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REVIEW ARTICLE

Inorganic hydrogen polysulfides: chemistry, chemical biology and detection

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Recent studies suggest that inorganic hydrogen polysulfides (H_2S_n, $n \geq 2$) 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 H_2S_n.

LINKED ARTICLES

This article is part of a themed section on Chemical Biology of Reactive Sulfur Species. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.4/issuetoc

Abbreviations

3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; BODIPY, boron difluoride dipyrromethene; CARS, cysteinyl-tRNA synthetase; d-PET, donor-excited photoinduced electron transfer; GSSH, glutathione persulfide; H_2S, hydrogen sulfide; H_2S_n, hydrogen polysulfides; HNO, nitroxyl; HSNO, nitrososulfide; ICT, intramolecular charge transfer; Keap1, Kelch ECH associating protein 1; mBB, mono-bromobimane; MPO, myeloperoxidase; NIR, near-IR; Nrf2, nuclear factor erythroid 2-related factor 2; RSS, reactive sulfur species; TP, two-photon
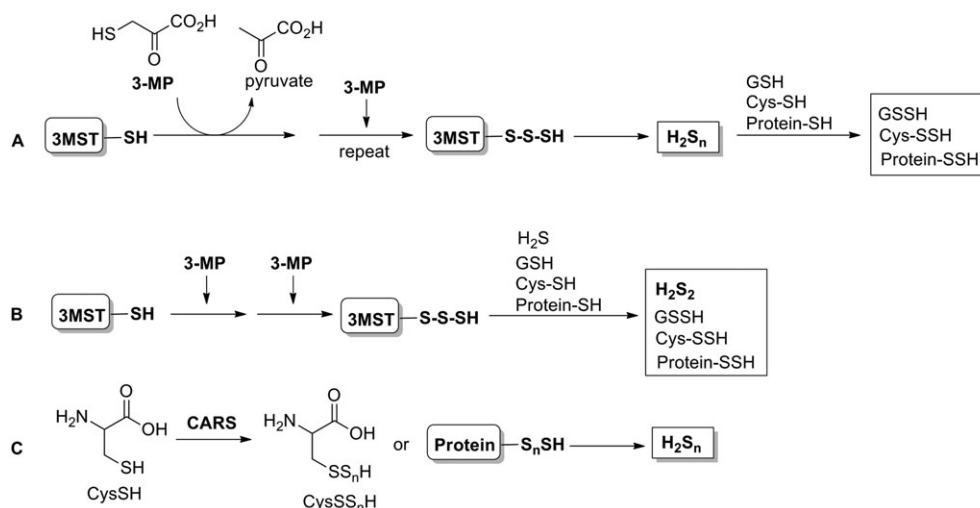
Introduction

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 *et al.*, 2001; Giles and Jacob, 2002; Gruhlke and Slusarenko, 2012; Paulsen and 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 (H_2S)** has been most well-studied as this gas molecule has recently been classified as a critical cell signalling molecule, much like **nitric oxide (NO)** (Li *et al.*, 2011; Wang, 2012; Módis *et al.*, 2014; Polhemus and Lefer, 2014; Szabo *et al.*, 2014). For example, H_2S 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 that focuses on a series of H_2S-related reactive sulfane sulfur species (Ono *et al.*, 2014; Toohey and Cooper, 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, the 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 signalling 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 mono-bromobimane (mBB) and further identified by LC-FL and LC-MS/MS analysis. Based on the identity and ratio of mBB-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 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^{-1} protein in the brain, which is comparable to the concentration of H_2S (4.8 nmol·g^{-1} 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 H_2S_n. Next, H_2S_n transfer 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 H_2S to form the corresponding persulfide species, including H_2S_n. It should be noted that in these studies, H_2S_n species were identified by mBB capturing experiments. An assumption in these conclusions is that all H_2S_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.

