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The Path to Controlled Delivery of Reactive Sulfur Species

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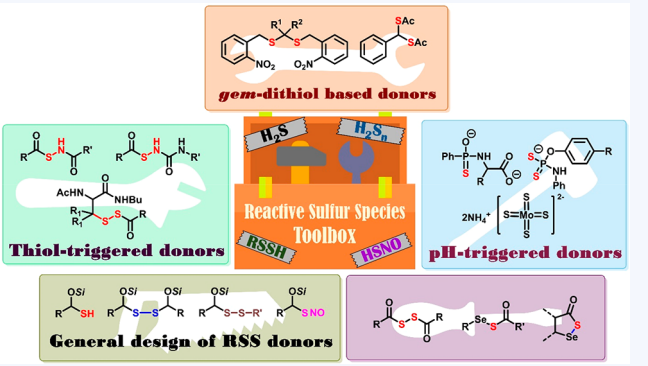
CONSPECTUS: Reactive sulfur species (RSS) play regulatory roles in many physiological and pathological processes. Since the discovery of hydrogen sulfide (H_2S) as a nitric oxide (NO)-like signaling molecule, understanding the chemical biology of H_2S and H_2S-related RSS, such as hydropersulfides (RSSH) and polysulfides (H_2S_n), has become a fast-growing research field. However, the research on these RSS has technical difficulties due to their high reactivity and instability. To solve this problem, considerable efforts have been put into the development of unique RSS releasing compounds (e.g., donors) or *in situ* RSS generation systems. This Account tells the story of our research group's effort to develop novel RSS donors.



We began with exploring molecular entities that were stable by themselves but could be triggered by biologically relevant factors, such as pH, thiols, light, or enzymes, to release H_2S in a controllable fashion. These studies led to the discovery of a series of novel H_2S donors. We later expanded our interests to other RSS including RSSH, H_2S_n, RSeSH, HSNO, RSOH, etc. The fundamental chemistry of these RSS was studied and applied to the development of the corresponding donors. In addition to small molecule donors, we also worked on H_2S-releasing biomaterials and their applications. This Account summarizes our work and systematically explains how each RSS donor template was proposed and evaluated. The Account covers the following key points: (1) rational chemistry design of each RSS donor template, (2) evaluation and mechanistic insights of each donor template, and (3) properties and biological applications of the donors.

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- Kang, J.; Li, Z.; Organ, C. L.; Park, C. M.; Yang, C. T.; Pacheco, A.; Wang, D.; Lefer, D. J.; Xian, M. pH-Controlled hydrogen sulfide release for myocardial ischemia-reperfusion injury. *J. Am. Chem. Soc.* **2016**, *138*, 6336−6339.[3] *These are the first pH-triggered H_2S donors, which have been used in many studies.*
- Kang, J.; Xu, S.; Radford, M. N.; Zhang, W.; Kelly, S. S.; Day, J. J.; Xian, M. O → S Relay deprotection: a general approach to controllable donors of reactive sulfur species. *Angew. Chem., Int. Ed.* **2018**, *57*, 5893−5897.[4] *This work provides a generic approach for the design of RSS donors. It can also be used in the design of sulfur-based sensors or prodrugs.*

INTRODUCTION

The physiological importance of H_2S has been recognized in the cardiovascular system and other organ systems throughout the body.[5−10] In blood vessels, cystathionine γ-lyase (CSE) is the major H_2S producing enzyme expressed in both smooth muscle and endothelium, as well as periadventitial adipose tissues.[11−14] Regulation of H_2S production from CSE is controlled by a complex integration of transcriptional, post-transcriptional, and post-translational mechanisms. The production of endogenous H_2S and the exogenous administration of H_2S have been demonstrated to exert protective

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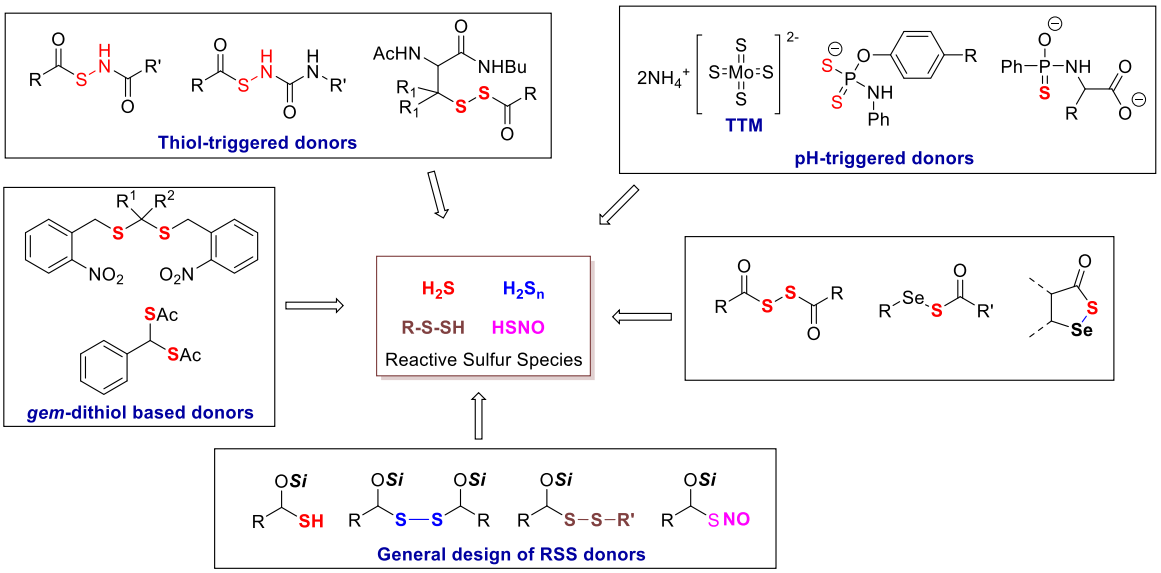

Figure 1. Summary of RSS donors developed by our laboratory.

