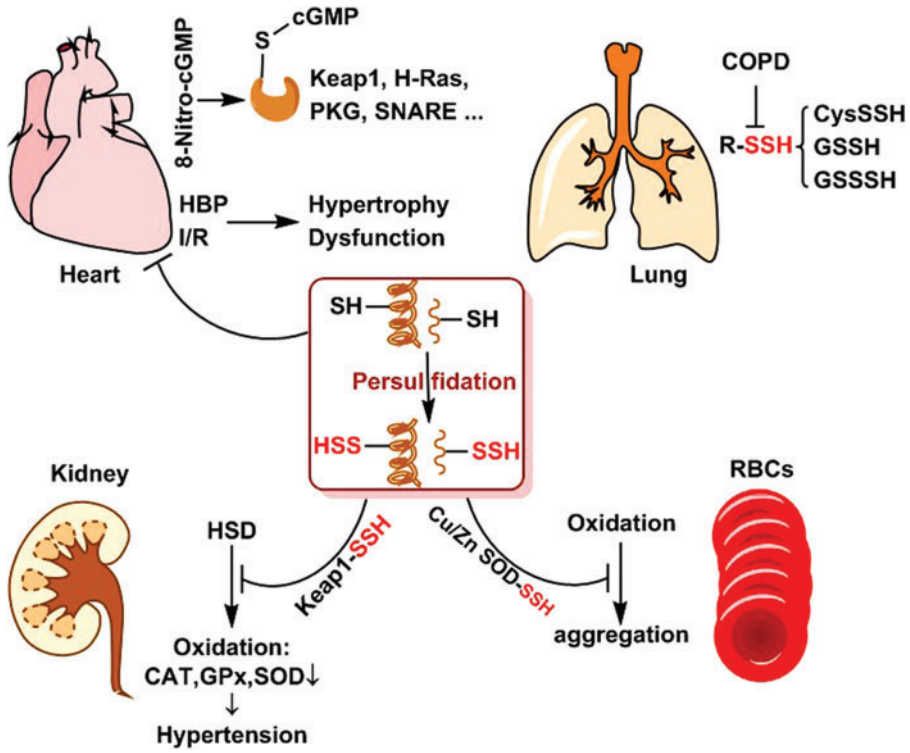


FIG. 2. Roles of S-persulfidation in the indicated organs. In hearts, S-persulfidation blocks 8-nitro-cGMP-mediated target protein S-guanylation and inhibits HBP and I/R-induced hypertrophy and dysfunction. In kidneys, S-persulfidation of Keap1 prevents HSD and oxidation-induced HBP. In RBCs, S-persulfidation of Cu/Zn SOD attenuates oxidation-induced aggregation. In lungs, COPD condition reduces LMW S-persulfide contents. 8-nitro-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; COPD, chronic obstructive pulmonary disease; HBP, hypertension; HSD, high-salt diet; I/R, ischemia/reperfusion; LMW, low molecular weight; RBCs, red blood cells; SOD, superoxide dismutase. Color images are available online.

cytoplasm Nrf2 content. Once modified by electrophiles, such as 8-nitro-cGMP, the Keap1-Nrf2 heterodimer will release Nrf2. The translocated Nrf2 will bind to antioxidant response element in nuclear DNA and regulate the expression of redox-related genes, such as heme oxygenase (HO)-1, NADPH quinone oxidoreductase, and glutamate-cysteine ligase modifier (Fig. 1). As a nucleophile, H_2S can directly react with some electrophiles, such as 8-nitro-cGMP, and thus abolish their oxidative injury (85). More importantly, H_2S can also induce S-persulfidation of Keap1, indirectly relieving nucleophiles-induced oxidative lesions (42, 119, 123). Furthermore, H_2S-mediated S-persulfidation of Keap1 at Cys151 residue significantly suppresses vascular atherosclerosis accelerated by diabetes (119). In addition, the aging of endothelial cells (ECs) and fibroblasts is of great importance to vascular dysfunction. In fact, the repair of DNA damages can retard the process and delay cellular senescence. It is reported that H_2S-indcued Cys341 S-persulfidation of MEK1 enhances ERK1/2 phosphorylation and nuclear translocation, thereby activating PARP-1 and initiating DNA ligase III-mediated DNA damage repair (132).