Very recently, Akaike *et al.* reported another interesting enzymic pathway that can contribute to the biosynthesis of
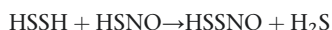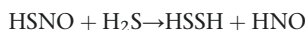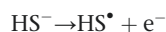

Scheme 1
3MST-mediated H_2S_n/persulfide biosynthetic pathways.

H_2S_n (Akaike *et al.*, 2017). They found that prokaryotic and mammalian cysteinyl-tRNA synthetases (CARS) can very effectively catalyse the production of cysteine persulfide (CysSSH) and polysulfides ($CysS_nSH$) using cysteine as the substrate (Scheme 1C). 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 H_2S_n. Therefore, H_2S_n biosynthesis is likely to be controlled by this CARS-mediated pathway. Overall, the mechanisms summarized in Scheme 1 indicate that CysSSH/GSSH and H_2S_n are normally formed together.

The crosstalk between H_2S and NO is interesting, and a synergistic effect of these two signalling molecules can be expected. Several groups have studied the direct reactions between H_2S 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 H_2S (using Na_2S as the equivalent) and NO (using a NO donor such as diethylamine NONOate or S-nitroso-N-acetyl-D,L-penicillamine) was generated, and the products were trapped and analysed by spectroscopy methods. Indeed, H_2S_n are found to be the products, as well as nitroxyl (HNO), nitrososulfide (HSNO) and even $SSNO^-$. The formation of these products can be possibly attributed to the following equations:

$$HS^- \rightarrow HS^\bullet + e^-$$

$$HS^\bullet + NO^\bullet \rightarrow HSNO$$

$$HSNO + H_2S \rightarrow HSSH + HNO$$

$$HSSH + HSNO \rightarrow HSSNO + H_2S$$

The fast reaction between H_2S and NO to form H_2S_n may have important biological implications. For example, H_2S producing enzymes (3MST and **cystathionine β-synthase**) and **NO synthase (NOS)** are localized to neurons and astrocytes in the CNS. Therefore, endogenously generated H_2S and NO can react with each other to produce H_2S_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 H_2S generated from these enzymes may interact and produce H_2S_n to activate **protein kinase G (PKG)1α** to induce vascular relaxation.

H_2S_n can also be generated from H_2S catalysed by haem proteins. For example, Olson *et al.* showed that cytosolic copper/zinc SOD catalysed the oxidation of H_2S to produce H_2S_2, as well as H_2S_3 and H_2S_5 (Olson *et al.*, 2018). O_2 or H_2O_2 was used as the electron acceptor in the reaction. Interestingly, SOD-catalysed H_2S oxidation is found to be inhibited by high H_2S concentration (>1 mM), and the reaction appears to be specific for dissolved H_2S (not the hydrosulfide anion HS^-). Nagy *et al.* recently studied the detailed mechanisms of **myeloperoxidase (MPO)**-catalysed H_2S oxidation (Garai *et al.*, 2017) and found the Compound III state is formed in the reactions of H_2S with MPO in the presence of oxygen. This enzymic reaction provides a slow flux of sulfane sulfur species generation (which likely involves H_2S_n) that may be important in endogenous signalling.

In addition to their biosynthesis, the degradation pathways of H_2S_n are also important, but these are much less studied. Recently, Nagy and co-workers investigated the fates of polysulfides under reducing environments (Dóka *et al.*, 2016). They studied how H_2S exposure affects the activity of thioredoxin reductase-1 (TrxR1). Instead of enzyme inhibition, TrxR1 was found to reduce H_2S_n 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**) was also found to catalyse 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 signalling.

Biological functions of H_2S_n

The oxidation state of the sulfane sulfur in H_2S_n is zero. H_2S_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 H_2S_n. For example, H_2S_n showed strong protective effects against oxidative damage caused by excessive ROS. This is due to H_2S_n promoting the release of nuclear factor erythroid 2-related factor 2 (**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). H_2S_n were also found to regulate the activity of the tumour suppressor protein, 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 co-workers showed that H_2S_n could suppress the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *via* persulfidation on Cys 152 (Jarosz *et al.*, 2015). Additionally, H_2S_n were found to induce Ca^{2+} 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 **PKG1α** 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, 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.

