

Inorganic Hydrogen Polysulfides: Chemistry, Chemical Biology, and Detection

H₂S_n formation, function, detection, and delivery

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Abstract

Recent studies suggest inorganic hydrogen polysulfides (H₂S_n, n≥2) play important regulatory roles in redox biology. Modulation of their cellular levels could have potential therapeutic value. This review article focuses on our current understanding of the biosynthesis, biofunctions, fundamental physical/chemical properties, detection methods, and delivery techniques of H₂S_n.

Abbreviations

mBB	mono-Bromobimane
BODIPY	boron difluoride dipyrromethene
CARS	cysteinyI-tRNA synthetase
CSE	cystathionine γ -lyase
CBS	cystathionine β -synthase
GSH	Glutathione
GSSH	Glutathione persulfide
HNO	Nitroxyl

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H ₂ S	Hydrogen sulfide
H ₂ S _n	Hydrogen polysulfide
HSNO	Nitrososulfide
ICT	Intramolecular charge transfer
Keap1	Kelch ECH associating protein 1
MPO	Myeloperoxidase
3MP	3-Mercaptopyruvate
3MST	3-Mercaptopyruvate sulfurtransferase
NIR	Near-infrared
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor 2
d-PET	donor-excited Photoinduced electron transfer
ROS	Reactive oxygen species
RSS	Reactive sulfur species
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SOD	Superoxide dismutase
TP	Two-photon

Introduction

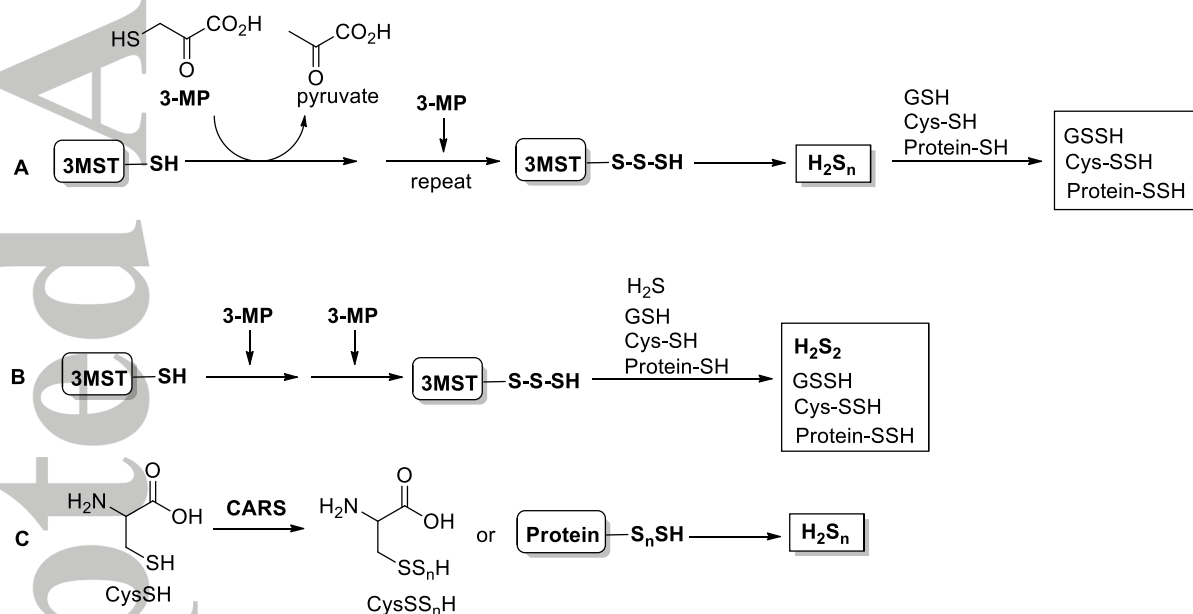
Reactive sulfur species (RSS) are a family of sulfur-containing molecules found in biological systems and are involved in a variety of biological processes (Giles, Tasker & Jacob, 2001; Giles & Jacob, 2002; Gruhlke & Slusarenko, 2012; Paulsen & Carroll, 2013; Yang *et al.* 2017). Representative RSS include thiols, hydrogen sulfide, persulfides, polysulfides, and *S*-modified cysteine derivatives such as *S*-nitrosothiols (SNO) and sulfenic acids (SOH). Among these molecules, hydrogen sulfide (H₂S) has been most well-studied as this gas molecule is recently classified as a critical cell signaling molecule, much like nitric oxide (NO) (Li, Rose *et al.*, 2011; Wang, 2012; Módis *et al.*, 2014; Polhemus & Lefer, 2014; Szabo *et al.*, 2014). For example, H₂S functions as an endothelial cell-derived relaxing factor

via direct activation of ATP-sensitive potassium (K_{ATP}) channels. 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, a hot new topic in this field has emerged which focuses on a series of H_2S -related reactive sulfane sulfur species (Ono *et al.*, 2014; Toohey *et al.*, 2014; Kimura, 2015; Park, *et al.*, 2015; Akaike *et al.*, 2017). Sulfane sulfur refers to sulfur atoms with six valence electrons but no charge (represented as S^0). H_2S -related sulfane sulfur compounds include persulfides ($R-S-SH$), polysulfides ($R-S_n-SH$ or $R-S-S_n-S-R$), inorganic hydrogen polysulfides (H_2S_n , $n \geq 2$), and protein-bound elemental sulfur (S_8). Among these, hydrogen polysulfides (H_2S_n) are especially attractive. Recent studies have suggested that H_2S_n exist endogenously and are linked to a number of physiological and pathological processes. H_2S_n can serve as the source of H_2S , as well as the sink of H_2S . Much of what is known as H_2S signaling may be actually due to H_2S_n . In this review, we summarize current knowledge about H_2S_n , focusing on their formation, functions, fundamental physical/chemical properties, detection methods, as well as releasing strategies.