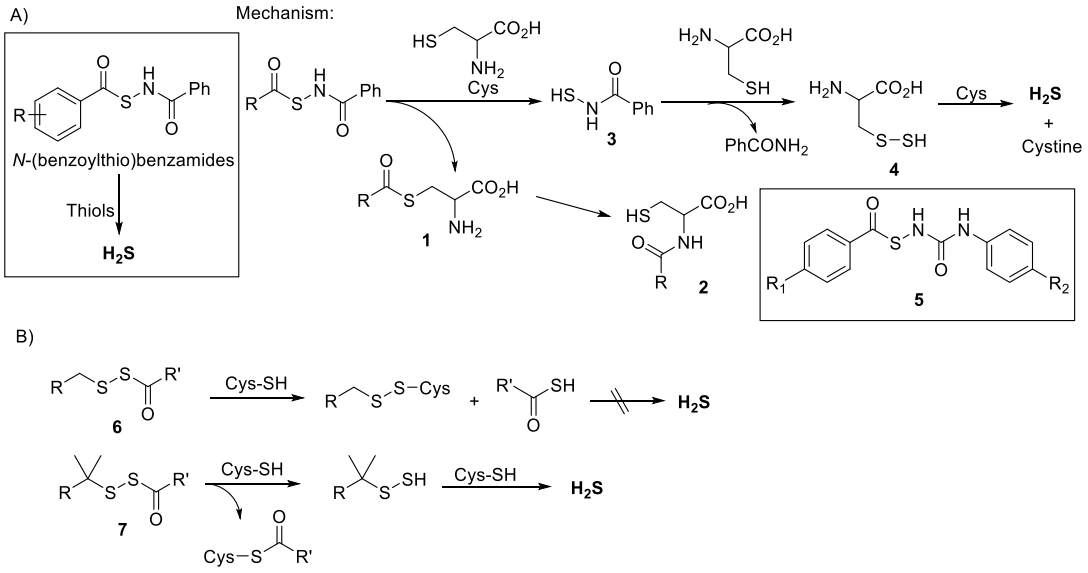

Figure 2. (A) H_2S release pathway from *N*-SH based donors; (B) H_2S release pathway from persulfide based donors.

effects in a number of pathologies.[5−14] For example, H_2S regulates diseases such as hypertension, atherosclerosis, and hypoxia-induced pulmonary hypertension. H_2S relaxes vascular smooth muscle to promote vasodilation and reduce blood pressure. While these results strongly suggest that modulation of H_2S levels could have potential therapeutic value for cardiovascular diseases, the underlying mechanisms of action of H_2S are still under investigation. In this research field, materials that can produce H_2S are essential research tools and potential therapeutic agents. In earlier works, researchers have typically used H_2S gas or sulfide salts (NaHS or Na_2S) to study H_2S *in vitro* and *in vivo*. However, the use of H_2S gas is problematic because of difficulties in obtaining accurate concentrations. Sulfide salts are short-lasting H_2S donors as they produce H_2S immediately upon dissolving in aqueous solutions. This rapid release of H_2S may cause acute changes in blood pressure and may exert toxic actions. In addition, H_2S concentration in aqueous solution can rapidly decrease due to volatilization. Because of these problems, the development of slow and controllable H_2S-releasing agents has become a fast-growing field, and many synthetic H_2S donors have been reported. In addition, several H_2S-related reactive sulfur species (RSS), in particular hydropersulfides (RSSH) and hydrogen polysulfides (H_2S_n), are also recognized as important cellular redox mediators. These RSS and H_2S are intimately linked biochemically. The research on RSSH and H_2S_n is also hindered by their inherent instability. Donor molecules for these RSS have started to emerge. Several excellent review articles have been published that summarize the progress in this exciting field and historical and current perspectives.[15−20]

This Account focuses on research from our own laboratory on the development of RSS donors. Our early work targeted H_2S donors. We have developed multiple strategies and structural templates for H_2S donors (thiol-triggered, pH-triggered, light-triggered, etc.). Later we explored donors for other RSS including RSSH, H_2S_n, RSeSH, HSNO, RSOH, etc. Our work has resulted in a diverse family of RSS donors, which will be discussed below (Figure 1).

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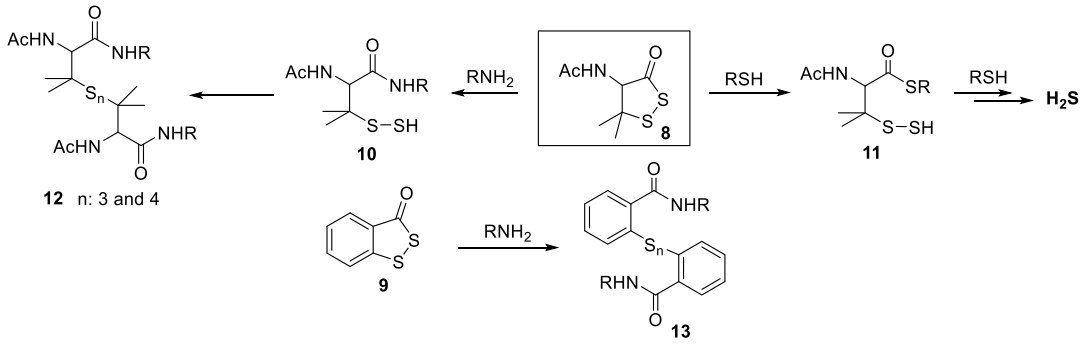

Figure 3. Reactions of cyclic acyl disulfides.

THIOL-TRIGGERED RSS DONORS

N-SH Based H_2S Donors and the Concept of Controllable Donors

Our work on H_2S donors started in 2008 when we recognized the need for such compounds. In our previous studies on *S*-nitrosothiols,[21,22] we recognized the instability of -S−N- bonds. We envisioned that *N*-mercapto (*N*-SH) compounds could serve as H_2S precursors. Since free *N*-SH compounds are unstable, -SH protection should afford stable products as the desired donors. Also, appropriate deprotection strategies can be used to control the release of H_2S. This design led to the first controllable H_2S donors, *N*-(benzoylthio)benzamides (Figure 2A).[1] These compounds are stable in aqueous solutions. They can release H_2S only in the presence of thiols (Cys or GSH). We demonstrated that H_2S release from the donors can be regulated upon structural modification. Electron withdrawing groups on the benzene ring caused faster H_2S generation, while electron donating groups slowed down H_2S release. Mechanistic studies suggest that the reaction is initiated by a thioester exchange between the donor and Cys. The resultant *S*-acylated cysteine, **1**, then undergoes a fast S to N acyl transfer to form a stable *N*-acylated cysteine, **2**. This process also produces a free *N*-SH intermediate, **3**, which further reacts with Cys to form cysteine persulfide, **4**. Finally, the reaction between **4** and cysteine produces cystine and H_2S. Later, this template was expanded to prepare a series of 1-mercapto-3-phenylurea based donors, **5**.[23] These donors showed increased H_2S peaking time (75−130 min) compared to *N*-(benzoylthio)benzamide donors (usually <60 min). Biological evaluation of these *N*-SH based donors demonstrated they have H_2S-like cytoprotective effects in both *in vitro* and *in vivo* models of myocardial ischemia−reperfusion (MI/R) injury.