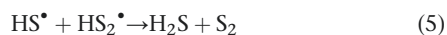## Fundamental physical/chemical properties of H_2S_n

There are several chemical methods to make mixtures of H_2S_n or relatively pure H_2S_2, H_2S_3 and H_2S_4 (Steudel, 2003). For example, liquid sulfur and H_2S can react to give a mixture of long-chain H_2S_n (Equation 1). As 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.

$$H_2S + nS_{liq} \rightarrow H_2S_{n+1} \qquad (1)$$

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 (Equation 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 catalysed by substances such as alkali, nucleophiles and metals.

$$H_2S_n \rightarrow H_2S + {}^{(n-1)}/_8 S_8 \qquad (2)$$
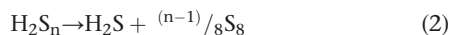
Thermolysis and photolysis of H_2S_n are believed to be radical chain reactions (Muller and Hyne, 1969; Gosavi *et al.*, 1973). Take H_2S_2, for example, the first step is assumed to be the homolytic cleavage of the S–S bond to form $HS^\bullet$ (Equation 3), which then abstracts hydrogen from H_2S_2 to form H_2S and $HS_2^\bullet$ (Equation 4). Recombination of $HS^\bullet$ and $HS_2^\bullet$ should form H_2S and elemental sulfur S_2 (Equation 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 signalling mechanisms.

$$H_2S_2 \rightarrow 2HS^\bullet \qquad (3)$$

$$HS^\bullet + H_2S_2 \rightarrow H_2S + HS_2^\bullet \qquad (4)$$

$$HS^\bullet + HS_2^\bullet \rightarrow H_2S + S_2 \qquad (5)$$

The pure forms of H_2S_n (n = 2–8) are found to be yellow liquids at 20°C. The intensity of their colour increases with the sulfur content. The freezing points of H_2S_2, H_2S_3 and H_2S_4 are −90°C, −53°C and −85°C, respectively; while their boiling points (at 1.013 bar) are estimated to be 70°C, 170°C and 240°C respectively.

The bond dissociation enthalpies for the S–S and S–H bonds of H_2S_n have been determined by high-level *ab initio* MO calculations. For H_2S_2 at 0 k, D(S–H) = 313 kJ·mol^{-1} and D(S–S) = 271 kJ·mol^{-1} were obtained by the G2(MP2) method (Antonello *et al.*, 2002). The S–S bond dissociation energies of H_2S_2, H_2S_3 and of the central bond of H_2S_4 were calculated to be 259.5, 211.8 and 168.5 kJ·mol^{-1}, 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 H_2S_n in cyclohexane have been reported (Fehér and Münzner, 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 that becomes more and more pronounced when sulfur atoms increase.

The estimated Gibbs energies of formation of H_2S_2 $\Delta_f G^o(H_2S_2)$ are −2 kJ·mol^{-1} in the gas phase and +5 kJ·mol^{-1} in water. From the $\Delta_f G^o$ values for HSSH, HSS$^-$ and SS^{2-}, a pK_a of 2.6 is estimated for HSSH, and a pK_a of 13 is estimated for HSS$^-$ (Koppenol and Bounds, 2017).

Redox reactions of H_2S/H_2S_n: H_2S can undergo both one- and two-electron oxidations. The HS radical (HS$^\bullet$) 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$^\bullet$/HS$^-$ redox couple is +920 mv versus NHE at pH 7 (Das *et al.*, 1999). HS$^\bullet$ can react with HS$^-$ to form hydrodisulfide radical anion, which can further react with oxygen (O_2) to form HSS$^-$. The bimolecular rate constants for H_2S (in the form of HS$^-$ in physiological buffers) with biologically important two-electron oxidants are 0.73 M^{-1}·s^{-1} for H_2O_2; 4.8 × 10^3 M^{-1}·s^{-1} for peroxynitrite (ONOOH); 8 × 10^7 M^{-1}·s^{-1} (Carballal *et al.*, 2011) or 2 × 10^9-M^{-1}·s^{-1} (Nagy and Winterbourn, 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 H_2S_2 (rate constant: 1 × 10^5 M^{-1}·s^{-1}) (Carballal *et al.*, 2011). The reactivities of H_2S towards these oxidants are comparable to the rate constants of low molecular weight thiols such as cysteine and GSH. Given the very low endogenous H_2S concentration (as compared to thiols) in tissues, it is unlikely these oxidations are physiologically significant. Therefore, it is unlikely H_2S oxidation plays an important role for H_2S_n formation under normal physiological conditions.