Summarily, in blood vessels, under physiological condition, S-persulfidation of K_{ATP} channels, TRPV4 and PDE5 causes vasorelaxation. Under physiopathological conditions, S-persulfidation of Keap1 exerts antioxidative effects and attenuates vascular dysfunction induced by diabetes and aging.

Endogenous redox imbalance is also a major risk for heart disease. Intracellular accumulation of 8-nitro-cGMP is a main cause of redox imbalance. Usually, 8-nitro-cGMP derives from reaction between reactive nitrogen species and guanine nucleotide. It was reported that 4-week myocardial infarction and 6-week pressure overload could significantly increase 8-nitro-cGMP generation in mouse hearts (85). Persistent 8-nitro-cGMP injury eventually leads to cardiac disorder or even chronic heart failure. It has been well documented that 8-nitro-cGMP can induce S-guanylation of special cysteine residues and weaken the activity of target proteins, such as Keap1, PKG, H-Ras, and soluble *N*-ethylmaleimide-sensitive factor attachment receptors (2, 84, 96). Through S-persulfidation, cysteines (both small molecules and proteins) can be converted into persulfides (RSSH). Notably, RSSH has much enhanced ability to eliminate 8-nitro-cGMP comparing with reduced cysteines (RSH), thereby preventing specific proteins from S-guanylation induced by 8-nitro-cGMP (84, 85). Apart from the LMW CysSSH, S-persulfidation of proteins at special cysteine residues is essential to the redox homeostasis. For instance, treatment of cardiomyocytes with GYY4137 (a H_2S donor) attenuates spontaneous hypertension-induced myocardial hypertrophy. The mechanism underlies H_2S-medicated inhibition of Kruppel-like factor 5 (KLF5) transcription activity *via* S-persulfidation of SP1 at Cys664 (76). Additionally, in I/R-induced myocardial injury, H_2S treatment during the reperfusion obviously lowers infarct size, thus improving myocardial contractile function. Further study showed that the levels of S-persulfidation and S-nitrosylation are significantly increased during the process (104). In summary, these studies suggest that S-persulfidation of LMW cysteine and cysteine residues of target proteins is essentially involved in cardioprotection (Fig. 2).

Apart from the heart and blood vessel, normal maintenance of cardiovascular function closely depends on other organs in the body, such as the lungs, kidneys, and even blood cells. Once these organs or cells are damaged or dysfunctional, secondary cardiovascular lesions will be initiated. In human chronic obstructive pulmonary disease (COPD), oxidative stress has been accepted as a major etiological factor driving the process of COPD (95). It was reported that the amounts of

reactive persulfides, including CysSSH and GSSH, were distinctively lowered in lung cells and epithelial lining fluid of the patients with COPD (86). Significantly, the persulfide amounts have a positive correlation with the degree of airflow limitation (86) (Fig. 2).

In human chronic kidney disease, ROS appear to be responsible for the high incidence of cardiovascular events. It has been found that high-salt diet triggers renal excessive oxidation, and resultant functional and structural injury, evidenced by the increased levels of serum creatinine and urea, and decreased levels of 24-h urine protein. Meanwhile, the cardiovascular function is abnormal showing as secondary hypertension. Importantly, the donation of H_2S improves renal function and structure and enhances urine volume, thereby alleviating the secondary hypertension of the patients with chronic kidney disease. Similar to the effects on other organs, the improvement of H_2S on renal function is still associated with S-persulfidation of Keap1 and, subsequent Nrf2 disassociation and nuclear translocation. These effects will consequently trigger the expression of antioxidant genes, such as CAT, GPx, and superoxide dismutase (SOD) (43) (Fig. 2).

