

# A Sweet H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> Dual Release System and Specific Protein S-Persulfidation Mediated by Thioglucose/Glucose Oxidase

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**ABSTRACT:** H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> are two redox regulating molecules that play important roles in many physiological and pathological processes. While each of them has distinct biosynthetic pathways and signaling mechanisms, the crosstalk between these two species is also known to cause critical biological responses such as protein S-persulfidation. So far, many chemical tools for the studies of H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> have been developed, such as the donors and sensors for H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub>. However, these tools are normally targeting single species (e.g. only H<sub>2</sub>S or only H<sub>2</sub>O<sub>2</sub>). As such, the crosstalk and synergistic effects between H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> can hardly be studied with those tools. In this work we report a unique H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> dual donor system by employing 1-thio- $\beta$ -D-glucose and glucose oxidase (GOx) as the substrates. This enzymatic system can simultaneously produce H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> in a slow and controllable fashion, without generating any bio-unfriendly by-products. This system was demonstrated to cause efficient S-persulfidation on proteins. In addition, we expanded the system to thiolactose and thioglucose-disulfide, therefore, additional factors ( $\beta$ -galactosidase and cellular reductants) could be introduced to further control the release of H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub>. This dual release system should be useful for future research on H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub>.

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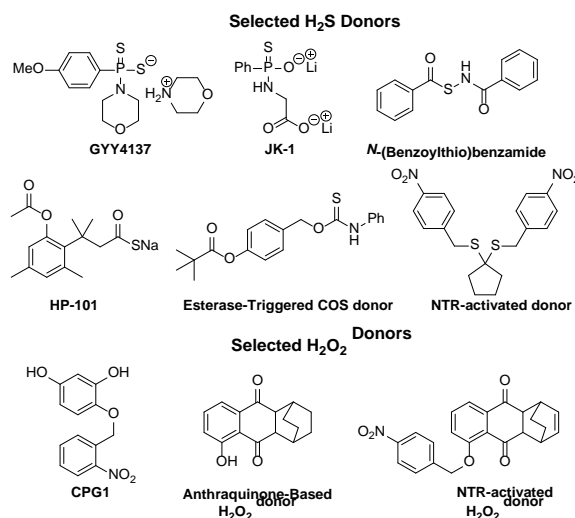
## INTRODUCTION

Reactive sulfur species (RSS) and reactive oxygen species (ROS) are two groups of molecules that play regulatory roles in redox biology. As the most well studied RSS H<sub>2</sub>S is endogenously produced by enzymes including cystathionine- $\beta$ -synthase (CBS), cystathionine- $\gamma$ -lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3-MST). Dysregulated H<sub>2</sub>S levels are associated with pathological processes like cancer, inflammation, hypertension, etc.<sup>1-2</sup> As the center species of ROS, H<sub>2</sub>O<sub>2</sub> is formed from spontaneous dismutation of superoxide (O<sub>2</sub><sup>•-</sup>), or under the catalysis of superoxide dismutase (SOD). Endogenous H<sub>2</sub>O<sub>2</sub> can also be produced by oxidases, such as NADP/H oxidases (NOX), lysyl oxidases (LOX), xanthine oxidase (XO), amine oxidase (AO), etc.<sup>3-4</sup> Aberrant production of H<sub>2</sub>O<sub>2</sub> leads to oxidative stress and damage, which is connected to aging and neurodegenerative diseases.<sup>5-6</sup> While H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> have their very distinct signaling pathways, they also interact with each other and work collectively in redox signaling.<sup>7</sup> The direct reaction between H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> under physiological conditions is known to be slow so their crosstalk is believed to through indirect effects on enzymes and/or other targets in signaling pathways.<sup>7</sup> For example, protein S-persulfidation is an important post-translational modification regulated by H<sub>2</sub>S. However, H<sub>2</sub>S cannot directly react with protein thiols to form persulfides and its reaction with disulfides is normally slow

and thermodynamically unfavored.<sup>8,9</sup> It is likely that persulfidation is the result of the reaction between H<sub>2</sub>S and sulfenic acid (S-OH), which is the oxidation product from H<sub>2</sub>O<sub>2</sub>.<sup>9</sup> As such, an appropriate level of H<sub>2</sub>O<sub>2</sub> is required to facilitate H<sub>2</sub>S signaling cascade, especially under oxidative stress. In another study H<sub>2</sub>S was found to significantly amplify H<sub>2</sub>O<sub>2</sub>-based therapeutic treatment for cancer in a mouse model.<sup>10</sup> Moreover, the mechanism of acute H<sub>2</sub>S intoxication is due to H<sub>2</sub>S serving as a substrate for complex II of the mitochondrial electron transport chain, thereby inducing high levels of ROS formation and oxidative stress.<sup>11</sup> These results indicate that concurrent presence of H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> is often needed to reveal their true biological importance.