If H_2S_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 H_2S_n (or their anions) in water. Nevertheless, inorganic salts of H_2S_n are often used as the equivalents of H_2S_n for biological studies. These salts (such as Na_2S_n and K_2S_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, Na_2S_2 and Na_2S_4 are relatively stable, but Na_2S_3 is unstable in the solid state. Na_2S_3 solid readily decomposes to form a eutectic mixture of Na_2S_2 and Na_2S_4 (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 (Equation 6).

$$S_n^{2-} + H_2O \leftrightarrow S_{n-1} + HS^- + OH^- \qquad (6)$$

This instant reaction has several implications: (i) when polysulfides are formed in aqueous environments, HS$^-$ and elemental sulfur will also be present; (ii) if HS$^-$ (or H_2S)

coexists with elemental sulfur, polysulfides are likely to form; (iii) precipitation of elemental sulfur may be the indicator of polysulfide presence; (iv) polysulfides in aqueous solutions are likely to be the combination of S_n^{2-}, HS^-, S_n and HS_n^-. The actual polysulfide anions (e.g. whether $n = 2$, 3 or 4 in S_n^{2-}) are difficult to determine. Because 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 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 polysulflides, 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 the current polysulfide studies, UV absorption peaks at ~300 and ~370 nm are often used to indicate the presence of polysulfides (Nagy and Winterbourn, 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 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 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 $\mu mol \cdot g^{-1}$ protein. As discussed previously, the use of the mBB assay is based on the assumption that all H_2S_n species and other RSS 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.

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 and Ting, 2008; Lin *et al.*, 2015). While a large number of fluorescent sensors for other RSS, such as cysteine, homocysteine, GSH 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 (i) H_2S_n-mediated aromatic substitution-cyclization reactions; (ii) H_2S_n-mediated ring-opening reaction of aziridine; (iii) H_2S_n-mediated reduction of nitro groups.

