

## REVIEW ARTICLE

# Inorganic hydrogen polysulfides: chemistry, chemical biology and detection

**Correspondence** Ming Xian, Key Laboratory of Molecular Clinical Pharmacology, School of Pharmaceutics Science, Guangzhou Medical University, Guangzhou, Guangdong 510436, China. E-mail: mxian@wsu.edu

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Heng Liu<sup>1,2</sup>, Miles N Radford<sup>2</sup>, Chun-tao Yang<sup>3</sup>, Wei Chen<sup>2</sup> and Ming Xian<sup>2,3</sup> 

<sup>1</sup>Hubei Collaborative Innovation Center for Advanced Organic Chemical Materials, Ministry of Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules and College of Chemistry and Chemical Engineering, Hubei University, Wuhan, Hubei, China,

<sup>2</sup>Department of Chemistry, Washington State University, Pullman, WA, USA, and <sup>3</sup>Key Laboratory of Molecular Clinical Pharmacology, School of Pharmaceutics Science, Guangzhou Medical University, Guangzhou, Guangdong, China

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  $H_2S_n$ .

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### 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

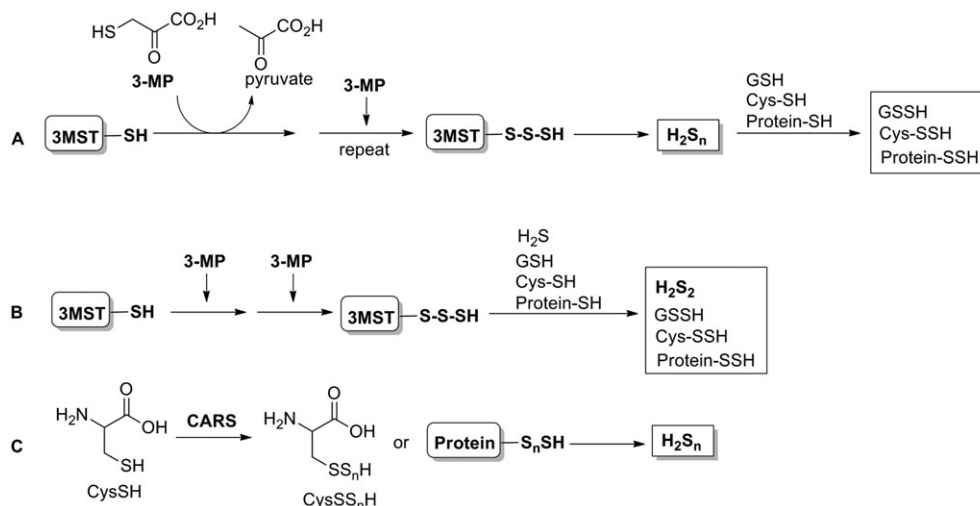
## Introduction

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<sub>2</sub>S)** 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<sub>2</sub>S functions as an endothelial cell-derived relaxing factor *via* direct activation of **ATP-sensitive potassium (K<sub>ATP</sub>)** channels. Deprivation of endogenous production of H<sub>2</sub>S contributes to the development of hypertension. Moreover, H<sub>2</sub>S has shown beneficial effects on oxidative stress, inflammation and fibrosis. While research on H<sub>2</sub>S is still actively ongoing, a hot new topic in this field has emerged that focuses on a series of H<sub>2</sub>S-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<sup>0</sup>). H<sub>2</sub>S-related sulfane sulfur compounds include persulfides (R-S-SH), polysulfides (R-S<sub>n</sub>-SH or R-S-S<sub>n</sub>-S-R), inorganic hydrogen polysulfides (H<sub>2</sub>S<sub>n</sub>, *n* ≥ 2) and protein-bound elemental sulfur (S<sub>8</sub>). Among these, the hydrogen polysulfides H<sub>2</sub>S<sub>n</sub> are especially attractive. Recent studies have suggested that H<sub>2</sub>S<sub>n</sub> exist endogenously and are linked to a number of physiological and pathological processes. H<sub>2</sub>S<sub>n</sub> can serve as the source of H<sub>2</sub>S, as well as the sink of H<sub>2</sub>S. **Much of what is known as H<sub>2</sub>S signalling may be actually due to H<sub>2</sub>S<sub>n</sub>.** In this review, we summarize current knowledge about H<sub>2</sub>S<sub>n</sub>, focusing on their formation, functions, fundamental physical/chemical properties, detection methods, as well as releasing strategies.