Persulfide Based H_2S Donors

From the reaction mechanism of *N*-SH based donors, we realized that cysteine persulfide **4** was the key intermediate responsible for H_2S generation. Cysteine persulfide is also the intermediate in CSE-catalyzed H_2S formation in living systems. We thus envisioned that *S*-protected persulfides could serve as biomimetic H_2S donors. A library of acylated persulfide compounds derived from cysteine and penicillamine were prepared and evaluated (Figure 2B).[2] These compounds were also expected to be thiol-triggered donors. Interestingly, primary persulfide based donors **6** (cysteine derivatives) and tertiary persulfide based donors **7** (penicillamine derivatives) behaved very differently in terms of their H_2S production. Cysteine-based donors **6** released very small amounts of H_2S (less than 10% donor-to-H_2S conversion). This was due to an unwanted disulfide cleavage of the donors by thiols, leading to thioacid formation but no H_2S production. In contrast, penicillamine-based donors **7** were more productive in H_2S generation (up to 80% donor-to-H_2S conversion). The two adjacent methyl groups prevented the cleavage of the disulfide bonds by thiols, so deacylation and subsequent H_2S generation dominated in the reaction. These persulfide based donors showed no toxicity in H9c2 cardiac myocytes (up to 100 μM). Their H_2S production upon interacting with myocytes could be detected by fluorescence imaging. They also exhibited potent myocardial protective effects in MI/R injury.

Cyclic Acyl Disulfides as H_2S Donors and Polysulfide Precursors

After we studied acyclic acyl disulfides (**6** and **7**) as H_2S donors, we next wondered if cyclic acyl disulfides would show different reactivity. To this end, two cyclic acyl disulfides (**8** and **9** in Figure 3) were tested.[24] Compound **8** was found to be highly reactive toward both amines and thiols. Both reactions should produce the corresponding persulfide (**10** and **11**) as the intermediate. With amines, **10** rapidly degrades to form polysulfides **12**. With thiols, the persulfide **11** could further react with excess RSH to produce H_2S. Therefore, **8** can be considered as a thiol-triggered H_2S donor. Interestingly, the benzene fused compound **9** showed much stronger stability compared to **8**. It showed reasonable reactivity to amines, to form polysulfides **13** via a similar persulfide intermediate. However, **9** reacted very poorly with thiols and therefore was not an efficient H_2S donor. The poor reactivity of **9** toward thiols may be attributed to its favorable 5-member cyclic structure. Even if it reacts with thiols, a ring-opened thioester would be formed, and this thioester linkage is still reactive, especially to a nearby -SH. As such, the following -SH triggered cyclization should re-form **9**.

SELENYLSULFIDE (RSeSH)-BASED RSS PRECURSORS

Having recognized that acyl disulfides (both cyclic and acyclic) are effective persulfide/H_2S precursors, we then turned our attention to their selenyl analogs. Selenocysteine (Sec) is known to be redox sensitive, and many selenoproteins are important redox enzymes. Compared to Cys, Sec has lower p*K*a (~5.3) and redox potential (−381 mV). We envisioned that Sec is a stronger nucleophile than Cys toward sulfane sulfurs. As such, selenylsulfides (RSeSH) should be readily generated from the reaction between Sec and cellular sulfane sulfurs. This may play a role in sulfur-related redox signaling. However, this has not been demonstrated in biological settings.

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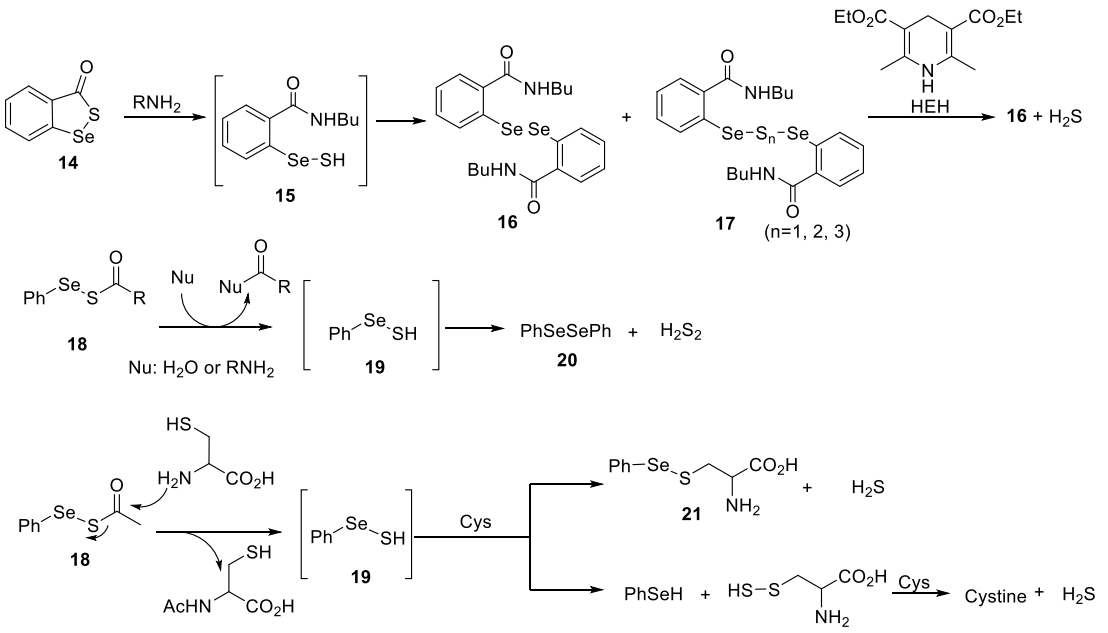

Figure 4. Reactions of acyl selenylsulfide toward amines or thiols regarding H_2S generation.