The blood system is a special flowable organ in the body, which consists of liquid plasma and tangible blood cells. The altered plasma components and dysfunctional blood cells can trigger secondary vascular lesions. As documented, S-persulfidation exists in red blood cells (RBCs) (14). When Cu/Zn SOD isolated from RBCs reacts with H_2S, S-persulfidation will be generated. Further study shows that the modification occurs at Cys111 residue of Cu/Zn SOD protein. The S-persulfidated Cu/Zn SOD protein exhibits a slower acid-induced unfolding and is more resistant to oxidation-induced aggregation, such as copper and H_2O_2 (14, 26) (Fig. 2). S-Persulfidation of Cu/Zn SOD may be conducive to endothelial protection and decrease the risk of venous thrombosis.

S-Persulfidation in neural health and disease

As a gaseous neuromodulator, physiological concentration of H_2S is essential for learning and memory by affiliating hippocampus long-term potentiation (LTP), which is one of the most important forms of synaptic plasticity (74). During the development of synaptic plasticity, glutamate receptor-mediated postsynaptic excitation, including LTP, plays a very important role. Among those glutamate receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor and *N*-methyl-D-aspartate (NMDA) receptor have been well documented. It was found that these receptors could be S--persulfidated during the process of synaptic plasticity (67, 74). These S-persulfidated proteins are seen in NR2A subunit of NMDA glutamate receptors and in postsynaptic proteins (67, 74). The postsynaptic proteins include protein kinase A (PKA), protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinases II, and they usually regulate the phosphorylation of glutamate receptors. In addition, D-serine in the brain acts as a neuronal signaling molecule and activates NMDA glutamate receptors, contributing to synaptic LTP generation. Endogenous D-serine is mainly produced due to the catalyzation of serine racemase (90). It has been shown that exogenous supplement of H_2S or boost of H_2S signal overtly restores S-persulfidation levels of serine racemase protein, followed by the improvement of age-related LTP deficit and hippocampus-dependent memory impairment (66). However, in inflammation-induced memory impairment, proinflammatory cytokine interleukin (IL)-1β can upregulate endogenous CBS and H_2S; nevertheless, they seem to exert adverse effects. It has been reported that H_2S can induce S-persulfidation of GAPDH in neuron dendrites. S-Persulfidation enhances GAPDHs binding ability to ubiquitin-protein ligase E3 (Siah protein). Then, E3 ligase Siah will link PSD95 synaptic scaffolding molecule, leading to PSD95s ubiquitination-dependent degradation and consequent memory impairment (77) (Fig. 3).

Apart from brain, spinal cord is another important part of central neural system. A very recent study showed that breathed H_2S could prevent ischemia-induced paraplegia