## Biosynthesis of H<sub>2</sub>S<sub>n</sub>

Currently, how H<sub>2</sub>S<sub>n</sub> are produced endogenously is still a topic under active investigation. The studies by Kimura *et al.* suggest that H<sub>2</sub>S<sub>n</sub> are mainly produced from **3-mercaptopyruvate (3MP)** by 3-mercaptopyruvate sulfurtransferase (3MST) (Kimura *et al.*, 2015). In their studies, the produced H<sub>2</sub>S<sub>n</sub> 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<sub>2</sub>S<sub>n</sub> species was H<sub>2</sub>S<sub>3</sub>, while H<sub>2</sub>S<sub>2</sub> and H<sub>2</sub>S<sub>5</sub> were minor products. Kimura *et al.* also found that H<sub>2</sub>S<sub>n</sub> were localized in the cytosol of cells, and H<sub>2</sub>S<sub>n</sub> could be produced from H<sub>2</sub>S by 3MST and rhodanese. The basal endogenous H<sub>2</sub>S<sub>3</sub> concentration was 3.4 nmol·g<sup>-1</sup> protein in the brain, which is comparable to the concentration of H<sub>2</sub>S (4.8 nmol·g<sup>-1</sup> 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<sub>2</sub>S<sub>n</sub> 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<sub>n</sub>SH), which then degrades to form H<sub>2</sub>S<sub>n</sub>. Next, H<sub>2</sub>S<sub>n</sub> 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<sub>n</sub>SH) is formed, it directly transfers the sulfane sulfur atom to other thiols or H<sub>2</sub>S to form the corresponding persulfide species, including H<sub>2</sub>S<sub>n</sub>. It should be noted that in these studies, H<sub>2</sub>S<sub>n</sub> species were identified by mBB capturing experiments. An assumption in these conclusions is that all H<sub>2</sub>S<sub>n</sub> 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

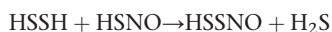
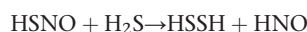
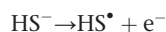


### Scheme 1

3MST-mediated H<sub>2</sub>S<sub>n</sub>/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<sub>n</sub>SH) 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<sub>n</sub>SH 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<sup>-</sup>. The formation of these products can be possibly attributed to the following equations:



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  $\beta$ -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  $\gamma$ -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)1 $\alpha$**  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 homologue (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 **PKG1 $\alpha$**  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<sub>2</sub>S<sub>n</sub>

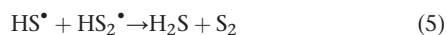
There are several chemical methods to make mixtures of H<sub>2</sub>S<sub>n</sub> or relatively pure H<sub>2</sub>S<sub>2</sub>, H<sub>2</sub>S<sub>3</sub> and H<sub>2</sub>S<sub>4</sub> (Steudel, 2003). For example, liquid sulfur and H<sub>2</sub>S can react to give a mixture of long-chain H<sub>2</sub>S<sub>n</sub> (Equation 1). As H<sub>2</sub>S and elemental sulfur occur together in hot underground deposits of natural gas, the formation of H<sub>2</sub>S<sub>n</sub> under these high-pressure conditions is very likely.



On the other hand, H<sub>2</sub>S<sub>n</sub> are unstable species and tend to decompose to elemental sulfur (S<sup>0</sup> or S<sub>8</sub>) and H<sub>2</sub>S (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<sub>2</sub>S<sub>n</sub> decomposition can be catalysed by substances such as alkali, nucleophiles and metals.



Thermolysis and photolysis of H<sub>2</sub>S<sub>n</sub> are believed to be radical chain reactions (Muller and Hyne, 1969; Gosavi *et al.*, 1973). Take H<sub>2</sub>S<sub>2</sub>, for example, the first step is assumed to be the homolytic cleavage of the S–S bond to form HS<sup>•</sup> (Equation 3), which then abstracts hydrogen from H<sub>2</sub>S<sub>2</sub> to form H<sub>2</sub>S and HS<sub>2</sub><sup>•</sup> (Equation 4). Recombination of HS<sup>•</sup> and HS<sub>2</sub><sup>•</sup> should form H<sub>2</sub>S and elemental sulfur S<sub>2</sub> (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.