Sensors based on H_2S_n-mediated aromatic substitution-cyclization (summarized in Scheme 3)
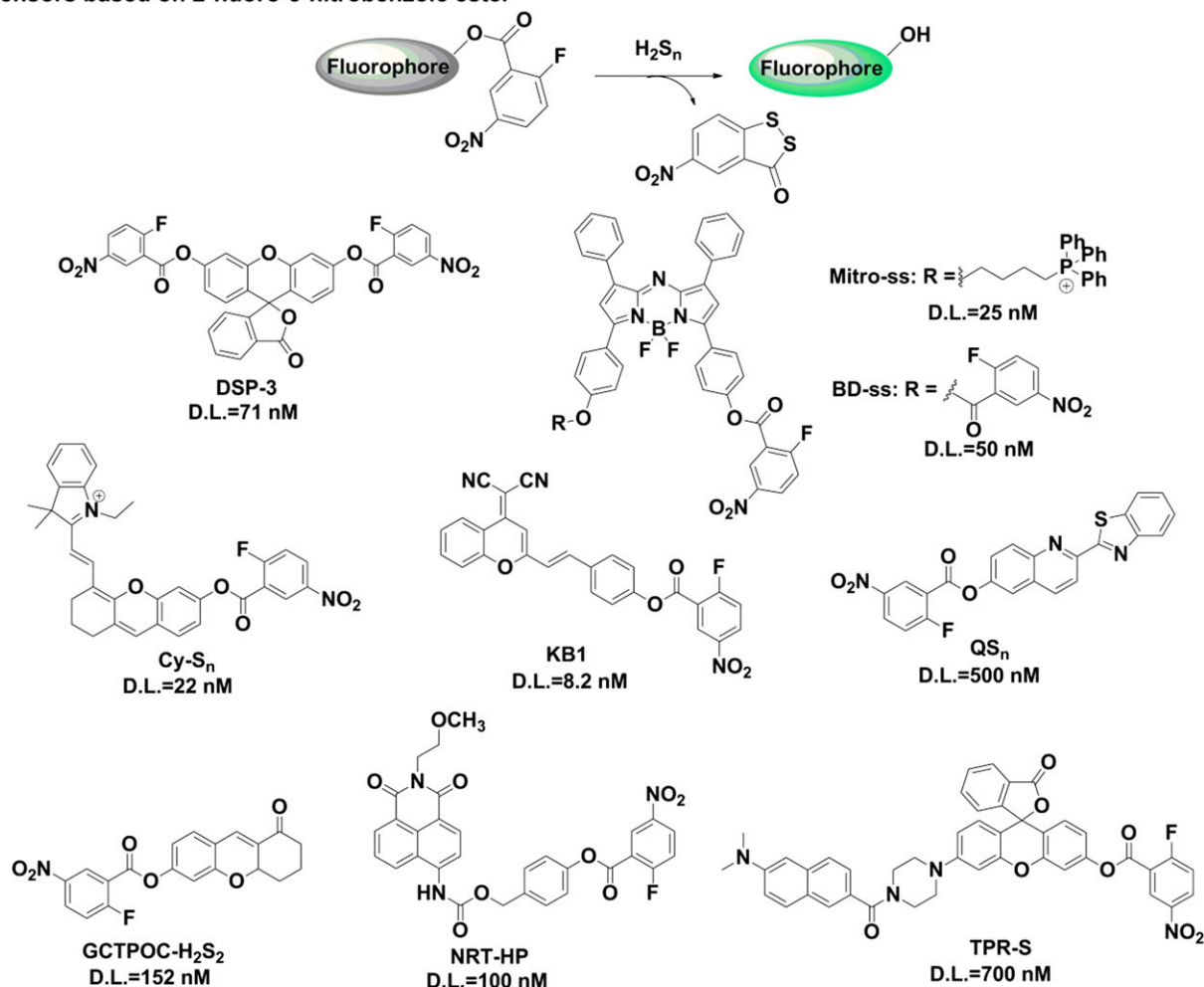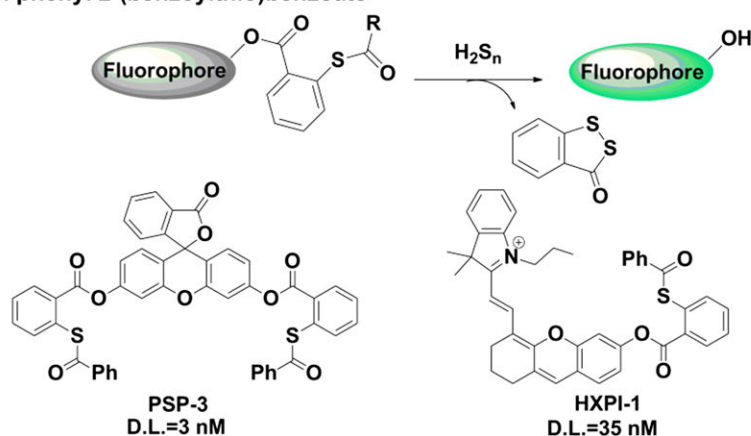
2-Fluoro-5-nitrobenzoic ester and phenyl-2-(benzoylthio) benzoate have been used as the specific recognition units for H_2S_n based on aromatic substitution–cyclization reaction. In 2014, our laboratory reported the first selective fluorescent sensors for H_2S_n, for example, DSP1-3, which employed the 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: (i) the 2-fluoro-5-nitrobenzoic ester is attacked by H_2S_2 *via* aromatic nucleophilic substitution to form a –SSH-containing intermediate and (ii) 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 H_2S_n. It was almost non-fluorescent in buffers but when treated with Na_2S_2, a 137-fold fluorescence increase was observed. The detection limit was 71 nM, and DSP-3 was successfully applied to image H_2S_n in HeLa cells.