We explored the methods for *in situ* generation of RSeSH and utilized these methods to understand the chemistry of RSeSH. We first tested cyclic compound **14** as the precursor of RSeSH (Figure 4).[24] Compound **14** was found to be completely inert to thiols. However, it could react with amines to form diselenide **16** and diselenylsulfides **17**, apparently via a RSeSH intermediate **15**. The formation of **15** was also demonstrated by a trapping experiment using methylsulfonyl benzothiazole. Interestingly, diselenylsulfides **17** were found to be unstable and could slowly degrade to form more stable diselenide **16**. Therefore, diselenylsulfides **17** belong to the sulfane sulfur family and can be considered precursors of H_2S. Indeed, the reaction between **17** and Hantzsch ester (HEH), a NADH model compound, led to effective production of H_2S. We also tested acyclic acyl selenyl sulfides **18**, which appeared be much more reactive than **14**.[25] These compounds undergo hydrolysis in water or react with amines to form stable diselenide **20** and H_2S_2, again via the selenylsulfide intermediate **19**. If Cys is present, the formation of H_2S can be observed. Mechanistic analysis shows that this reaction is initiated by acyl transfer from **18** to the -NH_2 of Cys, forming the selenylsulfide intermediate PhSeSH. The excess Cys further reacts with PhSeSH, on either Se or S atom. Both pathways should lead to the formation of H_2S and cystine as the final product. The unstable PhSeH is eventually converted to stable PhSeSePh. These results suggest that (1) selenylsulfides and diselenylsulfides could serve as a reserved H_2S pool and produce H_2S via redox regulation, (2) RSeSH may be important regulating molecules involved in Sec-related redox signaling, and (3) acyl selenylsulfides can be useful tools for better understanding the chemical biology of RSeSH.

■ *gem*-DITHIOL DERIVATIVES AS H_2S DONORS

In 2013, we had an idea to develop caged-H_2S donors based on the structure of geminal-dithiols. *gem*-Dithiols are unstable species that degrade rapidly in aqueous solutions to form H_2S.[26,27] Therefore, we envisioned that *gem*-dithiol is a useful template for the design of H_2S donors and -SH protection/deprotection would enable controlled H_2S release. We first tested a photoactivation strategy.[28] A photosensitive 2-nitrobenzyl group was used to prepare a series of *gem*-dithiol derivatives **22** (Figure 5). These compounds exhibited time-

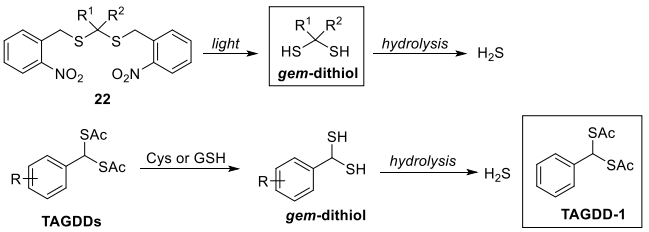

Figure 5. *gem*-Dithiol derivatives as H_2S donors.

dependent degradation under UV irradiation (365 nm). The corresponding *gem*-dithiol intermediate was produced, and subsequent hydrolysis liberated H_2S. We also found that the donors produced more H_2S under mildly acidic medium (pH = 5.5) than under neutral pH. This suggest that the hydrolysis of *gem*-dithiols is an acid-facilitated process. This work represents the first photocontrolled H_2S donors. Following this work, we applied an acyl group to stabilize the *gem*-dithiols and prepared a series of acylated benzyl *gem*-dithiol derivatives.[29] We expected they could react with cellular thiols (Cys or GSH) to form *gem*-dithiols and in turn to release H_2S. These are a new group of thiol-activated H_2S donors (named TAGDDs). Cys was found to be more active than GSH in promoting these donors to release H_2S. This can be explained by the fact that Cys is more reactive in the thioester transfer than GSH. Since H_2S exerts antiviral and anti-inflammatory activity, one of the donors (TAGDD-1) was tested as a potential antiviral agent.[30] In an *in vivo* (mouse) model of respiratory syncytial virus (RSV) infection, intranasal delivery of TAGDD-1 effectively reduced viral replication and lung inflammation. It also improved clinical disease parameters and pulmonary dysfunction. Mechanistic studies showed that TAGDD-1 had no effect on the initial steps of viral replication, as there was no reduction in RSV genome replication, viral

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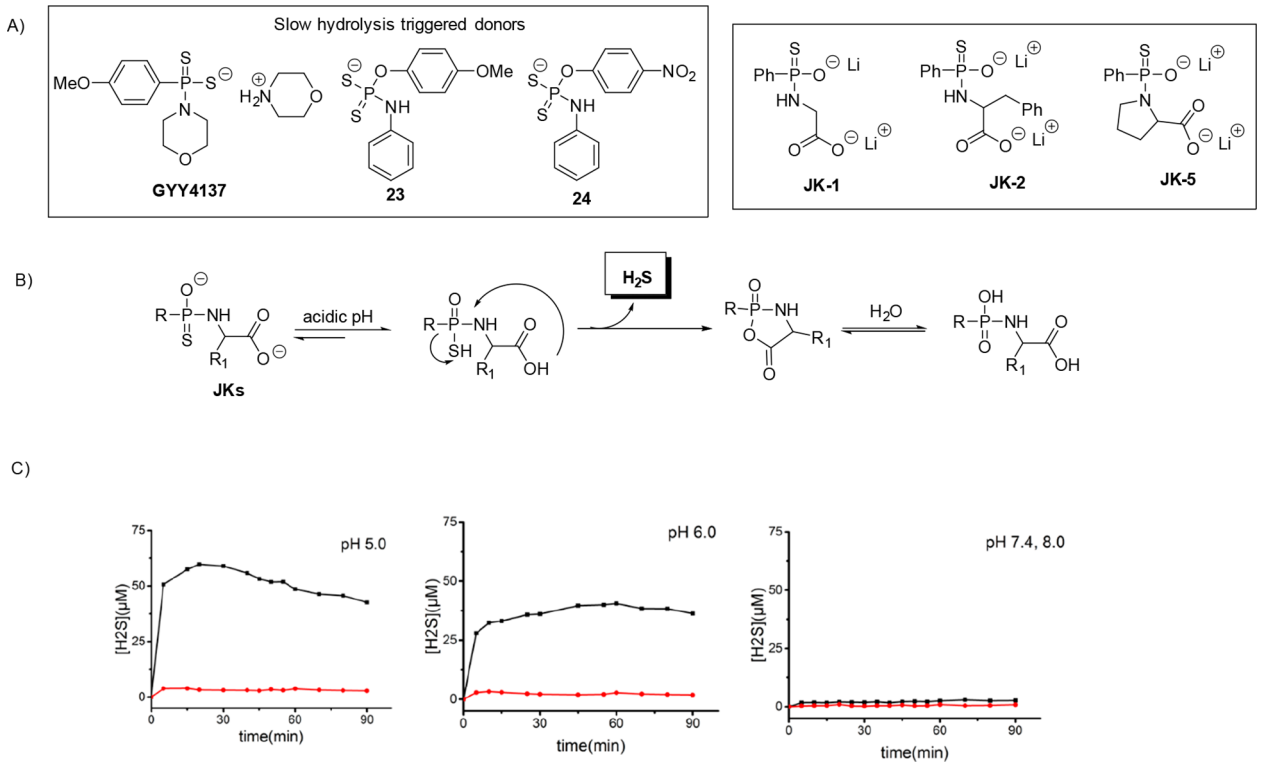