The pure forms of H<sub>2</sub>S<sub>n</sub> (*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<sub>2</sub>S<sub>2</sub>, H<sub>2</sub>S<sub>3</sub> and H<sub>2</sub>S<sub>4</sub> 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<sub>2</sub>S<sub>n</sub> have been determined by high-level *ab initio* MO calculations. For H<sub>2</sub>S<sub>2</sub> at 0 K, D(S–H) = 313 kJ·mol<sup>–1</sup> and D(S–S) = 271 kJ·mol<sup>–1</sup> were obtained by the G2(MP2) method (Antonello *et al.*, 2002). The S–S bond dissociation energies of H<sub>2</sub>S<sub>2</sub>, H<sub>2</sub>S<sub>3</sub> and of the central bond of H<sub>2</sub>S<sub>4</sub> were calculated to be 259.5, 211.8 and 168.5 kJ·mol<sup>–1</sup>, 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<sub>2</sub>S<sub>n</sub> 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<sub>2</sub>S<sub>2</sub> Δ<sub>f</sub>G<sup>o</sup>(H<sub>2</sub>S<sub>2</sub>) are –2 kJ·mol<sup>–1</sup> in the gas phase and +5 kJ·mol<sup>–1</sup> in water. From the Δ<sub>f</sub>G<sup>o</sup> values for HSSH, HSS<sup>–</sup> and SS<sup>2–</sup>, a pK<sub>a</sub> of 2.6 is estimated for HSSH, and a pK<sub>a</sub> of 13 is estimated for HSS<sup>–</sup> (Koppenol and Bounds, 2017).

Redox reactions of H<sub>2</sub>S/H<sub>2</sub>S<sub>n</sub>: H<sub>2</sub>S can undergo both one- and two-electron oxidations. The HS radical (HS<sup>•</sup>) produced by one-electron oxidation of sulfide (HS<sup>–</sup>) is a strong oxidant. When it is generated, it can participate in radical chain reactions. The reduction potential of the HS<sup>•</sup>/HS<sup>–</sup> redox couple is +920 mV versus NHE at pH 7 (Das *et al.*, 1999). HS<sup>•</sup> can react with HS<sup>–</sup> to form hydrodisulfide radical anion, which can further react with oxygen (O<sub>2</sub>) to form HSS<sup>–</sup>. The bimolecular rate constants for H<sub>2</sub>S (in the form of HS<sup>–</sup> in physiological buffers) with biologically important two-electron oxidants are 0.73 M<sup>–1</sup>·s<sup>–1</sup> for H<sub>2</sub>O<sub>2</sub>; 4.8 × 10<sup>3</sup> M<sup>–1</sup>·s<sup>–1</sup> for peroxyxynitrite (ONOOH); 8 × 10<sup>7</sup> M<sup>–1</sup>·s<sup>–1</sup> (Carballal *et al.*, 2011) or 2 × 10<sup>9</sup>·M<sup>–1</sup>·s<sup>–1</sup> (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<sup>–</sup> to form H<sub>2</sub>S<sub>2</sub> (rate constant: 1 × 10<sup>5</sup> M<sup>–1</sup>·s<sup>–1</sup>) (Carballal *et al.*, 2011). The reactivities of H<sub>2</sub>S 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<sub>2</sub>S concentration (as compared to thiols) in tissues, it is unlikely these oxidations are physiologically significant. Therefore, it is unlikely H<sub>2</sub>S oxidation plays an important role for H<sub>2</sub>S<sub>n</sub> formation under normal physiological conditions.

If H<sub>2</sub>S<sub>n</sub> 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<sub>2</sub>S<sub>n</sub> (or their anions) in water. Nevertheless, inorganic salts of H<sub>2</sub>S<sub>n</sub> are often used as the equivalents of H<sub>2</sub>S<sub>n</sub> for biological studies. These salts (such as Na<sub>2</sub>S<sub>n</sub> and K<sub>2</sub>S<sub>n</sub>) 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<sub>2</sub>S<sub>2</sub> and Na<sub>2</sub>S<sub>4</sub> are relatively stable, but Na<sub>2</sub>S<sub>3</sub> is unstable in the solid state. Na<sub>2</sub>S<sub>3</sub> solid readily decomposes to form a eutectic mixture of Na<sub>2</sub>S<sub>2</sub> and Na<sub>2</sub>S<sub>4</sub> (El Jaroudi *et al.*, 1999). When polysulfide salts are dissolved in water, a complex equilibrium mixture will be established with HS<sup>–</sup> and elemental sulfur being the products (Equation 6).