Several other research groups have adopted the fluoro-5-nitrobenzoic ester recognition unit and developed sensors
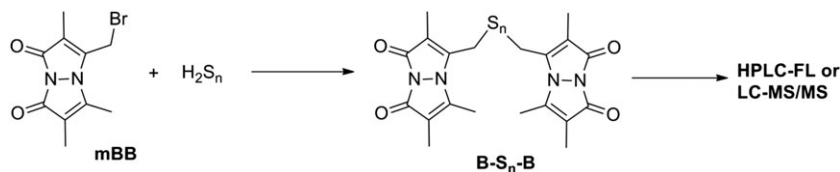
Scheme 2
Mono-bromobimane-based detection methods for H_2S_n.

A Sensors based on 2-fluoro-5-nitrobenzoic ester

DSP-3
D.L.=71 nM

Mitro-ss: R =
D.L.=25 nM

BD-ss: R =
D.L.=50 nM

Cy-S$_n$
D.L.=22 nM

KB1
D.L.=8.2 nM

QS$_n$
D.L.=500 nM

GCTPOC-H$_2$S$_2$
D.L.=152 nM

NRT-HP
D.L.=100 nM

TPR-S
D.L.=700 nM

B Sensors based on phenyl 2-(benzoylthio)benzoate

PSP-3
D.L.=3 nM

HXPI-1
D.L.=35 nM

Scheme 3

Fluorescent sensors based on H_2S_n-mediated aromatic substitution-cyclization. The detection limits (D.L.) for each sensor is shown below its structure.

with interesting fluorescence properties. For example, Chen *et al.* reported near-IR (NIR) fluorescent probes Mitro-ss and BD-ss (Gao *et al.*, 2015a,b). Both probes possessed boron difluoride dipyrromethene (BODIPY) as the fluorophore and 2-fluoro-5-nitrobenzoic ester as a response site. Mitro-ss bears a triphenylphoniummoiety as the mitochondria targeting group. The probes showed almost no fluorescence due to the donor-excited photoinduced electron transfer (d-PET) process

from the excited BODIPY to the 2-fluoro-5-nitrobenzoic ester. The addition of Na_2S_2 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 H_2S_n were further demonstrated. With one mechanism, H_2S_n could be generated from the reaction between H_2S and ROS, whereas with the other, H_2S_n could be produced from cystine by cystathionine γ-lyase and cystathionine β-synthase. 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 H_2S_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 H_2S_n in RAW264.7 macrophage cells and in mice. Han *et al.* also reported a NIR probe KB1, in which dicyanomethylene-benzopyran dye was employed as the fluorophore (Li *et al.*, 2018). KB1 showed more than 30-fold fluorescence increase (λ_{em} 682 nm) with H_2S_n and the detection limit of 8.2 nM. The probe was used for imaging H_2S_n in MCF-7 cells.