Biosynthesis of H_2S_n

Currently, how H_2S_n are produced endogenously is still a topic under active investigation. The studies by Kimura *et al.* suggest that H_2S_n are mainly produced from 3-mercaptopyruvate (3MP) by 3-mercaptopyruvate sulfurtransferase (3MST) (Kimura *et al.*, 2015). In their studies, the produced H_2S_n were trapped by monobromobimane (mBB) and further identified by LC-FL and LC-MS/MS analysis. Based on the identity and ratio of monobromobimane-trapped products, it was found that the major H_2S_n species was H_2S_3 , while H_2S_2 and H_2S_5 were the minor products. Kimura *et al.* also found that H_2S_n were localized in the cytosol of cells and H_2S_n could be produced from H_2S by 3MST and rhodanese. The basal endogenous H_2S_3 concentration was 3.4 nmol/g protein in the brain, which is comparable to the concentration of H_2S (4.8 nmol/g protein). Kimura *et al.* later found that 3MST also produces cysteine- and glutathione-persulfides (Cys-SSH and GSSH) (Kimura *et al.*, 2017). Based on these results two possible mechanisms describing 3MST-mediated H_2S_n and persulfide biosynthesis are shown in **Scheme 1**: A) 3MST

abstracts the sulfur from its substrate 3MP to form a persulfidated enzyme intermediate (3MST-S_nSH), which then degrades to form H₂S_n. Next, H₂S_n transfers the sulfane sulfur atom to other thiols (Cys, GSH, or protein-SH) to form persulfide species. B) After the persulfidated enzyme intermediate (3MST-S_nSH) is formed, it directly transfers the sulfane sulfur atom to other thiols or H₂S to form the corresponding persulfide species, including H₂S_n. It should be noted that in these studies H₂S_n species were identified by mBB capturing experiments. An assumption in these conclusions is that all H₂S_n species as well as other thiol species like CysSSH are captured by mBB with high effectiveness and the capturing rates are faster than the dynamic interchanges within these sulfur species. This assumption should be further evaluated before the conclusions can be well-accepted.

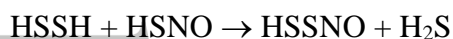
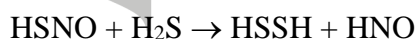
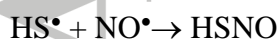


Scheme 1. 3MST-mediated H₂S_n/persulfide biosynthetic pathways

Very recently Akaike *et al.* reported another interesting enzymatic pathway which can contribute to the biosynthesis of H₂S_n (Akaike *et al.*, 2017). They found that prokaryotic and mammalian cysteinyl-tRNA synthetases (CARS) can very effectively catalyze the production of cysteine persulfide (CysSSH) and polysulfides (CysS_nSH) using cysteine as the substrate (Scheme 1-C). CARS was also found to integrate cysteine polysulfides into proteins during translational process. As one can imagine, CysSSH and CysS_nSH are valuable precursors of H₂S_n. Therefore, H₂S_n biosynthesis is likely controlled by this CARS-mediated pathway.

Overall the mechanisms summarized in Scheme 1 indicate that Cys-SSH/GSSH and H₂S_n are normally formed together.

The cross talk between H₂S and NO is interesting, and a synergistic effect of these two signaling molecules can be expected. Several groups have studied the direct reactions between H₂S and NO and found the chemistry is rather complicated (Eberhardt *et al.*, 2014; Cortese-Krott *et al.*, 2015; Miyamoto *et al.*, 2017). In these studies a mixture of H₂S (using Na₂S as the equivalent) and NO (using a NO donor such as diethylamine NONOate or SNAP) was generated and the products were trapped and analyzed by spectroscopy methods. Indeed H₂S_n are found to be the products, as well as HNO, HSNO, and even SSNO⁻. The formation of these products can be possibly attributed to the following equations:



The fast reaction between H₂S and NO to form H₂S_n may have important biological implications. For example, H₂S producing enzymes (3MST and cystathionine β-synthase) and NO synthase (NOS) are localized to neurons and astrocytes in the central nervous system. Therefore, endogenously generated H₂S and NO can react with each other to produce H₂S_n, which then activate TRPA1 channels to modify synaptic activity. In the cardiovascular system, eNOS and cystathionine γ-lyase are localized to vascular endothelium and smooth muscle, respectively. NO and H₂S generated from these enzymes may interact and produce H₂S_n to activate PKG1α to induce vascular relaxation.

H₂S_n can also be generated from H₂S catalyzed by heme proteins. For example, Olson *et al.* showed that cytosolic copper/zinc superoxide dismutase (Cu/Zn SOD) catalyzed the oxidation of H₂S to produce H₂S₂, as well as H₂S₃ and H₂S₅ (Olson *et al.*, 2018). O₂ or H₂O₂ were used as the electron acceptor in the reaction. Interestingly, SOD-catalyzed H₂S oxidation is found to be inhibited by high H₂S concentration (>1 mM) and the reaction appears to be specific for dissolved H₂S (not the hydrosulfide anion HS⁻). Nagy *et al.* recently studied the detailed mechanisms of myeloperoxidase (MPO)-catalyzed H₂S oxidation (Garai

et al., 2017) and found the Compound III state is formed in the reactions of H₂S with MPO in the presence of oxygen. This enzymatic reaction provides a slow flux of sulfane sulfur species generation (which likely involves H₂S_n), that may be important in endogenous signaling.

In addition to their biosynthesis, the degradation pathways of hydrogen polysulfides are also important but these are much less studied. Recently, Nagy and co-workers investigated polysulfides' fates under reducing environments (Dóka *et al.*, 2016). They studied how hydrogen sulfide exposure affects the activity of thioredoxin reductase-1 (TrxR1). Instead of enzyme inhibition, TrxR1 was found to reduce hydrogen polysulfides in a polysulfide concentration-dependent manner in the presence of NADPH. Polysulfides appear to be good substrates and not inhibitors of TrxR1. Moreover, the GSH system (with NADPH, GSH, and glutathione reductase (GR)) was also found to catalyze the reduction of polysulfides in a concentration-dependent manner. As can be expected, the Trx and GSH systems are critical in maintaining sulfane sulfur homeostasis and sulfide signaling.