This instant reaction has several implications: (i) when polysulfides are formed in aqueous environments, HS<sup>–</sup> and elemental sulfur will also be present; (ii) if HS<sup>–</sup> (or H<sub>2</sub>S)

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 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 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  $0.026 \mu\text{mol}\cdot\text{g}^{-1}$  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 $H_2S_n$

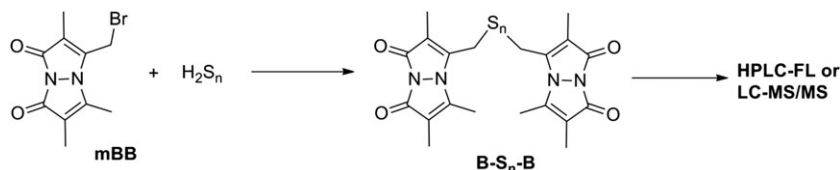
In order to better understand the functions of  $H_2S_n$ , methods that allow real-time and non-invasive detection of  $H_2S_n$  in biological systems are needed. In this regard, fluorescent sensors are ideal because of their high sensitivity and spatiotemporal resolution ability, as well as their ease 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.

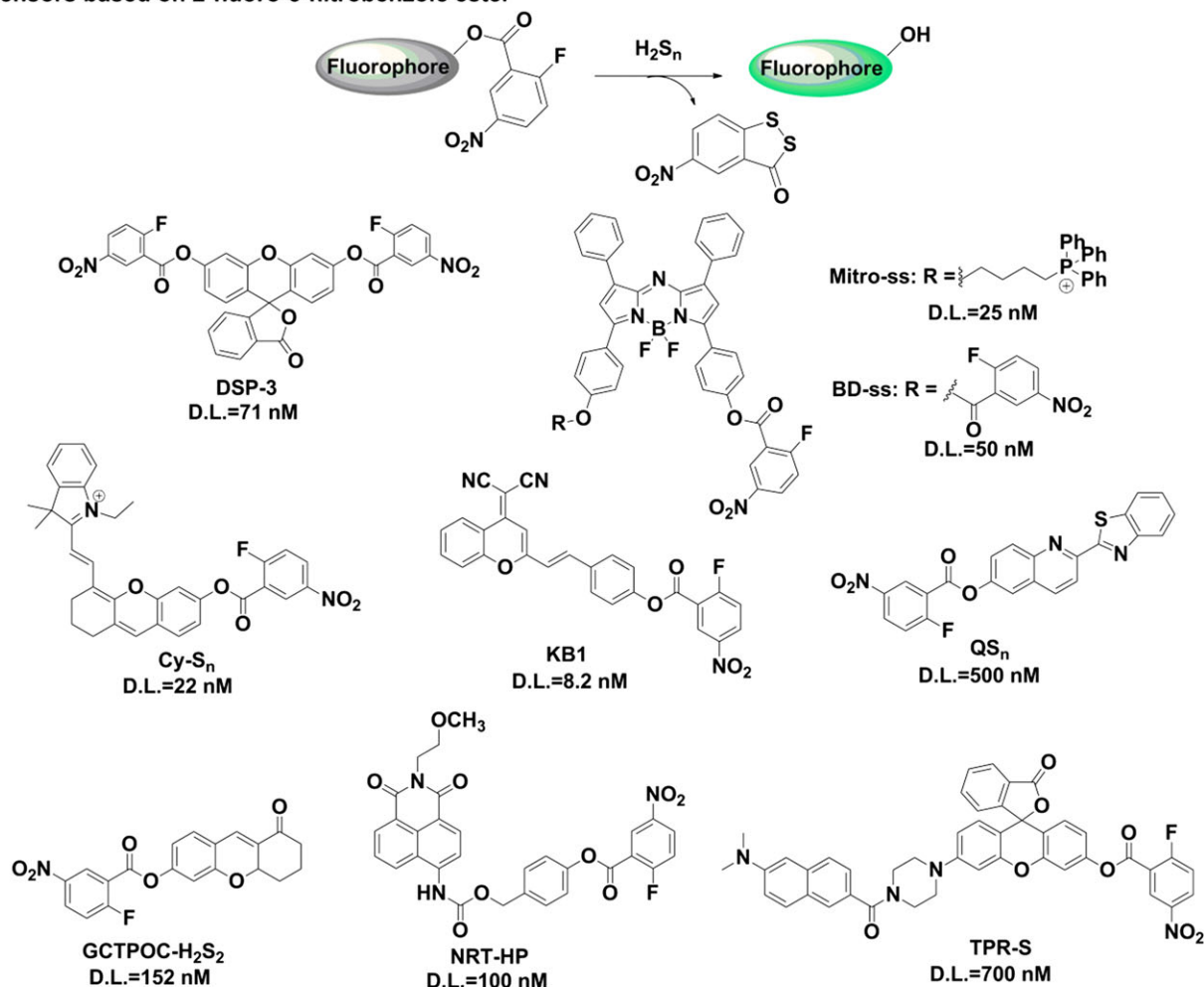
Several other research groups have adopted the fluoro-5-nitrobenzoic ester recognition unit and developed sensors