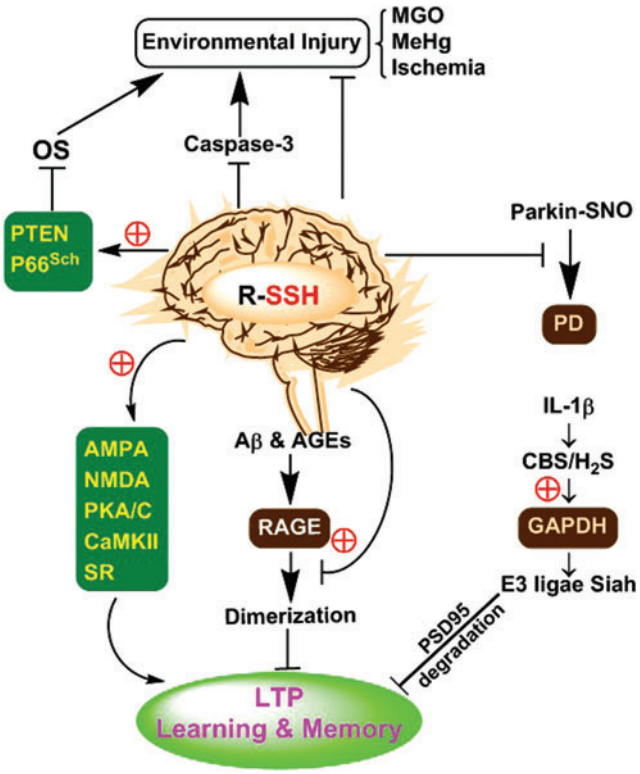


FIG. 3. Possible signaling roles of S-persulfidation in the nervous system. S-Persulfidation of glutamate AMPA and NMDA receptors, postsynaptic proteins (PKA/C, CaMKII) and serine racemase is involved in normal learning and memory. S-Persulfidation of RAGE blunts Aβ and AGEs-impaired learning and memory. Proinflammatory cytokine IL-1β-mediated S-persulfidation of GAPDH harms learning and memory through degradation of PSD95. S-Persulfidation of parkin blocks its S-nitrosylation and inactivation and thus eliminates PD lesions. S-Persulfidation increases PTEN and $P66^{Sch}$ activity and decreases caspase-3 activity, thereby reducing environmental stimuli-induced neuronal lesions. Aβ, Abeta 1–42 peptide; AGE, advanced glycation end product; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CaMKII, calmodulin-dependent protein kinases II; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; NMDA, *N*-methyl-D-aspartate; PD, Parkinson's disease; PKA, protein kinase A; PTEN, phosphatase and tensin homolog; RAGE, receptor for advanced glycation end product. Color images are available online.

Table 1. Representative Persulfidated Proteins and Their Functions

Target proteins	*Persulfide residues*	*Affected biological functions*
Cardiovascular system		
SUR1 subunit	C6/26	Activates K_{ATP} channels and dilates blood vessels
Kir6.1 subunit	C43	Activates K_{ATP} channels and dilates blood vessels
TRPV4	N/A (Not detected)	Enhances K_{Ca} channel-dependent vasodilation
PDE5A	N/A (Not detected)	Inhibits cGMP degradation and induces cavernous relaxation
SP1	C68/755	Enhances VEGFR2- and neuropilin1-dependent anti-aging
	C664	Inhibits KLF5 activation and secondary myocardial hypertrophy
Keap1	C151	Activates Nrf2 pathway and attenuates oxidative injuries
MEK1	C341	Activates ERK1/2 pathway and repairs damaged DNA
Cu/Zn-SOD	C111	Inhibits RBCs aggregation and venous thrombosis
Neural system		
NR2A	C87/320 (Speculated)	Activates NMDA glutamate receptors and improves memory
Serine racemase	C113	Prevents age-related LTP deficit and memory impairment
GAPDH	C150	Enhances Siah E3 ligase's binding, PSD95 degradation, and memory impairment
RAGE	C259/301	Blocks RAGEs dimerization and persistent oxidation-induced neural lesions
Parkin	C95/182/C59	Inhibits Parkinson's disease-related lesions
PTEN	C71/124	Prevents from oxidative injury in nerve cells
Caspase-3	C163	Attenuates apoptotic death
Digestive system		
SUR2B subunit	C24/1455	Attenuates K_{ATP} channel Kir6.1's tyrosine nitration
Bone		
RUNX2	C123/132	Improves fracture healing
Metabolic disorder and diabetes		
Pyruvate carboxylase	C265	Enhances gluconeogenesis
ATP synthase	C244/294	Boosts ATP generation in liver cells
PPARγ	C139	Promotes lipid storage
Inflammation and cancer		
NF-κB p65 subunit	C38	Increases NF-κB activity and attenuates macrophage apoptosis
HuR	C13	Reduces monocyte adherence and inflammation
c-Jun	C269	Inhibits ROS production and NLRP3 inflammasome activation
LDHA	C163	Enhances colonic carcinoma energy synthesis and growth

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; K_{ATP}, ATP-sensitive potassium channel; Keap1, Kelch-like ECH-associated protein 1; LDHA, lactate dehydrogenase A; LTP, long-term potentiation; NF, nuclear factor; NMDA, *N*-methyl-D-aspartate; Nrf2, nuclear factor-like 2; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin homolog; RAGE, receptor for advanced glycation end product; RBC, red blood cell; ROS, reactive oxygen species; RUNX2, runt-related transcription factor 2; SOD, superoxide dismutase; SP1, specificity protein 1; VEGF, vascular endothelial growth factor.

through inhibition of inflammation and apoptosis. The mechanism was associated with induction of nuclear factor (NF)-κB p65 subunit S-persulfidation from S-nitrosylation (50).