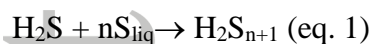
Biological functions of H₂S_n

The oxidation state of the sulfane sulfur in H₂S_n is zero. H₂S_n can readily react with protein cysteine residues to form protein persulfides (P-S-SH). This reaction, named as S-persulfidation (also known as S-perthiolation or S-sulfhydration), is believed to be the major contributor of the biological functions of H₂S_n. For example, H₂S_n showed strong protective effects against oxidative damage caused by excessive ROS. This is due to H₂S_n promoting the release of Nrf2 and induces the translocation of Nrf2 into the nucleus by persulfidating its binding partner Kelch-like ECH-associated protein 1 (Keap1), subsequently causing an increase in intracellular GSH levels and the expression of HO-1 (a Nrf2-regulatory gene) (Koike *et al.*, 2013). H₂S_n were also found to regulate the activity of the tumor suppressor the lipid phosphatase and tensin homolog (PTEN) by introducing the sulfane sulfur into the active site cysteine of PTEN (Greiner *et al.*, 2013). The study by Mutus and coworkers showed that H₂S_n could suppress the activity of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) via persulfidation on Cys 152 (Jarosz *et al.*, 2015). Additionally, H₂S_n was found to induce Ca²⁺ influx in astrocytes and stimulate mouse sensory neurons by

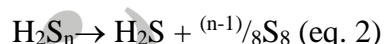
activating TRPA1 channels. The molecular mechanism is due to persulfidation of two cysteine residues at the amino terminus of the TRPA1 channels (Yukari *et al.*, 2015). Eaton *et al.* found that in the presence of oxidants, such as H_2O_2 or O_2 , H_2S can be converted into H_2S_n very quickly and afterwards activate protein kinase G (PKG) 1 α by thiol-disulfide exchange reactions. The PKG disulfide formation could then effectively relax vascular smooth muscle (Stubbert *et al.*, 2014). Finally, the recent discovery by Akaike *et al.* about CARS-mediated persulfide formation is very interesting (Akaike *et al.*, 2017). Polysulfides generated by CARS (including CysSSH and H_2S_n) contribute significantly to polysulfidation on proteins (via both post-translational and co-translational processes). This new pathway plays an important role in the mitochondrial electron transport chain (ETC), suggesting a new role of polysulfides/persulfides in maintaining mitochondrial bioenergetics. This unique sulfur respiration is expected to be linked to oxidative stress related diseases.

Fundamental physical/chemical properties of H_2S_n

Chemical methods to make mixtures of H_2S_n or relatively pure H_2S_2 , H_2S_3 , H_2S_4 exist (Steudel, 2003). For example, liquid sulfur and H_2S can react to give a mixture of long-chain H_2S_n (eq. 1). Since H_2S and elemental sulfur occur together in hot underground deposits of natural gas, the formation of H_2S_n under these high-pressure conditions is very likely.

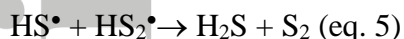


On the other hand, H_2S_n are unstable species and tend to decompose to elemental sulfur (S^0 or S_8) and H_2S (eq. 2). In the natural gas field, when the gas is produced and the pressure and temperature are lowered, the decomposition reaction takes place. The precipitated elemental sulfur solid may cause serious problems when it clogs pipelines and valves. H_2S_n decomposition can be catalyzed by substances like alkalines, nucleophiles, and metals.



Thermolysis and photolysis of H_2S_n are believed to be radical chain reactions (Muller *et al.*, 1969; Gosavi *et al.*, 1973). Take H_2S_2 for example, the first step is assumed to be the homolytic cleavage of the S-S bond to form HS^\bullet (eq. 3), which then abstract hydrogen from H_2S_2 to form H_2S and HS_2^\bullet (eq. 4). Recombination of HS^\bullet and HS_2^\bullet should form H_2S and

elemental sulfur S₂ (eq. 5). The reaction progress and intermediates can be detected by UV, IR, or luminescence spectroscopy. Thiyl radicals generated in this process may also react with other molecules (Dénès *et al.*, 2014) such as NO to form SNO adducts (Madej *et al.*, 2008). This further complicates the signaling mechanisms.



The pure forms of H₂S_n (n=2~8) are found to be yellow liquids at 20 °C. The intensity of their color increases with the sulfur content. The freezing points of H₂S₂, H₂S₃, and H₂S₄ are -90 °C, -53 °C, -85 °C, respectively; while their boiling points (at 1.013 bar) are estimated to be 70 °C, 170 °C, 240 °C, respectively.

The bond dissociation enthalpies for the S-S and S-H bonds of H₂S_n have been determined by high-level ab initio MO calculations. For H₂S₂ at 0 K, D(S-H)=313 kJ/mol and D(S-S)=271 kJ/mol were obtained by the G2(MP2) method (Antonello *et al.*, 2002). The S-S bond dissociation energies of H₂S₂, H₂S₃, and of the central bond of H₂S₄ were calculated to be 259.5, 211.8, 168.5 kJ/mol, respectively, by the CCSD(T)/6-311++G(2df,p)//MP2/6-311++G** level of theory (Steudel *et al.*, 2001).

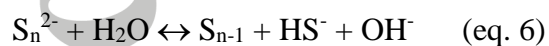
The UV-Vis spectra (200-400 nm) of H₂S_n in cyclohexane have been reported (Fehér *et al.*, 1963). These molecules show strong absorption in the region 200-230 nm and the extinction coefficients decrease with longer wavelengths. Also, longer sulfur chains resulted in a higher molar absorbance at a given wavelength and more red-shifted spectra. There is an absorption maximum or a broad plateau at 260-330 nm which becomes more and more pronounced when sulfur atoms increase.

The estimated Gibbs energies of formation of H₂S₂ Δ_fG°(H₂S₂) are -2 kJ/mol in the gas phase and +5 kJ/mol in water. From the Δ_fG° values for HSSH, HSS⁻, and SS²⁻, a pK_a of 2.6 is estimated for HSSH, and a pK_a of 13 is estimated for HSS⁻ (Koppenol *et al.*, 2017).

Redox reactions of H₂S/H₂S_n: H₂S can undergo both one- and two-electron oxidations. The HS radical (HS[•]) produced by one-electron oxidation of sulfide (HS⁻) is a strong oxidant. When it is generated, it can participate in radical chain reactions. The reduction potential of

the HS[•]/HS⁻ redox couple is +920 mv vs NHE at pH 7 (Das *et al.*, 1999). HS[•] can react with HS⁻ to form hydrodisulfide radical anion, which can further react with oxygen (O₂) to form HSS⁻. The bimolecular rate constants for H₂S (in the form of HS⁻ in physiological buffers) with biologically important two-electron oxidants are: 0.73 M⁻¹ s⁻¹ for H₂O₂; 4.8×10³ M⁻¹ s⁻¹ for peroxynitrite (ONOOH); 8×10⁷ M⁻¹s⁻¹ (Carballal *et al.*, 2011) or 2×10⁹ M⁻¹s⁻¹ (Nagy *et al.*, 2010) for hypochlorite (HOCl) (at pH 7.4 and 37 °C). The immediate products are HSOH or HSCl, which can further react with HS⁻ to form H₂S₂ (rate constant: 1×10⁵ M⁻¹s⁻¹) (Carballal *et al.*, 2011). The reactivities of H₂S toward these oxidants are comparable to the rate constants of low molecular weight thiols such as cysteine and glutathione. Given the very low endogenous H₂S concentration (as compared to thiols) in tissues, it is unlikely these oxidations are physiologically significant. Therefore, it is unlikely H₂S oxidation plays an important role for H₂S_n formation under normal physiological conditions.