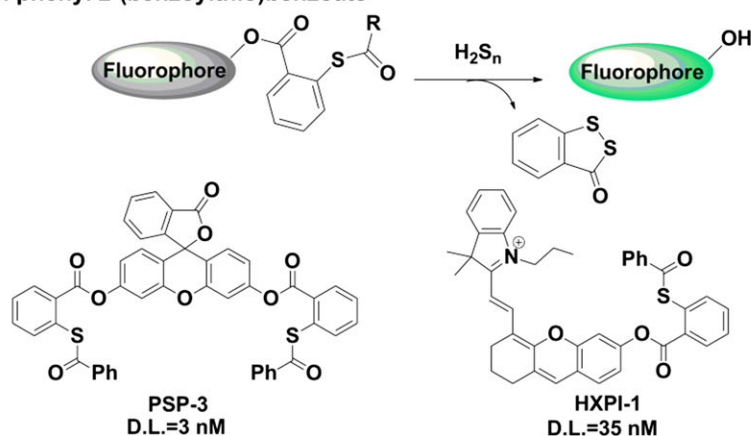
### Scheme 2

Mono-bromobimane-based detection methods for  $H_2S_n$ .

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



## B Sensors based on phenyl 2-(benzoylthio)benzoate



## Scheme 3

Fluorescent sensors based on H<sub>2</sub>S<sub>n</sub>-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 triphenylphosphonium moiety 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  $\text{Na}_2\text{S}_2$  triggered a significant fluorescence enhancement ( $\lambda_{\text{em}}$  730 nm), suggesting the d-PET process was suppressed by deprotection. With the aid of Mitro-ss, the two possible generation mechanisms of  $\text{H}_2\text{S}_n$  were further demonstrated. With one mechanism,  $\text{H}_2\text{S}_n$  could be generated from the reaction between  $\text{H}_2\text{S}$  and ROS, whereas with the other,  $\text{H}_2\text{S}_n$  could be produced from cystine by cystathionine  $\gamma$ -lyase and cystathionine  $\beta$ -synthase. Another NIR probe Cy- $\text{S}_n$  using semiheptamethine as the fluorophore was reported by Peng *et al.* (Ma *et al.*, 2017). With this probe, the presence of  $\text{H}_2\text{S}_n$  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  $\text{H}_2\text{S}_n$  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 ( $\lambda_{\text{em}}$  682 nm) with  $\text{H}_2\text{S}_n$  and the detection limit of 8.2 nM. The probe was used for imaging  $\text{H}_2\text{S}_n$  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 photo-damage to biological specimens. To date, there are several TP fluorescent probes developed for sensing  $\text{H}_2\text{S}_n$  by utilizing the 2-fluoro-5-nitrobenzoic ester moiety as the recognition site. In 2015, Liu *et al.* reported a TP probe  $\text{QS}_n$  using 2-benzothiazol-2-yl-quinoline-6-ol as the fluorophore (Zeng *et al.*, 2015). The addition of  $\text{H}_2\text{S}_n$  to the  $\text{QS}_n$  solution resulted in a 24-fold enhancement in fluorescence intensity at 534 nm under both one-photon ( $\lambda_{\text{ex}}$  368 nm) and TP ( $\lambda_{\text{ex}}$  730 nm).  $\text{QS}_n$  was not only used to visualize exogenous and endogenous  $\text{H}_2\text{S}_n$  in HeLa cells but also applied to investigate the distribution of  $\text{H}_2\text{S}_n$  in living zebrafish embryos. Another TP probe GCTPOC- $\text{H}_2\text{S}_2$  was reported by Lin *et al.* (Shang *et al.*, 2016). The reaction between GCTPOC- $\text{H}_2\text{S}_2$  and  $\text{H}_2\text{S}_n$  produced a product that exhibited larger TP action cross-section up to 500 GM ( $\lambda_{\text{ex}}$  780 nm). With this probe, imaging of  $\text{H}_2\text{S}_n$  in MCF-7 cells and *in situ* detecting  $\text{H}_2\text{S}_n$  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  $\text{H}_2\text{S}_n$  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  $\text{H}_2\text{S}_n$  in different cell lines (MCF-7, A549) by TP microscopy. Furthermore, upon excitation at 800 nm, probe NRT-HP could detect  $\text{H}_2\text{S}_n$  in tissue up to 300  $\mu\text{M}$  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  $\text{H}_2\text{S}_n$ , 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  $\text{H}_2\text{S}_n$ , 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  $\text{H}_2\text{S}_n$  in live HeLa cells, rat liver slices and LPS-induced acute organ injury upon excitation at 740 nm with femtosecond pulses.