Figure 6. (A) Structures of phosphinodithioate based H_2S donors, (B) H_2S release mechanism of JK donors, and (C) comparison of H_2S release from JK1 (dark line) vs GYY4137 (red line).

mRNA, or protein synthesis. It is likely that the donor inhibits viral replication in part at the level of virus assembly, but mostly at the level of virus release. In addition to our work, Chakrapani et al. applied the *gem*-dithiol template in the design of bacterial nitroreductase (NTR) activated H_2S donors, which were used to study the modulatory role of H_2S in antibiotic resistance.[31]

GYY4137-INSPIRED H_2S DONORS

GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino)-phosphinodithioate) is one of the first small molecule H_2S donors reported.[32] It has excellent water solubility. In aqueous solutions, H_2S release from GYY4137 occurs at a slow and sustained rate (lasting for days). GYY4137 is perhaps the most popular H_2S donor and has been used by many researchers. However, recent research questions GYY4137 as a suitable H_2S donor.[33,34] It was proposed that GYY4137 produces H_2S upon hydrolysis. ^{31}P NMR studies revealed that barely detectable GYY4137 decomposition was noted after days in neutral pH buffers. Since the administration of GYY4137 to biological samples (cells, tissues, etc.) indeed induced detectable H_2S formation, it was suggested that GYY4137 might interact with certain biomolecules to facilitate its H_2S production, while the mechanism is still unclear. The observed biological responses from GYY4137 might not be solely due to H_2S; they could also come from the phosphinodithioate structure. Because of these problems, we attempted to modify its core structure (phosphinodithioate) with the hope of modulating the donors' H_2S release profile. In 2013, we replaced the C−P bond of GYY4137 with an O−P bond and prepared a series of O-alkyl and -aryl substituted phosphino-dithioates (such as **23** and **24** in Figure 6).[35] H_2S release measurements showed that O-aryl analogs exhibited slow and sustainable H_2S generation similar to GYY4137, while O-alkyl analogs' H_2S release ability was even worse. It is likely that O-alkyl substitutions increased the donor's stability and made hydrolysis more difficult. The observed very slow and unproductive H_2S generation from GYY4137 and **23**/**24** suggested that the hydrolysis reaction between water and these phosphinodithioates was slow. In theory, the hydrolysis is a bimolecular reaction between the donor and water. We then wondered if this process could be enhanced by an intramolecular process. A series of phosphonamidothioate donors (JK donors) were designed to test this idea.[3] In this template, a free -CO_2H group was tethered to the phosphonamidothioate core. Under neutral or slightly acidic pH, the donors should be protonated to form phosphorothiols, and the intramolecular nucleophilic addition of the -CO_2H should favor H_2S release. We expected this cyclization could enhance H_2S release. Indeed, JK donors showed much enhanced H_2S releasing ability compared with GYY4137. We also found that H_2S release from JKs was pH-dependent, with faster and more H_2S release under acidic pH. For instance, JK1 released barely detectable H_2S at pH 7.4 and 8 but significant amounts of H_2S at pH 5 or 6. In addition, structural modifications could tune their H_2S release profiles. The use of a benzyl group at the α-position made the donor JK-2 release detectable H_2S even under neutral or weakly basic pH. JK-5, however, showed almost no H_2S release, likely because the rigid proline ring inhibited the intramolecular cyclization.

The unique pH-triggered H_2S release from JK donors can have interesting applications as it is known that some pathological conditions cause acidic microenvironments and H_2S is known to attenuate such situations. For example, acute myocardial ischemia is associated with the acidification of the extracellular and intracellular space (pH ≈ 6), resulting from

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metabolic reactions producing acid equivalents as well as from the accumulation of acid products like CO_2 after cessation of perfusion. JKs were first tested in a murine model of MI/R injury, which was induced by subjecting mice to 45 min left ventricular ischemia, followed by 24 h reperfusion. Compared to vehicle-treated mice, mice treated with JKs showed significantly reduced infarct size per area-at-risk (INF/AAR). In another preclinical study, delayed H_2S therapy with JK1 was investigated on a murine model of transverse aortic constriction (TAC)-induced heart failure (HF).[36] Treatment with JK1 initiated at 3 weeks post-TAC could significantly attenuate left ventricle (LV) chamber dilatation and preserve LV ejection fraction (LVEF) accompanied by reduction in cardiac fibrosis. Delayed treatment with JK1 effectively ameliorated cardiorenal syndrome evidenced by reduced plasma creatinine levels and renal fibrosis. Additionally, JK1 was demonstrated to attenuate endothelium dysfunction. Such protection in multiple organs ultimately led to improved exercise capacity. The role of JK1 in nonsteroidal anti-inflammatory drug (NSAID) related gastric lesions was also studied *in vivo*.[37] Intragastric (IG) administration of 200 mg/kg aspirin (ASP) markedly induced gastric mucosal injury and enhanced pro-inflammatory factors interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α), which were significantly alleviated by preadministration of 150 μg/kg JK1. Moreover, ASP-induced MPO release, COX-2 up-regulation, and GSH depletion were partially reversed by the preadministration of JK1, indicating the protective effect of JK1 against ASP triggered oxidative stress.

Dymock et al. also reported some GYY4137 analogues as H_2S donors.[38] The H_2S release profiles and antiproliferative activities of these compounds against several solid tumor cell lines were evaluated. A cyclic phosphorane derivative, FW1256, showed the best H_2S releasing capacity and anticancer activity in cells. Two protonated GYY4137 analogues, AP67 and AP72, were studied by Ohia et al.[39] These donors could relax precontracted isolated bovine posterior ciliary arteries (PCAs), which may contribute to endogenous biosynthesis of NO and the action of K_{ATP} channels.