If H₂S_n are generated in physiological conditions, it is expected that their aqueous solutions are formed. However, the exact species present in the solutions are still unclear. This is due to the instability and high reactivity of H₂S_n (or their anions) in water. Nevertheless, inorganic salts of H₂S_n are often used as the equivalents of H₂S_n for biological studies. These salts (such as Na₂S_n and K₂S_n) are yellow (or orange-yellow) hygroscopic crystalline substances, which show a pronounced thermochromic effect. It is worth noting that these salts have different stability. For example, Na₂S₂ and Na₂S₄ are relatively stable but Na₂S₃ is unstable in the solid state. Na₂S₃ solid readily decomposes to form a eutectic mixture of Na₂S₂ and Na₂S₄ (El Jaroudi *et al.*, 1999). When polysulfide salts are dissolved in water, a complex equilibrium mixture will be established with HS⁻ and elemental sulfur being the products (eq. 6).



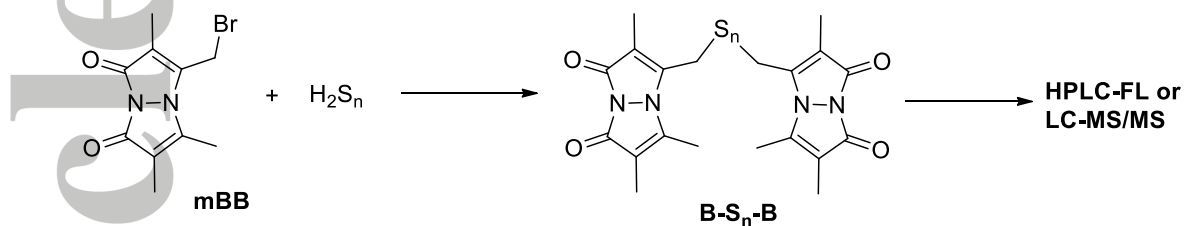
This instant reaction has several implications: 1) when polysulfides are formed in aqueous environments, HS⁻ and elemental sulfur will also be present; 2) if HS⁻ (or H₂S) coexists with elemental sulfur, polysulfides are likely to form; 3) precipitation of elemental sulfur may be the indicator of polysulfide presence; 4) polysulfides in aqueous solutions are likely to be the combination of S_n²⁻, HS⁻, S_n, HS_n⁻. The actual polysulfide anions (e.g. whether

$n = 2, 3, \text{ or } 4$ in S_n^{2-}) are difficult to determine. Since there is no direct method to determine single species either analytically or spectroscopically, the results of identification of specific polysulfides are somewhat speculative and rest on certain assumptions. For example, when monobromobimane (mBB) is used to trap polysulfides, the obtained bimane-polysulfide adducts (B- S_n -B) may not actually reflect the true identity of the polysulfide species presented, as the formation of B- S_n -B adducts may be stepwise and thiol exchange reactions could occur. Currently, it is impossible to completely exclude HS^- and elemental sulfur from the solutions of polysulfides. This should be taken into consideration when studying the chemistry and biological functions of polysulfides. HS^- and elemental sulfur are stable species and their corresponding clean solutions (without the presence of polysulfides) could be obtained. Therefore, control experiments using HS^- and elemental sulfur should always be carried out to confirm the results of polysulfides, especially for *in vitro* studies.

Detection methods for H_2S_n

UV-Vis absorption spectroscopy of aqueous polysulfide solutions have been studied (Giggenbach, 1972). Absorption bands in the range of 240-420 nm are assigned to polysulfide anions (S_n^{2-}). In current polysulfide studies, UV absorption peaks at ~300 nm and ~370 nm are often used to indicate the presence of polysulfides (Nagy *et al.*, 2010; Greiner *et al.*, 2013). However, this method is not particularly sensitive. In recent studies of H_2S_n in biological samples, especially in tissue samples, H_2S_n are normally derivatized with monobromobimane (mBB) to form the corresponding alkylated products, such as B- S_2 -B (from H_2S_2) or B- S_3 -B (from H_2S_3) (**Scheme 2**). These adducts can then be analysed and quantified by high performance liquid chromatography (HPLC) with a scanning fluorescence detector and tandem MS (MS/MS). The appropriate derivatizing conditions are to incubate H_2S_n with mBB for 30 min in 0.1 M phosphate buffer (pH 7.0) (Koike *et al.*, 2017). Using this method, endogenous H_2S_2 in mouse brain tissues was measured to be 0.026 $\mu\text{mol/g}$ protein. As discussed previously, the use of mBB assay is based on an assumption that all H_2S_n species and other reactive sulfur species such as CysSSH are alkylated by mBB with high efficiency and the alkylation rates are faster than the dynamic interchanges within these

sulfur species. This assumption should be carefully validated before it can be considered as the standard method for the identification of H_2S_n .



Scheme 2. Monobromobimane-based detection methods for H_2S_n

Fluorescent sensors for H_2S_n

In order to better understand the functions of H_2S_n , methods that allow real-time and non-invasive detection of H_2S_n in biological systems are needed. In this regard, fluorescent sensors are ideal because of their high sensitivity and spatiotemporal resolution ability, as well as their ease of use (Fernández-Suárez *et al.*, 2008; Lin *et al.*, 2015). While a large number of fluorescent sensors for other RSS, such as cysteine, homocysteine, glutathione, and H_2S , have been developed, only a small selection of H_2S_n sensors have been reported in the last several years (Gupta *et al.*, 2017; Takano *et al.*, 2017). These reported sensors are summarized in this section. Up to date, all known H_2S_n sensors are reaction-based fluorescent sensors. These sensors react with H_2S_n through certain specific chemical reactions to change their fluorescence properties, thus achieving the selective detection of H_2S_n . So far, three main strategies have been used in the design of H_2S_n sensors, which take advantage of the strong nucleophilicity and reduction ability of H_2S_n . These strategies are: (1) H_2S_n -mediated aromatic substitution-cyclization reactions; (2) H_2S_n -mediated ring-opening reaction of aziridine; (3) H_2S_n -mediated reduction of nitro groups.