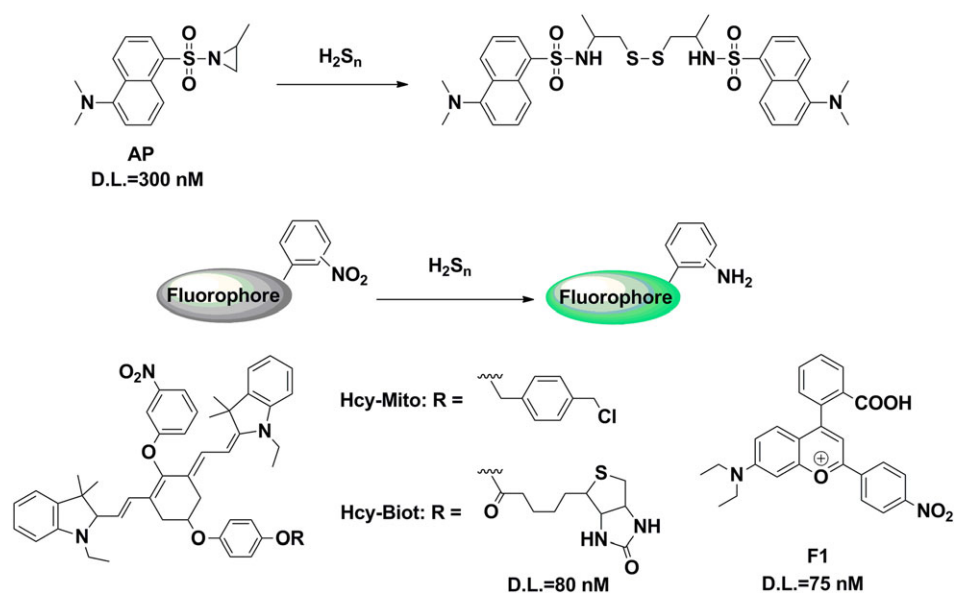
Although 2-fluoro-5-nitrobenzoic ester has been widely used in the design of  $\text{H}_2\text{S}_n$  probes, this recognition site could also react with biothiols to form thioether adducts. This problem does not affect the selectivity of the probes but could lead to the consumption of the probes in biological systems. High probe loading is usually required in actual applications. To solve this problem, our laboratory designed another strategy for  $\text{H}_2\text{S}_n$  sensing, which utilized phenyl 2-(benzoylthio)benzoate moiety as the recognition unit. This design takes the advantage of the dual reactivity of  $\text{H}_2\text{S}_n$ . It is known that  $\text{H}_2\text{S}_n$  are stronger nucleophiles than biothiols and  $\text{H}_2\text{S}$  under physiological pH. Moreover,  $\text{H}_2\text{S}_n$  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,  $\text{H}_2\text{S}_n$  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  $\text{H}_2\text{S}_n$  (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  $\text{H}_2\text{S}_n$ , excellent selectivity and high sensitivity with a detection limit of 3 nM. It was used in the detection of exogenous and endogenous  $\text{H}_2\text{S}_n$  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 ( $\lambda_{\text{em}}$  708 nm) and was used to visualize  $\text{H}_2\text{S}_n$  in mice (Fang *et al.*, 2017). The detection limits of each sensor are provided below its structure in Scheme 3.