In summary, S-persulfidation of proteins is significant in learning and memory of central neuronal system. It is not only involved in the development of normal learning and memory but also mediates inflammation-induced learning impairment.

In chronic neurodegenerative diseases, S-persulfidation of specific proteins is also of great significance (Fig. 3). Alzheimer's disease (AD) starts slowly and worsens over time, characterized by a gradual loss of short-term memory. In AD cellular models, the treatment of human SH-SY5Y neuroblastoma cells with Abeta 1–42 peptide (Aβ) or advanced glycation end products induces receptor for advanced glycation end products (RAGEs) dimerization and cellular senescence. It has been suggested that H_2S can induce S-persulfidation of RAGE at Cys259/301 residues. By this mechanism, H_2S blocks RAGEs dimer formation and persistent oxidation-induced neural lesions (138). Unlike the impaired memory of AD, Parkinson's disease (PD) mainly causes lesions in motor nervous system. It has been demonstrated that the mutation of parkin gene is one of the leading causes of PD. Normally, parkin protein acts as an ubiquitin E3 ligase, and its activity becomes low or even lost in the pathogenic process of inherited and sporadic PD (25). Notably, S-nitrosylation is a common reason for parkin's loss of ubiquitin ligase activity, which weakens its neuroprotective function (22). Furthermore, parkin's S-persulfidation abundance is significantly decreased in PD patients' brains. Interestingly, through upregulation of S-persulfidation, H_2S is able to restore parkin's catalytic activity, consequently inhibiting PD-related lesions (113).

Akin to parkin, PTEN and $P66^{Shc}$ proteins can also be S-persulfidated, which is useful for the prevention of nerve cells against oxidation related injury. It is known that H_2O_2 and/or NO-induced modifications of specific cysteine residues of PTEN and $P66^{Shc}$ abolish the enzymatic activity. However, CBS/H_2S can shift PTENs S-nitrosylation to S-persulfidation at Cys71 and Cys124 sites, followed by the improvement of enzymatic activity and downstream signaling in SH-SY5Y cells (Fig. 3) (38, 87, 120).

Sometimes, S-persulfidation of proteins may have inhibitory effects on enzymatic activities. For instance, in ischemic murine primary cortical neurons, due to S-persulfidation at Cys163, the apoptotic effector protein caspase-3 will lose activity and apoptotic death will decrease (75). In addition, under the condition of neuronal carbonyl stress induced by methylglyoxal (MGO), persulfide (Na_2S_2) as well as polysulfides (Na_2S_3 and Na_2S_4) exert significant neuroprotection by reducing the formation of MGO-modified proteins in SH-SY5Y cells (61, 62). It has also been found that induction of endogenous H_2S suppresses MGO-induced peripheral neural injury (130).

In environmental electrophile-related neurotoxicity, exposure of cerebellar granule neurons to methyl mercury (MeHg) triggers S-guanylation and activation of H-Ras. However, MeHg-mediated neurotoxicity can be dramatically eliminated by the pretreatment with persulfide (46). Overall, in neurodegeneration and environmental stimuli-related lesions, S-persulfidation of proteins and LMW persulfides usually have significant protective effects.

S-Persulfidation and digestive system diseases

In I/R-induced gastric epithelial cell injury, the increased ROS levels significantly cause inflammatory response through NF-κB and mitogen-activated protein kinases signaling pathways. As aforementioned, Keap1-Nrf2 is essential to endogenous antioxidation. H_2S-induced S-persulfidation of Keap1 leads to Nrf2 disassociation and activation, thereby inducing antioxidation gene expressions. The effects of H_2S may have potential therapeutic value in acute gastric mucosal inflammatory lesions (39, 122).