SULFUR CONTAINING INORGANIC COMPLEXES AS H_2S DONORS

In 2015, we recognized that almost all H_2S donors reported at that time were based on small molecule organic compounds. We wondered if certain sulfur-containing inorganic compounds (or metal complexes) could serve as H_2S donors. This idea was inspired by the fact that sodium nitroprusside ($Na_2[Fe(CN)_5NO]$) is a well-known NO donor now but its NO releasing capability was not appreciated before NO was identified as the important signaling molecule. We suspected that the same situation might exist in H_2S. After some literature review, we turned our attention to ammonium tetrathiomolybdate (TTM, Figure 7). TTM is known as an excellent copper chelator and has been used clinically in the treatment of copper toxicosis, especially for Wilson's disease. TTM is prepared via the reaction between MoO_4^{2-} and H_2S. We envisioned that the reverse reaction (i.e., H_2S release) could occur in aqueous solutions. We then tested the hydrolysis of TTM under different pH conditions.[40] As expected, we observed H_2S formation from TTM, and the release was pH-dependent. Under neutral pH, the release was a slow but sustained process. We also demonstrated TTM

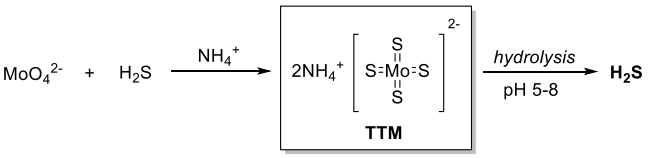

Figure 7. Ammonium tetrathiomolybdate (TTM) as a possible H_2S donor.

exhibited H_2S-like cytoprotection against oxidative damage in a cell model. Singer et al. later did a more comprehensive study on TTM.[41] They found that temperature and thiol contents could control H_2S generation from TTM. This sulfide generation reduced metabolism both *in vivo* (in awake rats) and *ex vivo* (in skeletal muscle tissues), with a superior safety profile compared to other sulfide donors. In animal models, TTM protected against reperfusion injury and organ damage following heart attack, stroke, and hemorrhage, with significant improvements in outcome and a good safety profile. These studies revealed a new H_2S-related perspective on TTM. This property should be considered when utilizing TTM in clinic studies. For example, TTM has been used as an anticancer agent due to its copper chelation ability as elevated copper promotes tumor growth. On the other hand, H_2S generation from TTM is carcinogenic through YTHDF1-dependent PRPF6 m^6A methylation in lung adenocarcinoma cells.[42] Removing H_2S may be needed in order to apply TTM in anticancer therapy.

OTHER PERSULFIDE AND H_2S_N DONORS

9-Fluorenylmethyl (Fm) Disulfides as Persulfide Donors

Traditionally the standard method for the preparation of small molecule persulfides (RSSH) is hydrolysis (using HCl/MeOH) of acylated disulfides. However, this is a slow process (usually >12 h), which might be suitable for relatively stable RSSH (such as triphenylmethyl persulfide and adamantyl persulfide) but not for most RSSH. This slow RSSH formation would cause problems in the study of RSSH due to their instability. In addition, RSSH are known to have lower pK_a (~6) than RSH (~8). Under physiological pH, RSSH should exist mainly as RSS^-, and this form would dominate their properties and reactivity. As such, we decided to explore base-triggered RSSH generation methods as we believed direct formation of RSS^- would be ideal for biomimetic studies of RSSH. To this end, we prepared two Fm-pyridinyl disulfides (**25** and **26**, Figure 8).[43] The carboxylate on **26** was used to improve its water solubility. These reagents showed high reactivity toward thiols (RSH). The resulting 9-fluorenylmethyl disulfides (RSSFm) were demonstrated to be base-triggered RSSH donors. Upon the treatment of DBU (2 equiv), Fm was completely removed in 20 min, and the isolated products were the corresponding polysulfides and elemental sulfur. If thiol-blocking reagents such as IAM were present, the generated RSSH could be trapped in high yields. Using BSA as an example, we further demonstrated that this strategy (with **26** and DBU) could induce persulfide formation on proteins. Fm-disulfides can also serve as RSS^- precursors in organic medium, making them useful in the study of persulfide-related chemistry. For example, we have developed a method to synthesize unsymmetrical trisulfides using Fm-disulfides.[44]

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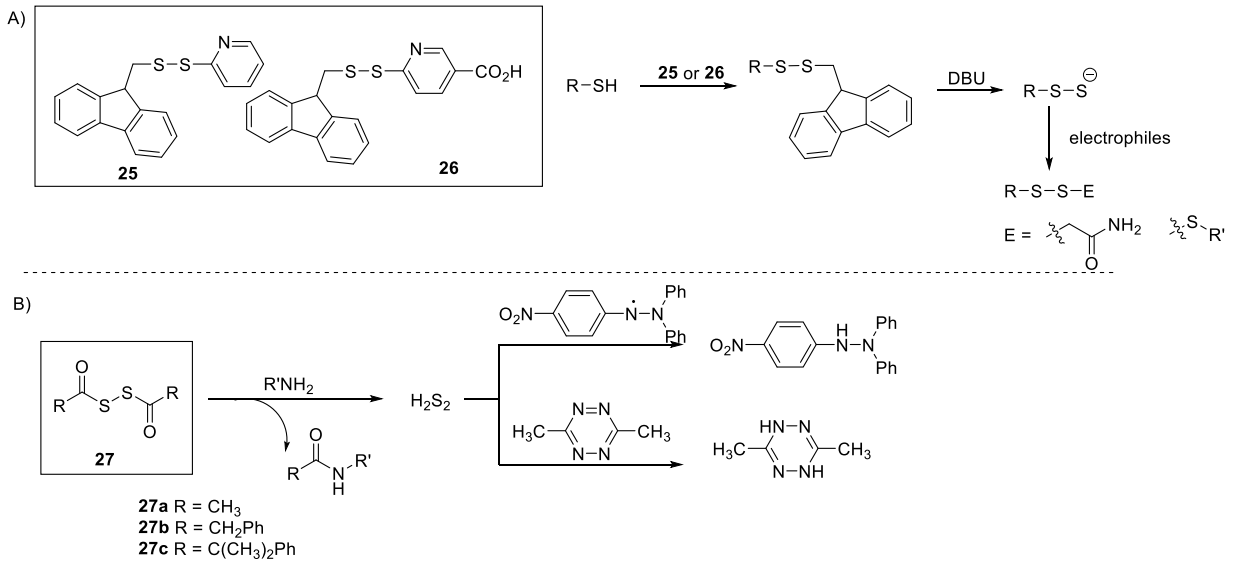

Figure 8. (A) Base triggered persulfide formation from 9-fluorenylmethyl disulfides and (B) H_2S_2 formation from diacyl disulfides and subsequent reactions.