### Sensors based on $\text{H}_2\text{S}_n$ -mediated ring-opening reaction of aziridine (Scheme 4)

$\text{H}_2\text{S}_n$  are known to be strong nucleophiles and expected to be more reactive in certain nucleophilic reactions than  $\text{H}_2\text{S}$  or thiols. Our group has explored some reactions of  $\text{H}_2\text{S}_n$  and found that  $\text{H}_2\text{S}_n$  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  $\text{H}_2\text{S}_n$ , the fluorescence intensity at 530 nm increased due to the suppress of the TICT effect. The reaction product of AP with  $\text{H}_2\text{S}_n$  showed not only good TP photophysical properties but also high luminescence efficiency in solid state. However, attempts to use AP for imaging  $\text{H}_2\text{S}_n$  in live cells were not successful.

### Sensors based on $\text{H}_2\text{S}_n$ -mediated reduction of nitro groups (Scheme 4)

Based on the strong reducibility of  $\text{H}_2\text{S}_n$ , Chen *et al.* designed two NIR fluorescent probes Hcy-Mito and Hcy-Biot that could be used for monitoring  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{S}_n$  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<sub>2</sub><sup>•-</sup> 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<sub>2</sub>S<sub>n</sub> 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<sub>2</sub>S<sub>n</sub> was estimated to be 80 nM. Hcy-Mito was used for *in situ* detection of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>S<sub>n</sub> levels in mitochondria and endogenous O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>S<sub>n</sub> 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 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<sub>2</sub>S<sub>n</sub>, 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<sub>2</sub>S 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<sub>2</sub>S and H<sub>2</sub>S<sub>n</sub>

H<sub>2</sub>S<sub>n</sub> and H<sub>2</sub>S 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<sub>2</sub>S<sub>n</sub> and H<sub>2</sub>S. We recently developed such a probe, DDP-1 (Scheme 5). It employs two recognition sites in one molecule: phenyl 2-(benzoylthio)benzoate for H<sub>2</sub>S<sub>n</sub> and azide for H<sub>2</sub>S. Rhodol and coumarin were used as the fluorophores and

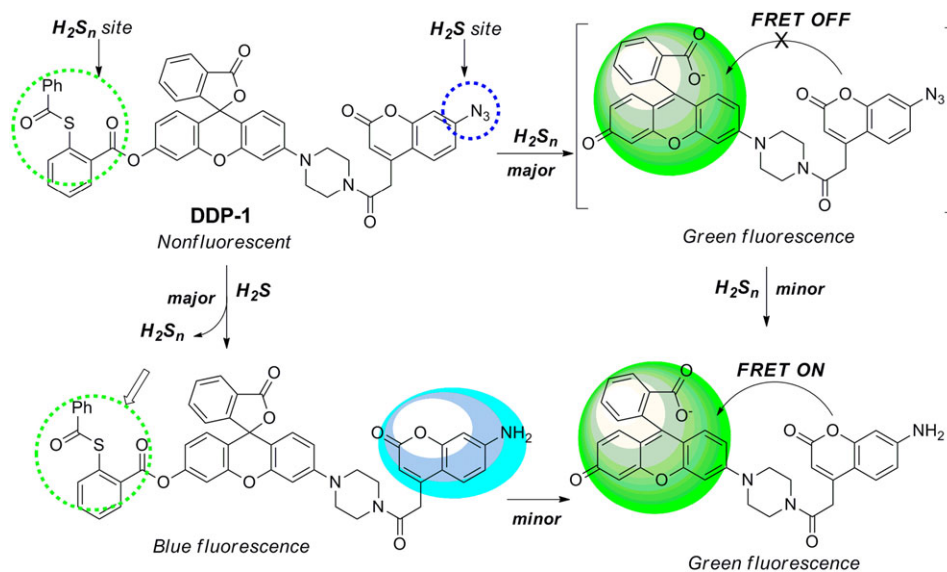
tethered *via* a rigid piperazine linker (Chen *et al.*, 2016). H<sub>2</sub>S<sub>n</sub> selectively reacted with the phenyl 2-(benzoylthio)benzoate moiety and induced a remarkable green fluorescence increase of rhodol. However, H<sub>2</sub>S promoted the reduction reaction of azide –N<sub>3</sub> to –NH<sub>2</sub>, which was accompanied by the oxidation of H<sub>2</sub>S to H<sub>2</sub>S<sub>n</sub>. This would lead to blue fluorescence of coumarin. Meanwhile, the resultant H<sub>2</sub>S<sub>n</sub> would also react with 2-(benzoylthio)benzoate to release rhodol and trigger the FRET from coumarin to rhodol. As a result, H<sub>2</sub>S could be identified by both fluorescence signals in blue and green channels. The detection limit of H<sub>2</sub>S<sub>n</sub> and H<sub>2</sub>S was calculated to be 100 and 24 nM respectively. DDP-1 is the first fluorescent probe capable of selective discrimination of H<sub>2</sub>S<sub>n</sub> and H<sub>2</sub>S.