In murine gastric smooth muscles, exogenous H_2S can trigger S-persulfidation in voltage-dependent potassium channel (KV) protein, instead of K_{ATP} channel protein, and enhance the tonic contraction, which can be abolished by DTT (a reducing agent) (71). Oppositely, in colonic smooth muscles, H_2S exhibits inhibitory effects on contraction, due to S-persulfidation-mediated inhibition of RhoA/Rho kinase (83). Additionally, in experimental colitis model, H_2S apparently S-persulfidates K_{ATP} channel SUR2B subunit at Cys24/1455 sites, and such an effect attenuates NO-induced tyrosine nitration in another K_{ATP} channel subunit Kir6.1 (53). In summary, S-persulfidation has effects on epithelial cells, as well as smooth muscles, which seems to be tissue specific.

S-Persulfidation and bone health

Under physiological conditions, normal amount of H_2S is a critical regulator of bone homeostasis. Bone marrow-derived mesenchymal stem cells (MSCs) are a common precursor of both adipocytes and osteoblasts. In fact, endogenously produced H_2S in MSCs affects osteogenic differentiation and MSCs self-renewal. The effects of H_2S are associated with the activation of TRPV3/V6/M4 channels-induced Ca^{2+} influx. H_2S-induced channel protein activation is linked to S-persulfidation of cysteine residues. The increased Ca^{2+} content in cells consequently activates Wnt/beta-catenin signaling and in turn enhances osteogenic differentiation (72).

In addition, during bone fracture healing, CSE/H_2S is capable of activating runt-related transcription factor 2 (RUNX2) and improves the fracture healing. Further study showed that H_2S-mediated activation of RUNX2 relied on S-persulfidation at Cys123 and Cys132 (136). However, if the critical cysteine residues are S-nitrosylated, for instance, in peroxisome proliferator-activated receptor (PPAR)γ, the activation of target proteins will be inhibited, which is harmful for osteoblast differentiation (19). Under such conditions, S-persulfidation of specific proteins may have potential therapeutic effects.

S-Persulfidation in metabolic disorder and diabetes

Endogenous H_2S possesses diverse roles in metabolisms of nutrients, such as glucose, lipid, and protein, in an insulin-dependent or -independent manner. H_2S can regulate mitochondrial biogenesis *via* ATP synthase (121). Such effects rely on S-persulfidation on specific proteins. For example, in the liver, overexpressed CSE and exogenously applied H_2S enhance the activity of pyruvate carboxylase (PC) and gluconeogenesis, through S-persulfidation of Cys265 in PC proteins (49). Beyond gluconeogenesis, H_2S is able to facilitate hepatic glucose production from glucogen breakdown and thus increase blood glucose levels. The effects of H_2S on glucogen breakdown are associated with S-persulfidation-mediated activation of glucose-6-phosphatase and fructose-1,6-bisphosphatase (112).

In addition to the regulation of nutrient metabolisms, the liver plays a role in alcohol metabolism. It has been known that drinking too much alcohol increases the risk of cancer. Disulfiram can be used to treat chronic alcoholism by inducing unpleasant effects when even small amounts of alcohol are consumed. The mechanism is due to the inhibition of aldehyde dehydrogenase (ALDH). A recent study by Iciek *et al.* showed that some reactive sulfur species (including GSSH, K_2S_X, and garlic-derived allyl trisulfide) also effectively inhibited the activity of ALDH. This inhibition could be easily reversed by reducing agents such as DTT and GSH, indicating that S-persulfidation mediates the regulation of ALDH activity (44).

In hepatocellular cells, H_2S can improve the activity of ATP synthase through S-persulfidation on Cys244 and Cys294, thereby boosting mitochondrial ATP generation. It was pointed out that S-persulfidation-mediated activation was significant to maintain physiological ATP synthase activity and mitochondrial bioenergetics (79). However, during mitochondrial biogenesis and bioenergetics, CARSs can catalyze a reaction from cysteine to CysSSH. Importantly, this S-persulfidation occurs in cysteine residues of dynamin-related protein 1 (Drp1) and also in free form cysteines used for Drp1 protein translation (1). As a mediator of mitochondrial fission, the inactivation of Drp1 induced by S-persulfidation may recover mitochondrial function and ATP synthesis.