Two-photon (TP) fluorescent probes, with low-energy NIR wavelength excitation, are very useful for bio-imaging. These probes have advantages such as deep penetration in tissues, excellent 3D imaging capability and minimum photodamage to biological specimens. To date, there are several TP fluorescent probes developed for sensing H_2S_n by utilizing the 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 TP (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 that exhibited larger TP 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 co-workers 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 TP microscopy. Furthermore, upon excitation at 800 nm, probe NRT-HP could detect H_2S_n in tissue up to 300 μM of penetration depth. In order to get larger emission shift to improve the measuring accuracy, Yin *et al.* reported a 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 slices 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 H_2S_n sensing, which utilized phenyl 2-(benzoylthio)benzoate moiety as the recognition unit. This design takes the advantage of the dual reactivity of H_2S_n. It is known that H_2S_n are stronger nucleophiles than biothiols and H_2S under physiological pH. Moreover, H_2S_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, H_2S_n have a unique dual-reactivity and can be both a nucleophile and an electrophile. Therefore, we predicted phenyl 2-(benzoylthio)benzoate-based probes (PSP) should be specific for H_2S_n (Scheme 3B) (Chen *et al.*, 2015a, b). 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 a fast response to H_2S_n, excellent selectivity and high sensitivity with a detection limit of 3 nM. It was used in the detection of exogenous and endogenous H_2S_n in different cells. Based on this design, Ma *et al.* further developed an NIR probe HXPI-1, which employed a hemicyanine derivative as the fluorophore (λ_{em} 708 nm) and was used to visualize H_2S_n in mice (Fang *et al.*, 2017). The detection limits of each sensor are provided below its structure in Scheme 3.

### 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.*, 2015a,b). 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 TP 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.

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
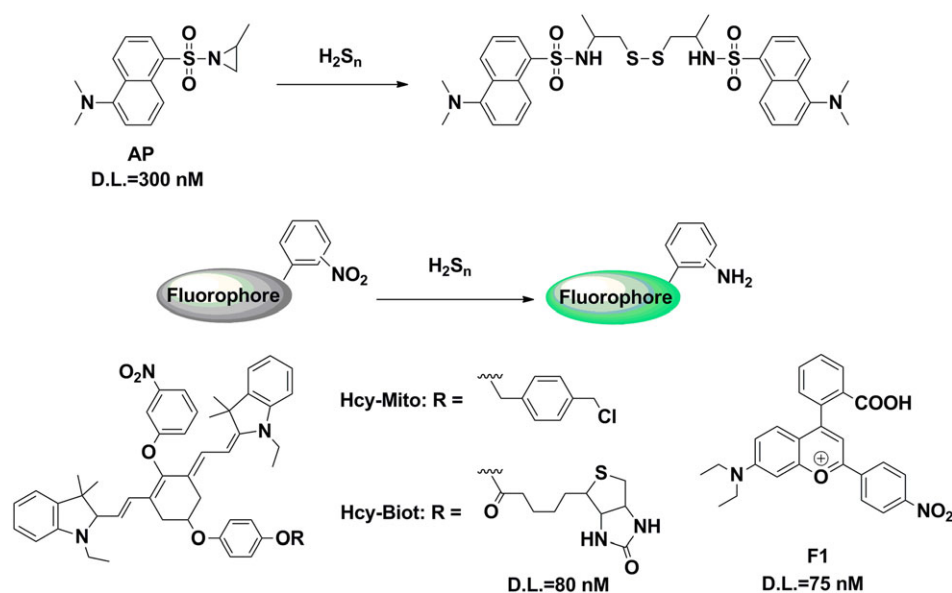
Scheme 4

Aziridine-based sensor AP and nitro-reduction based sensors. The detection limits (D.L.) for each sensor is shown below its structure.

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 0.1 μM. 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^{-} and H_2S_n crosstalk in living cells. Hcy-Biot showed high tumour-targeting ability and excellent tissue penetration in live animal models. Yang *et al.* reported another nitro-based probe F1 based on the flavylium fluorophore (Gong *et al.*, 2016). The fluorescence of F1 was quenched because attaching a nitro group to flavylium 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 flavylium. 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 crosstalks, 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 a 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 was calculated to be 100 and 24 nM respectively. DDP-1 is the first fluorescent probe capable of selective discrimination of H_2S_n and H_2S.