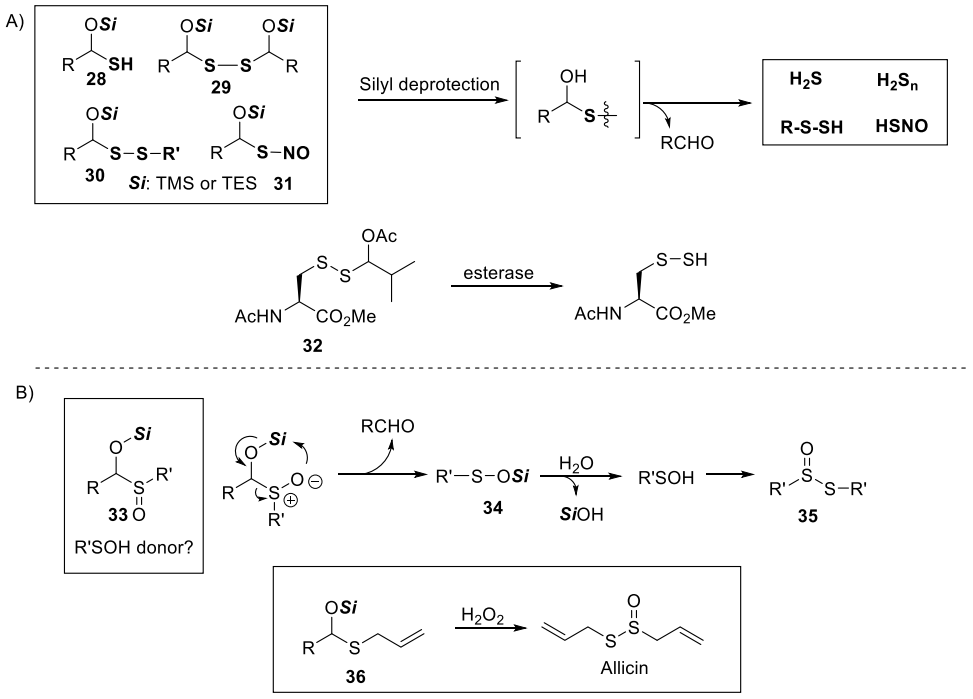

Figure 9. (A) Design of O to S relay deprotection based RSS donors and (B) O to O silyl migration induced thiosulfinate formation.

Diacyl Disulfides as H_2S_2 Donors

Hydrogen persulfide (or polysulfides, H_2S_n) are the oxidation products of H_2S. Their regulatory roles are closely linked to H_2S. Recent studies suggest that some biological activities originally attributed to H_2S may come from H_2S_n.[45−48] The study of H_2S_n is very challenging because these species are very unstable and can rapidly degrade or react with other biomolecules. Specific H_2S_n donors can be useful tools for illustrating their chemical biology. However, very few such donors have been developed so far. We envisioned diacyl disulfides could serve as an H_2S_2-releasing moiety. However, this structure template is known to be highly reactive. Previous studies showed that diacyl disulfides readily react with biothiols (to release H_2S)[49] or amines (to form amides).[50] Thus, it is unlikely acyl disulfides can serve as useful H_2S_2 donors in biological systems. However, they may be useful in simplified systems (without unwanted nucleophiles) to produce and study H_2S_2. To this end, we tested the possibility of a series of diacyl disulfides (**27a−c**) as H_2S_2 donors.[51] These molecules were found to be stable in organic solvents. However, they slowly degraded in aqueous solutions due to hydrolysis. The hydrolysis could proceed by two pathways: (1) bis-deacylation to form H_2S_2 and (2) mono-deacylation to form thioacid, H_2S, and S_8. With the presence of excess nucleophiles such as amines, these molecules produced H_2S_2 in both aqueous and organic solvents (Figure 8). This method was used to explore the redox chemistry of H_2S_2, such as scavenging 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and reducing tetrazines. H_2S_2

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effectively outperformed H_2S at reducing DPPH and 3,6-dimethyl-1,2,4,5-tetrazine in these experiments. This proved H_2S_2 is a stronger one- and two-electron reductant. Diacyl disulfides were used by Wang et al. to develop enzyme-activated H_2S_2 donors.[52] However, it is unclear if those donors could react with nucleophilic residues on proteins.

A GENERAL TEMPLATE FOR THE DESIGN OF RSS DONORS

In previous studies on RSS donors, researchers normally employ specific sulfur (S-) protecting groups to cage the sulfur containing molecules. These protecting groups also serve as the triggers for the desired RSS release. It should be noted that biocompatible *S*-protecting groups are rather limited, and therefore, the corresponding triggering strategies are also limited. On the other hand, biocompatible oxygen (*O*-) protecting groups have been well developed. Many *O*-protection/deprotection strategies have been employed in prodrug and sensor developments. We envisioned that if *O*-protection/deprotection could be used in *S*-cage/decage, it would significantly increase the options for RSS donor design. To this end, we designed an O to S relay deprotection.[4] As shown in Figure 9, a geminal thiol-hydroxyl moiety was used as the key template. The removal of the *O*-protection group on the substrates should generate a geminal thiol-hydroxyl derivative, which should readily degrade to form the SH-containing molecules. Based on this idea, four series of α-siloxy sulfur derivatives (**28**−**31**) were prepared and evaluated. We found that these compounds could undergo pH-dependent silyl-deprotection and release the corresponding H_2S, H_2S_n, RSSH, and HSNO. As one can imagine, this method can be expanded to other sulfur species, so it can be considered as a general method for the design of RSS donors. Of particular interest are compounds **31**, which were proven to be clean HSNO donors. This has some advantage compared to previous HSNO formation methods, which rely on *in situ* generation via H_2S + GSNO, or $H_2S(g)$ + $NO(g)$.[53,54] We also demonstrated that the silyl protecting groups could be switched to esters. Such compounds (like **32**) are esterase-triggered RSSH donors. A drawback of this method is the generation of aldehyde as the byproduct. Ketone-derived templates might be a better option as ketones are less harmful compared to aldehydes. The study of ketone-derived templates is currently ongoing in our lab.

Very recently we attempted to expand this relay deprotection to develop sulfenic acid (RSOH) donors using α-siloxy sulfoxides **33**.[55] Interestingly, **33** were found to be extremely unstable and could not be obtained. This is due to a rapid O to O silyl migration to generate the corresponding aldehyde and silyl-protected sulfenic acid **34**, which is known to be highly susceptible to hydrolysis and solvolysis by protic reagents. As such, sulfenic acids (RSOH) would be formed, which in turn self-conjugate to from thiosulfinates **35** as the isolated products. Based on this mechanism, α-siloxy sulfides can be considered as oxidation triggered donors for sulfenic acids (RSOH) or thiosulfinates. For example, a series of allyl sulfides **36** were proven to be H_2O_2-triggered allicin donors.