Besides livers, adipose tissues are another important organ related to nutrient metabolisms. It was reported that CSE/H_2S promotes glucose uptake and lipid storage in adipocytes of fatty tissues, and the action relies on S-persulfidation of Cys139 in PPARγ (17). Actually, many functions in both the livers and fatty tissues, including metabolisms of glucose and lipid, are controlled by insulin from pancreatic islets. It has been shown that persulfides, such as CysSSH, cause tRNAs methylthiolation and stimulate insulin secretion *in vitro* and *in vivo* to regulate glucose and lipid homeostasis (107).

S-Persulfidation in immune system and inflammation

Besides barrier function, ECs in vascular lumen regulate the process of inflammation (125). It has been shown that

CSE/H_2S-induced S-persulfidation of RNA binding protein HuR at Cys13 prevents HuRs homodimerization and activity, thereby downregulating CD62E and cathepsin S. Through these effects, H_2S can inhibit monocyte adherence and inflammatory response, thus delaying the atherogenesis process in $ApoE^{-/-}$ mice (9). Sirtuin-1, a histone deacetylase, exerts antiatherogenic effects by regulating the acetylation of proteins, such as P65, P53, and sterol response element binding protein, thereby reducing endothelial and macrophage inflammation. Notably, endogenous CSE/H_2S can directly S-persulfidate Sirtuin-1 and enhance its activity (30), thus eliminating inflammation. In macrophages, H_2S attenuates oxidative stress-induced mitochondrial ROS production and NLRP3 inflammasome activation by S-persulfidating c-Jun's Cys269 (70). On the condition of bacterial infection, the infected macrophages can remove the bacteria through generation of 8-nitro-cGMP in an autophagy-dependent manner. However, the smart bacteria, such as *Salmonella*, have a powerful ability to release reactive persulfides to block the antibacterial autophagy of macrophages, ensuring the bacteria's survival (55). Additionally, it was reported that endogenous CSE expression and H_2S production were decreased in the allergic asthma of young patients. The decrease may induce type-2 immunoreaction. Notably, H_2S-induced S-persulfidation of GATA3 protein attenuates type-2 immunoreaction, therefore eliminating allergic asthma symptom (115).

S-Persulfidation and cancer

Numerous studies have shown that H_2S plays roles in cancer development and progression, through stimulation of angiogenesis, regulation of intracellular signaling, and cellular bioenergetics, while some reports are controversial (32). However, the available studies on the roles of S-persulfidation in cancer are very limited. In colonic carcinoma, endogenous CBS expression and H_2S production are distinctively upregulated. The upregulation can enhance cellular bioenergetics and tumor growth. Lactate dehydrogenase A (LDHA) is a direct regulator of glycolysis and bioenergetics. Like the expression profile of CBS/H_2S in colonic carcinoma, endogenous LDHA is overexpressed and can also enhance tumor growth. Treatment of human colon cancer (HCT116) cells with H_2S boosts mitochondrial function and glycolysis, characterized by increased oxygen consumption and ATP production. Further study revealed that the effects of H_2S were associated with LDHAs S-persulfidation of Cys163 and consequent activation (111).

In lung carcinoma, endogenous H_2S is essential to tumor cell growth through repairing mitochondrial DNA damage. In fact, the induction of DNA damage is common action of chemotherapeutic drugs. Tumor cells repair the damaged DNA usually relying on exo/endonuclease gene (EXOG). It was found that H_2S can activate EXOG, thereby enhancing tumor cells resistance to chemotherapeutic drugs. Further study uncovered that H_2S-induced activation of EXOG is related to S-persulfidation at Cys76 residue (106).

Interestingly, a recent study showed that H_2S could inhibit the efflux of doxorubicin (Dox) from liver cancer cells and enhance Doxs chemotherapy activity. The authors found that the treatment of H_2S induced the S-persulfidation of retinoid X receptor (RXR) β and then inhibited the heterodimer formation between RXR β and liver X receptor α. Such effects eventually weakened ABCA1- and ABCG8-mediated efflux transportation of chemotherapeutic agents, thereby reversing drug resistance of live cancer cells (103).