Sensors based on H_2S_n -mediated aromatic substitution-cyclization (summarized in Scheme 3)

2-Fluoro-5-nitrobenzoic ester and phenyl-2-(benzoylthio)benzoate have been used as the specific recognition units for H_2S_n based on aromatic substitution-cyclization reaction. In 2014, our laboratory reported the first selective fluorescent sensors for H_2S_n , e.g. DSP1-3, which employed 2-fluoro-5-nitrobenzoic ester moiety as the H_2S_n recognition unit (Liu *et al.*, 2014). The sensing mechanism is based on two-step reactions: 1) the 2-fluoro-5-nitrobenzoic ester is attacked by H_2S_2 via aromatic nucleophilic substitution to form a -SSH-containing

intermediate; 2) the –SSH moiety undergoes an intramolecular cyclization leading to the release of the fluorophore. Among the DSP probes, DSP-3 exhibited high sensitivity and selectivity for H₂S_n. It was almost non-fluorescent in buffers; however, when treated with Na₂S₂, a 137-fold fluorescence increase was observed. The detection limit was 71 nM and DSP-3 was successfully applied to image H₂S_n in HeLa cells.

Several other research groups have adopted the fluoro-5-nitrobenzoic ester recognition unit and developed sensors with interesting fluorescence properties. For example, Chen *et al.* reported NIR fluorescent probes Mitro-ss and BD-ss (Gao *et al.*, 2015; Gao *et al.*, 2015). Both probes possessed boron difluoride dipyrromethene (BODIPY) as the fluorophore and 2-fluoro-5-nitrobenzoic ester as a response site. Mitro-ss bears a triphenylphosphonium moiety (TPP) as the mitochondria targeting group. The probes showed almost no fluorescence due to the d-PET process from the excited BODIPY to the 2-fluoro-5-nitrobenzoic ester. The addition of Na₂S₂ triggered a significant fluorescence enhancement (λ_{em} 730 nm), suggesting the d-PET process was suppressed by deprotection. With the aid of Mitro-ss, the two possible generation mechanisms of H₂S_n were further evidenced: a) H₂S_n could be generated from the reaction between H₂S and ROS; b) H₂S_n could be produced from cystine by cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS). Another NIR probe Cy-S_n using semiheptamethine as the fluorophore was reported by Peng *et al.* (Ma *et al.*, 2017). With this probe, the presence of H₂S_n resulted in a remarkable fluorescence enhancement while other RSS or ROS did not induce obvious fluorescence enhancement. The probe was successfully used to image endogenous H₂S_n in RAW264.7 macrophage cells and in mice. Han *et al.* also reported a NIR probe KB1, in which dicyanomethylene-benzopyran (DCMB) dye was employed as the fluorophore (Li *et al.*, 2018). KB1 showed more than 30-fold fluorescence increase (λ_{em} 682 nm) with H₂S_n and the detection limit of 8.2 nM. The probe was used for imaging H₂S_n in MCF-7 cells.

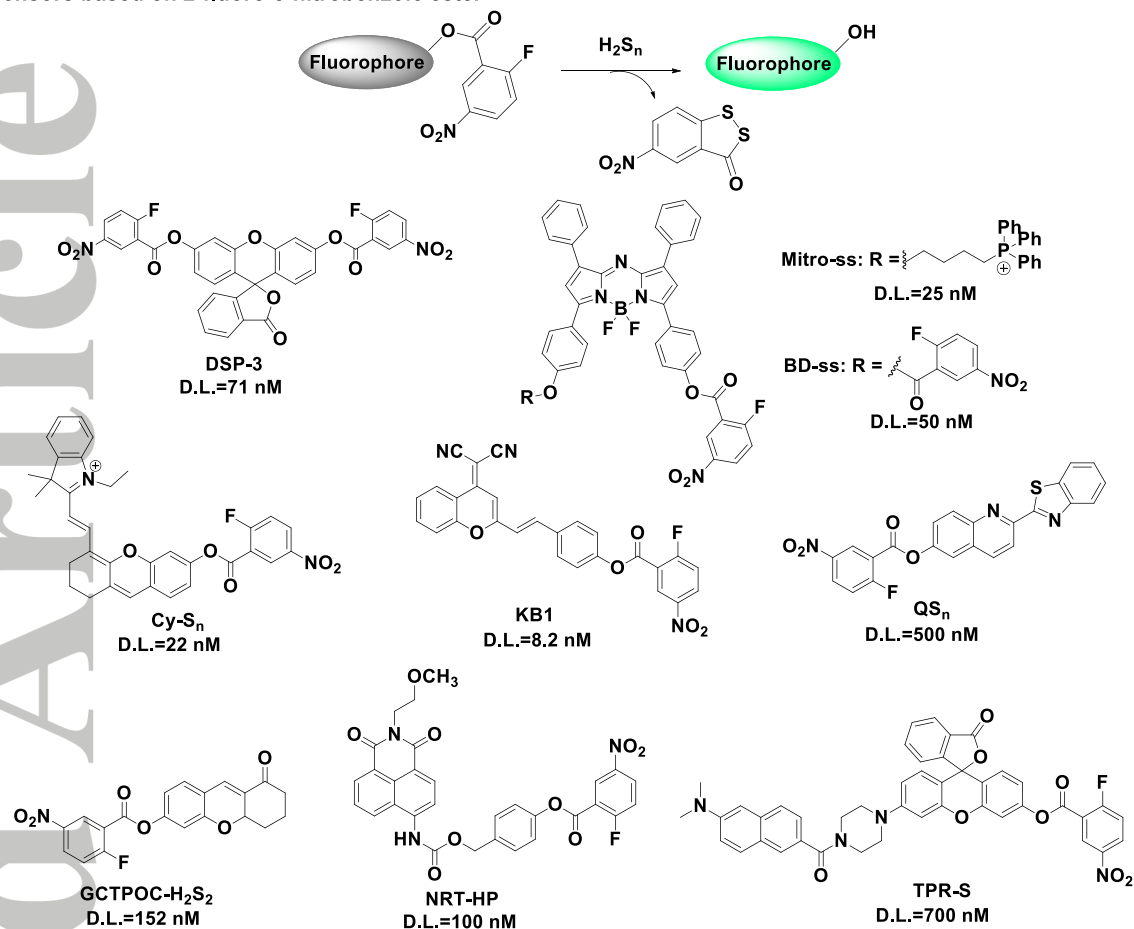
Two-photon (TP) fluorescent probes, with low-energy near-infrared wavelength excitation, are very useful for bio-imaging. These probes have advantages such as deep penetration in tissues, excellent 3D imaging capability, and minimum photo-damage to biological specimens. To date, there are several two-photon fluorescent probes developed for