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), is not well-defined. As one can imagine, Cys-S_nSH and H_2S_n are likely to coexist, so one should not expect the probes to 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 the 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
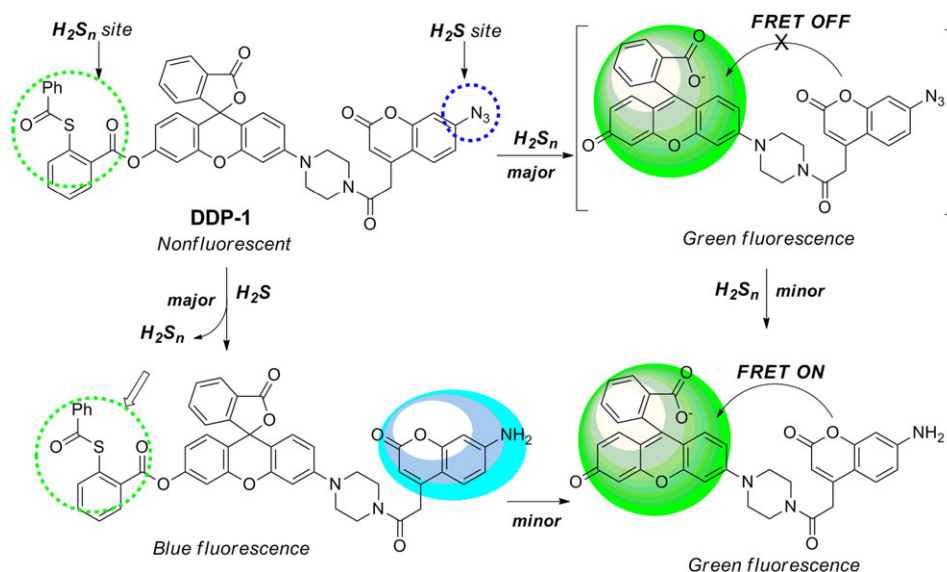
Scheme 5

Dual detection probe DDP-1.
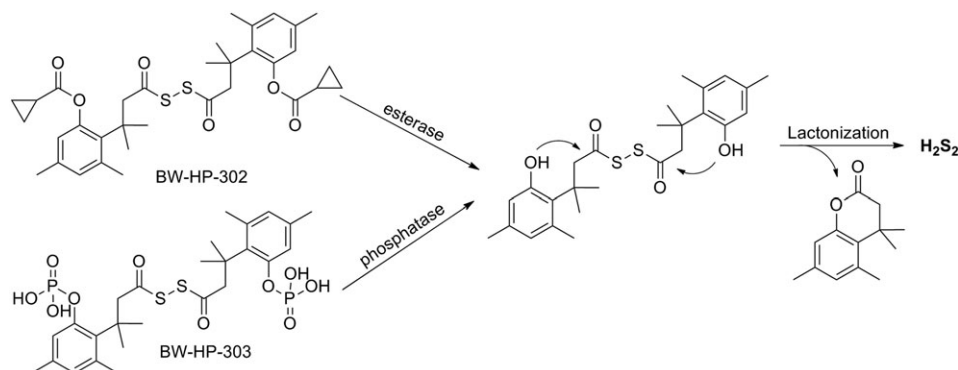
Scheme 6

Enzyme-triggered H_2S_n donors.

problematic as it is difficult to attribute the observed bioactivity solely to 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., 2018). In this work, H_2S_2 is caged as two thiol acid groups linked by a disulfide bond, for example, 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 have been prepared. Their enzyme-catalysed H_2S_2 productions have been demonstrated. Initial studies showed that these donors could induce protein S-persulfidation on 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 and 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 explored in future studies.

Concluding remarks

Increasing numbers of publications 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 evidence. In terms of their chemistry, H$_2$S$_n$ are highly reactive and unstable molecules. Tracking their formation and their fates can be very difficult. Moreover, H$_2$S$_n$ and H$_2$S often coexist, as H$_2$S$_n$ are found to be the common impurities in H$_2$S solutions (Greiner *et al.*, 2013). Therefore, differentiation of the functions of H$_2$S and H$_2$S$_n$ 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$_2$S$_n$ should be valuable. It is gratifying to see the development of H$_2$S$_n$ detection sensors has made some progress in recent years and the development of H$_2$S$_n$ 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.*, 2017a,b).

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

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