Conclusions

The discovery of S-persulfidation in living organisms and its regulatory roles in redox biology are very attractive. S-Persulfidation leads to the formation of both small-molecule-based persulfides (RSSH) and protein persulfides. These species are highly reactive and thus quite unstable. Their presence or fates in biological environments are sometimes difficult to track. This review presents current understandings of persulfides (and S-persulfidation) chemistry, biochemistry, and their links with health and disease. In current studies, researchers commonly use thiol-blocking reagents, such as IAM, NEM, and monobromobimane, to convert unstable persulfides to stable derivatives for further analysis. However, these methods are not always reliable. As recently discovered by Nagy and colleagues, sulfur alkylation can alter sulfur speciation and sometimes can even change the identity of alkylated products (especially for polysulfide derivatives) (11). As such, the actual readouts do not necessarily represent true original (such as intracellular) speciation of sulfur species.

As discussed in this review, the specific target proteins of S-persulfidation are essential in organismal physiological health and human disease states (Table 1). Current studies are mainly focused on the cardiovascular system and neuronal system. Studies in other systems or organs are still limited. In addition, some reports are still contradictory, for instance, the roles of S-persulfidation in cancer growth. Apparently, further clarifications on these issues are needed, perhaps with the help of better understanding persulfide chemistry/biochemistry and more reliable detection methods. There is little doubt that the general area of investigation of S-persulfidation will be a topic of significant research interest in the coming years.

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Abbreviations Used

3MP = 3-mercaptopyruvate
3MST = 3-mercaptopyruvate sulfurtransferase
8-nitro-cGMP = 8-nitroguanosine 3′,5′-cyclic monophosphate
Aβ = Abeta 1–42 peptide
AD = Alzheimer's disease
ALDH = aldehyde dehydrogenase
BDP-NAC = bpin-disulfide prodrug-N-acetyl cysteine
CARS = cysteinyl-tRNA synthetase
CBS = cystathionine β-synthase
COPD = chronic obstructive pulmonary disease
CSE = cystathionine γ-lyase
DADS = diallyl disulfide
DATS = dially trisulfide
Dox = doxorubicin
Drp1 = dynamin-related protein 1
DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid)
DTT = dithiothreitol
ECs = endothelial cells
ER = endoplasmic reticulum
EXOG = exo/endonuclease gene
FRET = fluorescence resonance energy transfer
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GPx = glutathione peroxidase
GSH = glutathione
GSSH = glutathione persulfide
HO = heme oxygenase
IAA = iodoacetic acid
IAM = iodoacetamide
IAP = iodoacetamide-linked biotin
IL = interleukin
I/R = ischemia/reperfusion
K_{ATP} = ATP-sensitive potassium channel
K_{Ca} = calcium-activated potassium channel
Keap1 = Kelch-like ECH-associated protein 1
LDHA = lactate dehydrogenase A
LMW = low molecular weight
LTP = long-term potentiation
MeHg = methyl mercury
MGO = methylglyoxal
MMTS = methyl methanethiosulfonate
MSBT = methylsulfonyl benzothiazole
MSCs = mesenchymal stem cells
NEM = *N*-ethyl maleimide
NF = nuclear factor
NMDA = *N*-methyl-D-aspartate
Nrf2 = nuclear factor-like 2

Abbreviations Used (Cont.)

PC = pyruvate carboxylase
PD = Parkinson's disease
PDE5 = phosphodiesterase type 5
PKA = protein kinase A
PKG = cGMP-dependent protein kinase
PPAR = peroxisome proliferator-activated receptor
PTEN = phosphatase and tensin homolog
RAGE = receptor for advanced glycation end product
RBC = red blood cell
redox = reduction/oxidation
ROS = reactive oxygen species
RUNX2 = runt-related transcription factor 2
RXR = retinoid X receptor
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SILAC = stable isotope labeling with amino acid in culture
SOD = superoxide dismutase
SP1 = specificity protein 1
SQR = sulfide quinone oxidoreductase
SR = sarcoplasmic reticulum
TRP = transient receptor potential
VEGF = vascular endothelial growth factor