sensing H_2S_n by utilizing 2-fluoro-5-nitrobenzoic ester moiety as the recognition site. In 2015, Liu *et al.* reported a TP probe QS_n using 2-benzothiazol-2-yl-quinoline-6-ol as the fluorophore (Zeng *et al.*, 2015). The addition of H_2S_n to the QS_n solution resulted in a 24-fold enhancement in fluorescence intensity at 534 nm under both one-photon (λ_{ex} 368 nm) and two-photon (λ_{ex} 730 nm). QS_n was not only used to visualize exogenous and endogenous H_2S_n in HeLa cells, but also applied to investigate the distribution of H_2S_n in living zebrafish embryos. Another TP probe GCTPOC- H_2S_2 was reported by Lin *et al.* (Shang *et al.*, 2016). The reaction between GCTPOC- H_2S_2 and H_2S_n produced a product which exhibited larger two-photon action cross-section up to 500 GM (λ_{ex} 780 nm). With this probe, imaging of H_2S_n in MCF-7 Cells and *in situ* detecting H_2S_n levels in live mice were achieved. Liu and coworkers developed a naphthalimide-based TP ratiometric probe NRT-HP based on the ICT mechanism (Han *et al.*, 2016). Upon addition of H_2S_n to the NRT-HP solution, the fluorescence emission at 460 nm decreased, and a new red-shifted emission at 542 nm increased simultaneously. Probe NRT-HP was successfully applied to image H_2S_n in different cell lines (MCF-7, A549) by two-photon microscopy. Furthermore, upon excitation at 800 nm, probe NRT-HP could detect H_2S_n in tissue up to 300 μ M of penetration depth. In order to get larger emission shift to improve the measuring accuracy, Yin *et al.* reported a fluorescence resonance energy transfer (FRET)-based TP probe TPR-S based on a naphthalene-rhodol dye (Zhang *et al.*, 2016). Before adding H_2S_n , the rhodol moiety of TPR-S was in spiro ring-closing form and the FRET process was off, so the probe emitted only naphthalene emission at 448 nm. With the addition of H_2S_n , the fluorescence emission at 448 nm decreased and a new emission at 541 nm increased. The reason for this phenomenon was that rhodol moiety was deprotected to form spiro ring-opening and the FRET was on. The efficiency of FRET from naphthalene to rhodol was estimated to be 91.3%. The probe TPR-S was able to image H_2S_n in live HeLa cells, rat liver slice, and LPS-induced acute organ injury upon excitation at 740 nm with femtosecond pulses.

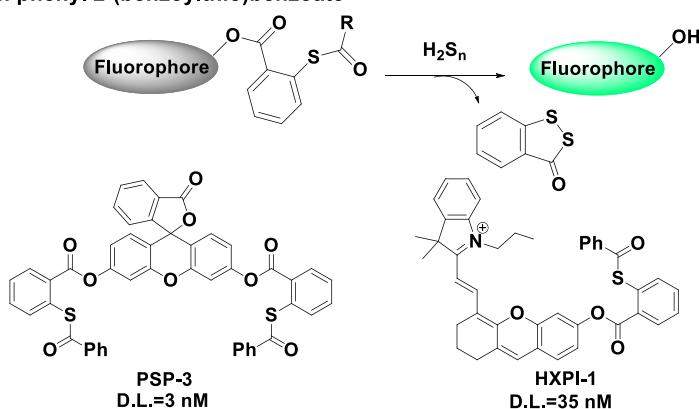
Although 2-fluoro-5-nitrobenzoic ester has been widely used in the design of H_2S_n probes, this recognition site could also react with biothiols to form thioether adducts. This problem does not affect the selectivity of the probes but could lead to the consumption of the

probes in biological systems. High probe loading is usually required in actual applications. To solve this problem, our laboratory designed another strategy for H₂S_n sensing, which utilized phenyl 2-(benzoylthio)benzoate moiety as the recognition unit. This design takes the advantage of the dual reactivity of H₂S_n. It is known that H₂S_n are stronger nucleophiles than biothiols and H₂S under physiological pH. Moreover, H₂S_n belong to the sulfane sulfur family. A characteristic reaction of sulfane sulfurs is that they can act as electrophiles and react with certain nucleophiles (Chen *et al.*, 2013). Overall H₂S_n have a unique dual-reactivity and can be both a nucleophile and an electrophile. Therefore, we envisioned phenyl 2-(benzoylthio)benzoate-based probes PSP should be specific for H₂S_n (**Scheme 3b**) (Chen *et al.*, 2015). We found the substitution group (R) of the thioester moiety was critical for the probes' selectivity and PSP3 was the most promising probe. PSP3 showed fast response to H₂S_n, excellent selectivity, and high sensitivity with a detection limit of 3 nM. It was used in the detection of exogenous and endogenous H₂S_n in different cells. Based on this design, Ma *et al.* further developed a NIR probe HXPI-1, which employed a hemicyanine derivative as the fluorophore (λ_{em} 708 nm) and was used to visualize H₂S_n in mice (Fang *et al.*, 2017).

(a) Sensors based on 2-fluoro-5-nitrobenzoic ester



(b) Sensors based on phenyl 2-(benzoylthio)benzoate



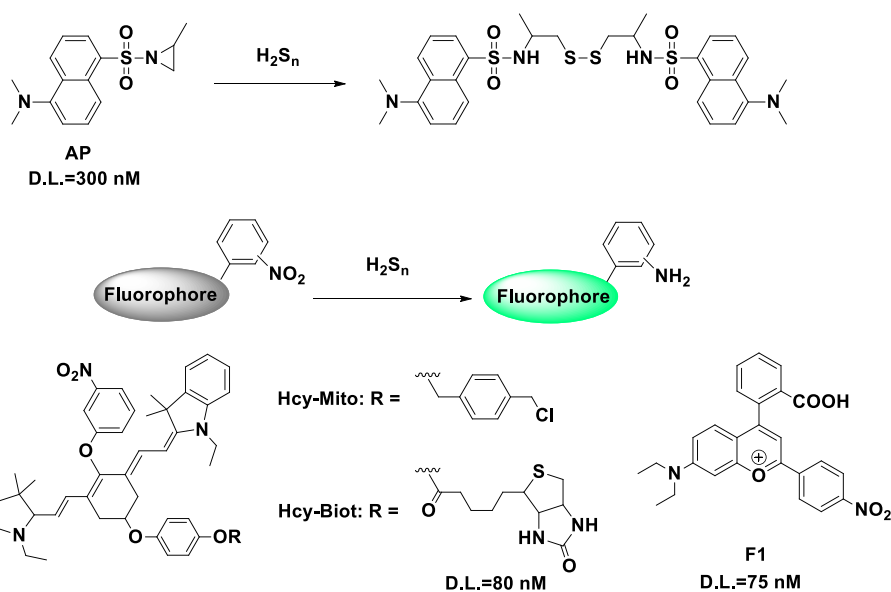
Scheme 3. Fluorescent sensors based on H_2S_n -mediated aromatic substitution-cyclization.