So far, a handful of H<sub>2</sub>S<sub>n</sub> fluorescent probes have been reported, and we expect to see more being developed in the coming years. In the study of H<sub>2</sub>S<sub>n</sub> probes, researchers usually would validate their specificity for H<sub>2</sub>S<sub>n</sub> (vs. other thiols and H<sub>2</sub>S). However, their selectivity for some other important sulfur species, especially recently recognized cysteine hydropolysulfide Cys-S<sub>n</sub>SH (Ida *et al.*, 2014; Akaike *et al.*, 2017), is not well-defined. As one can imagine, Cys-S<sub>n</sub>SH and H<sub>2</sub>S<sub>n</sub> 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<sub>n</sub>SH and H<sub>2</sub>S<sub>n</sub> should have similar biological functions.

#### Releasing agents of H<sub>2</sub>S<sub>n</sub>

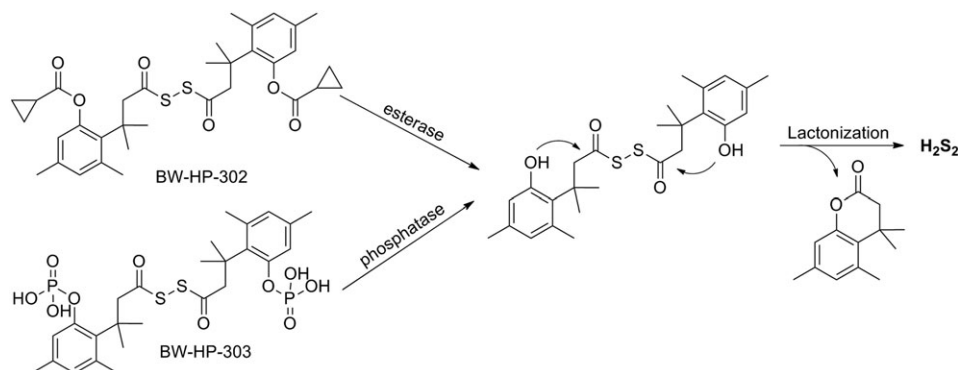
In the current studies of H<sub>2</sub>S<sub>n</sub>, researchers always use their inorganic salts (Na<sub>2</sub>S<sub>2</sub>, Na<sub>2</sub>S<sub>3</sub>, etc.) as the standard H<sub>2</sub>S<sub>n</sub> equivalents. These salts (Na<sub>2</sub>S<sub>2</sub>, Na<sub>2</sub>S<sub>3</sub>, Na<sub>2</sub>S<sub>4</sub>) 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<sup>2-</sup>) and elemental sulfur (S<sub>8</sub>). 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<sub>2</sub>S<sub>n</sub> are highly reactive and unstable molecules. Tracking their formation and their fates can be very difficult. Moreover, H<sub>2</sub>S<sub>n</sub> and H<sub>2</sub>S often coexist, as H<sub>2</sub>S<sub>n</sub> are found to be the common impurities in H<sub>2</sub>S solutions (Greiner *et al.*, 2013). Therefore, differentiation of the functions of H<sub>2</sub>S and H<sub>2</sub>S<sub>n</sub> 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<sub>2</sub>S<sub>n</sub> should be valuable. It is gratifying to see the development of H<sub>2</sub>S<sub>n</sub> detection sensors has made some progress in recent years and the development of H<sub>2</sub>S<sub>n</sub> 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).

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### Conflict of interest

The authors declare no conflicts of interest.

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