H_2S-Releasing Biomaterials

While these small-molecule donors are useful tools, we also expected RSS-releasing materials could offer some advantages such as improved bioavailability and releasing profile. So far, we have developed several H_2S-releasing biomaterials using the monomeric donors discovered in our lab. In 2015, we reported the first H_2S-releasing electrospun microfibers (H_2S-fibers).[56] Biodegradable polycaprolactone (PCL) solutions with concentrations ranging from 6% to 12% were doped with a thiol-activated N-SH donor (NSHD1), and homogeneous H_2S-fibers with diameters ranging from 0.5 to 1.5 μm were fabricated by electrospinning. The half-life of H_2S release from these microfibers was significantly longer than the donor itself. H_2S-fibers effectively protected H9C2 and NIH 3T3 cells from H_2O_2 induced oxidative damage and supported 3T3 fibroblast proliferation. In 2016, the pH-controllable H_2S donor JK1 was used to fabricate a H_2S releasing nanofiber PCL-JK1.[57] This hybrid fibrous scaffold showed notably slower releasing rate compared to JK1. The full-thickness cutaneous wound model on C57BL/6 mice was used to evaluate wound healing efficacy of PCL-JK1. Compared with undoped PCL fiber, PCL-JK1 could significantly promote wound recovery and regeneration over 20 days.

In addition to electrospun fibers, we also incorporated JK1 into hydrogels to test its therapeutic potential. In one study, JK1 was encapsulated into a collagen hydrogel (Col-JK1) for the treatment of intervertebral disc degeneration (IDD).[58] Collagen hydrogel can be degraded by the overexpressed matrix metalloproteinases (MMPs) in IDD. The subsequent release of JK1 in the low pH environment leads to *in situ* generation of H_2S, making Col-JK1 a pH and enzyme dual responsive system. As expected, Col-JK1 exhibited a notably slower H_2S release rate compared to free JK1. The addition of MMP-9 caused a much faster generation of H_2S from Col-JK1 at pH 6.0. In *in vivo* studies Col-JK1 effectively restored disc degeneration in a puncture-induced IDD rat model. Mechanistic studies revealed that Col-JK1 inhibited the apoptosis of nucleus pulposus (NP) cells and attenuated the degradation of the disc extracellular matrix (ECM) through the regulation of the NF-κB signaling pathway. In another study, we encapsulated JK1 into hyaluronic acid (HA) hydrogel to form a HA-JK1 hybrid system.[59] *In vitro* and *in vivo* results indicated that HA-JK1 could induce the macrophage polarization to M2 anti-inflammatory phenotype. Additionally, HA-JK1 significantly accelerated wound tissue regeneration.

For wounds with heavy exudate, a dressing that can absorb wound exudate and maintain a moist wound environment will facilitate the wound healing process. Very recently, an H_2S-releasing sponge (SA/JK1) was developed by incorporating JK1 into an alginate sponge.[60] SA/JK1 exhibited similar pH-dependent H_2S releasing behavior as JK1. However, SA/JK1 showed prolonged and significantly higher H_2S concentration profile than that of JK1 at pH 6.0, which may be attributed to the highly porous structure of the sponge matrix. *In vitro* study indicated that SA/JK1 was nontoxic to L929 fibroblast and could accelerate cell proliferation and migration. Moreover, SA/JK1 could significantly improve wound healing process in an *in vivo* full thickness dermal defect model, making this hybrid sponge dressing a potential candidate for treatment of nonhealing wounds.

CONCLUSIONS

Over the past decade, our group has developed a variety of chemical tools for RSS delivery. These RSS include H_2S, RSSH, RSeSH, RSOH, H_2S_n, and HSNO. These donor compounds can be used to further understand RSS biology and some of them have been shown to confer positive medicinal benefits in cell- and animal-based models. To truly

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harness the potential of RSS based therapy, some challenges must yet be overcome. For instance, while H_2S appears promising for cardiovascular diseases, this approach will not be clinically feasible unless appropriate H_2S delivery techniques are available. Appropriate techniques are judged by their (1) triggering mechanism, (2) tunability, (3) biocompatibility, and (4) convenience of use. It is now recognized that the therapeutic window of H_2S is narrow. It is expected that slow and sustained H_2S release is optimal. However, what the optimal H_2S release rate or profile needs to be is still unclear. Another problem is that most donors also produce large amounts of organic byproducts in addition to RSS, and this can cause unwanted side effects. Therefore, new donor templates that can minimize production of byproducts or just produce benign byproducts are needed. On the other hand, donors for H_2S_n and persulfides are still underdeveloped. Compared to H_2S, the chemical biology of these RSS is more complicated due to their inherent instability. The presence of cellular "biothiols" together with H_2S_n and persulfides should quickly result in a collection of RSS species including polysulfides and H_2S. It might be critical to understand such equilibria and RSS distributions, as well as to appreciate RSS bioactivities as a whole, rather than each RSS individually. Finally, RSS are not the only regulatory molecules in redox signaling. They have been shown to exert interdependent regulatory functions with other signaling molecules such as ROS and RNS. These interdependencies, termed "crosstalk", may unlock key insights into the gasotransmitter/RSS domain. Dual donors (which simultaneously release both RSS and ROS or RNS) could facilitate acquisition of these insights. To this end, we recently discovered a controllable H_2S/H_2O_2 dual release system based on thioglucose and glucose oxidase.[61] This system could lead to efficient S-persulfidation on proteins. We expect more interesting donor molecules that address the aforementioned challenges will emerge in the foreseeable future.

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Notes

The authors declare no competing financial interest.

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Xiang Ni received his Ph.D. in Organic Chemistry from Nankai University in 2016. He is currently a postdoctoral researcher at Brown University in the lab of Prof. Ming Xian. His research focuses on the development of small molecular H_2S donors and scavengers.

Shane S. Kelly received his B.S. with a double major in chemical engineering and chemistry from Washington State University in 2017. He then joined the Washington State University Ph.D. program, where he studied organic chemistry before becoming a visiting research fellow at Brown University in 2020. He is currently completing Ph.D. study at Pacific Northwest National Laboratory through the Distinguished Graduate Research Program with a focus on proteomics. His research centers around the study of reactive sulfur species chemical biology through the development of chemical tools and proteomics methodology.

Shi Xu obtained his bachelor and master's degrees from Sichuan University and Nankai University, respectively. In 2019, he completed his Ph.D. at Washington State University with Prof. Ming Xian. He now works as a postdoctoral researcher in the Xian Group at Brown University. His research interest lies in chemical biology of hydropersulfide and polysulfides. He is especially interested in developing methods for their delivery and detection.

Ming Xian is currently a Professor of Chemistry at Brown University. He started his academic career at Washington State University (WSU) in 2006 and moved to Brown in 2020. He was the Ralph G. Yount Distinguished Professor at WSU and served as Associate Chair in the Chemistry Department from 2016 to 2020. His research focuses on the development of novel chemistry and chemical tools to facilitate biological studies of reactive sulfur species.

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