The detection limits (D.L.) of each sensor are provided.

Sensors based on H_2S_n -mediated ring-opening reaction of aziridine (Scheme 4)

H_2S_n are known to be strong nucleophiles and expected to be more reactive in certain nucleophilic reactions than H_2S or thiols. Our group has explored some reactions of H_2S_n and found that H_2S_n could effectively react with aziridines. Based on this reaction, the first off-on

twisted intramolecular charge transfer (TICT)-based fluorescent probe AP was developed (Chen *et al.*, 2015). The probe was constructed by incorporating an aziridine group on dansyl dye to quench the fluorescence. When reacted with H_2S_n , the fluorescence intensity at 530 nm increased due to the suppress of the TICT effect. The reaction product of AP with H_2S_n showed not only good two-photon photophysical properties, but also high luminescence efficiency in solid state. However, attempts to use AP for imaging H_2S_n in live cells were not successful.



Scheme 4. Aziridine-based sensor AP and Nitro-reduction based sensors

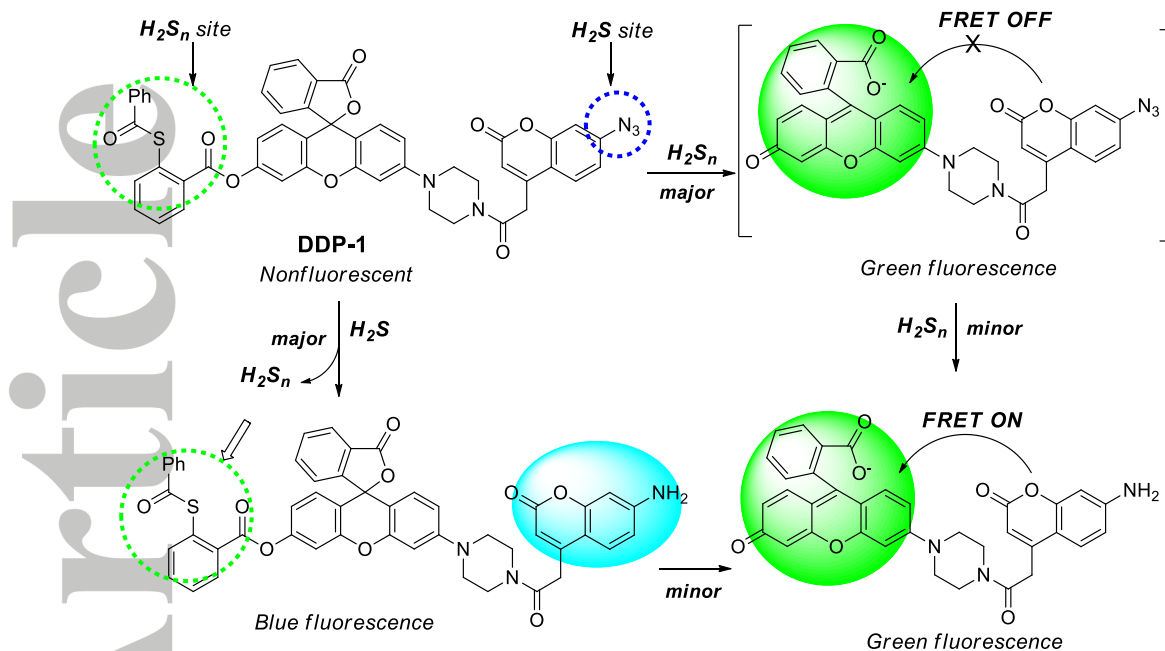
Sensors based on H_2S_n -mediated reduction of nitro groups (Scheme 4)

Based on the strong reducibility of H_2S_n , Chen *et al.* designed two NIR fluorescent probes Hcy-Mito and Hcy-Biot that could be used for monitoring $O_2^{\bullet-}$ and H_2S_n in cells and *in vivo* (Huang *et al.*, 2016). Two different targeting groups were used on these probes. Hcy-Mito bearing a benzyl chloride moiety could target the mitochondria and Hcy-Biot bearing a biotin moiety could target carcinoma tissues. These probes displayed almost no fluorescence due to the destroyed polymethine conjugated system and the d-PET process from heptamethine cyanine dye to *m*-nitrophenol group. Addition of $O_2^{\bullet-}$ would restore the polymethine conjugated systems of Hcy-Mito and Hcy-Biot, resulting in a weak fluorescence at 780 nm, and the detection limit was measured to be 0.1 μ M. Subsequently, the addition of H_2S_n would reduce the nitro group to amine and trigger strong fluorescence enhancement due

to the block of the d-PET process. The detection limit of H_2S_n was estimated to be 80 nM. Hcy-Mito was used for in situ detection of $O_2^{\bullet-}$ and H_2S_n levels in mitochondria and endogenous $O_2^{\bullet-}$ and H_2S_n cross-talk in living cells. Hcy-Biot showed high tumor-targeting ability and excellent tissue penetration in live animal models. Yang *et al.* reported another nitro-based probe F1 based on the flavylum fluorophore (Gong *et al.*, 2016). The fluorescence of F1 was quenched because attaching a nitro group to flavylum blocked the ICT process. Upon treatment with H_2S_n , the nitro group would be reduced to amine that unblocked the ICT process, leading to restore the strong fluorescence of flavylum. It should be noted that H_2S could also induce some fluorescence enhancement for these nitro-based probes. Therefore, the selectivity of these probes may not be ideal.

Sensors for dual detection of both H_2S and H_2S_n

H_2S_n and H_2S are redox partners. They should work collectively to maintain a sulfur-related redox balance. In order to better understand their functions and cross-talks, it is highly desirable to develop sensors that can simultaneously detect H_2S_n and H_2S . We recently developed such a probe DDP-1 (**Scheme 5**). It employs two recognition sites in one molecule: phenyl 2-(benzoylthio)benzoate for H_2S_n and azide for H_2S . Rhodol and coumarin were used as the fluorophores and tethered via a rigid piperazine linker (Chen *et al.*, 2016). H_2S_n selectively reacted with the phenyl 2-(benzoylthio)benzoate moiety and induced a remarkable green fluorescence increase of rhodol. However, H_2S promoted the reduction reaction of azide- N_3 to $-NH_2$, which was accompanied by the oxidation of H_2S to H_2S_n . This would lead to blue fluorescence of coumarin. Meanwhile the resultant H_2S_n would also react with 2-(benzoylthio)benzoate to release rhodol and trigger the FRET from coumarin to rhodol. As the result, H_2S could be identified by both fluorescence signals in blue and green channels. The detection limit of H_2S_n and H_2S were calculated to be 100 nM and 24 nM respectively. DDP-1 is the first fluorescent probe capable of selective discrimination of H_2S_n and H_2S .



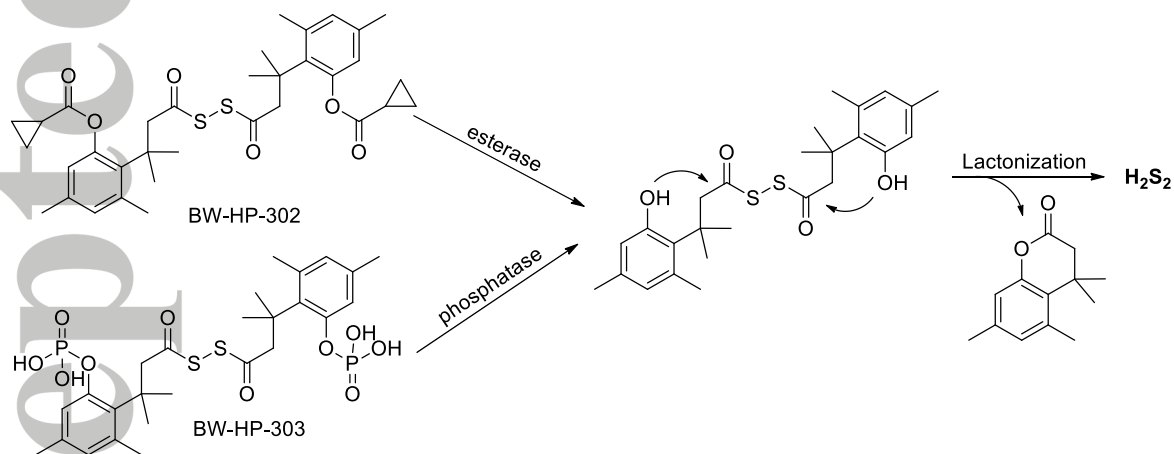
Scheme 5. Dual detection probe DDP-1

So far a handful of H_2S_n fluorescent probes have been reported and we expect to see more being developed in the coming years. In the study of H_2S_n probes, researchers usually would validate their specificity for H_2S_n (vs other thiols and H_2S). However, their selectivity for some other important sulfur species, especially recently recognized cysteine hydropolysulfide $Cys-S_nSH$ (Ida *et al.*, 2014; Akaike *et al.*, 2017) can hardly be defined. As one can imagine, $Cys-S_nSH$ and H_2S_n are likely to co-exist so one should not expect the probes can differentiate these species. Nevertheless, this should not cause a problem as $Cys-S_nSH$ and H_2S_n should have similar biological functions.

Releasing agents of H_2S_n

In current studies of H_2S_n , researchers always use their inorganic salts (Na_2S_2 , Na_2S_3 , etc.) as the standard H_2S_n equivalents. These salts (Na_2S_2 , Na_2S_3 , Na_2S_4) are commercially available by vendors like Dojindo Molecular Technologies, Inc. Due to their instability, these salts often contain other sulfur-based impurities like sulfide (S^{2-}) and elemental sulfur (S_8). This problem makes the use of these salts problematic as the observed bioactivity can hardly be attributed to only H_2S_n . Moreover, due to the high reactivity of H_2S_n , their biological production is expected to be a slow and steady process, which cannot be mimicked using Na_2S_n (these salts produce H_2S_n immediately upon dissolving in buffers and are considered

fast and uncontrollable H_2S_n donors). Due to these concerns, slow and controllable H_2S_n donors should be useful research tools in this field. This is similar to the field of H_2S donors. However, donors of H_2S_n are very underdeveloped. Recently Wang and co-workers developed several such donors (**Scheme 6**) (Yu *et al.*, 2017). In this work, H_2S_2 is caged as two thiol acid groups linked by a disulfide bond, e.g. acyl disulfides. A masked phenol hydroxyl acts as a latent nucleophile for initiation of H_2S_2 release through lactonization. A ‘trimethyl lock’ is also employed to facilitate lactonization. Esterase-triggered donors like BW-HP-302 and phosphatase-triggered donors like BW-HP-303 are prepared. Their enzyme-catalysed H_2S_2 productions have been demonstrated. Initial studies showed that these donors could induce protein *S*-persulfidation on glyceraldehyde 3-phosphate dehydrogenase (GAPDH). It should be noted that acyl disulfides are used as the core of these donors. It is known that acyl disulfides are highly reactive species and can easily react with nucleophiles like amines (Mali & Gopi, 2014). In biological systems these donors may react with naturally existing nucleophiles (like amino acids) to cause off-target effects and undesired H_2S_2 release. This possibility needs to be figured out in future studies.



Scheme 6. Enzyme-triggered H_2S_n donors

Concluding remarks

Increasing literature reports have suggested H_2S_n are potent physiological mediators and play important roles in sulfur-related redox biology. Nevertheless, the actual chemical species involved in those biological processes are still unclear as current conclusions are mostly based on derivatization methods, which are viewed as indirect evidences. From the

chemistry point-of-view, H_2S_n are highly reactive and unstable molecules. Trapping their formation and fates can be very difficult. Moreover, H_2S_n and H_2S often co-exist, as H_2S_n are found to be the common impurities in H_2S solutions (Greiner *et al.*, 2013). Therefore, differentiation of the functions of H_2S and H_2S_n can also be challenging. Along with further understanding their biological functions, it is also important to explore their chemical properties and reactions in biological systems. To this end, the development of chemical tools or methods for the delivery and detection of H_2S_n should be valuable. It is gratifying to see the development of H_2S_n detection sensors has made some progress in recent years and the development of H_2S_n donors has started emerging. We expect these will continue to be active research topics and more interesting work will appear in the future.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

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Conflict of interest

The authors declare no conflict of interest.

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