So far, many chemical tools have been developed for RSS/ROS studies. In particular, donor molecules for these reactive species have received considerable attention. For example, a variety of H<sub>2</sub>S donors have been reported and they can be triggered by different cellular factors to release H<sub>2</sub>S.<sup>12-14</sup> Figure 1 shows a few examples: GYY4137 and JK1 are pH-triggered donors. *N*-Benzoylthiobenzamides are thiol-triggered donors. A few enzyme-triggered donors are also reported, including the esterase-activated donor (HP-101), esterase and carbonic anhydrase (CA)-triggered COS/H<sub>2</sub>S donors, and nitroreductase-activated donors.<sup>15-17</sup> On the other hand, H<sub>2</sub>O<sub>2</sub> donors are much less developed, and researchers still tend to use H<sub>2</sub>O<sub>2</sub> directly in studies. However, it has been demonstrated that bolus

delivery of H<sub>2</sub>O<sub>2</sub> is often problematic due to rapid consumption of H<sub>2</sub>O<sub>2</sub> in biological systems such as in cells.<sup>18,19</sup> This justifies the need of slow and continuous H<sub>2</sub>O<sub>2</sub> releasing methods. Currently only a few hydroquinone and anthraquinone derivatives are reported as H<sub>2</sub>O<sub>2</sub> donors (Figure 1).<sup>20-22</sup>



**Figure 1.** Representative examples of synthetic H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> donors

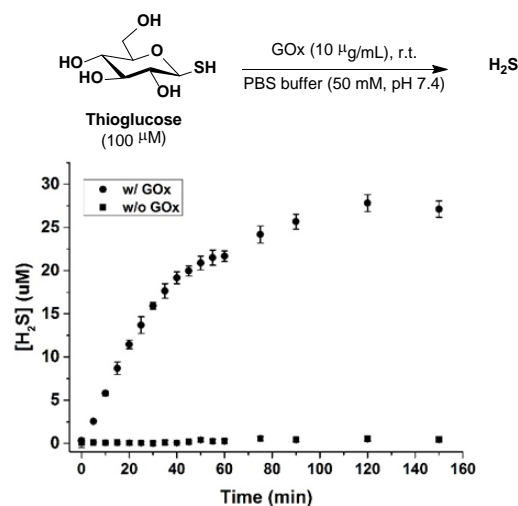
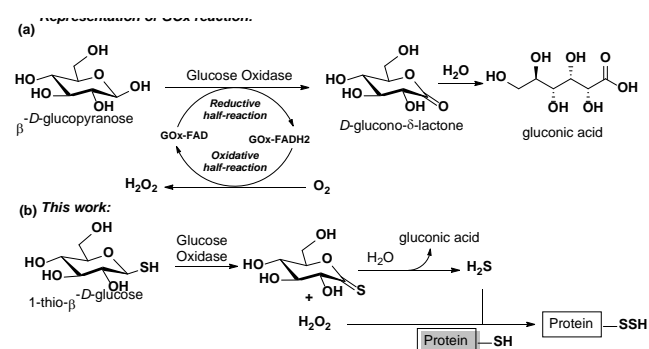
While these donor compounds have advanced our understanding of H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub>, there are still limitations: 1) All these donors are synthetic materials. Inevitably they will also produce significant amounts of organic byproducts (in addition to H<sub>2</sub>S or H<sub>2</sub>O<sub>2</sub>). This could cause unwanted side effects. 2) Enzyme-triggered donors (especially H<sub>2</sub>O<sub>2</sub> donors) are still very limited. 3) All available donors can only produce one species (H<sub>2</sub>S or H<sub>2</sub>O<sub>2</sub>), which can hardly mimic the concurrent presence of H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub>, nor allow the study of their crosstalk and synergistic effects. Herein, we would like to report a new H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> dual donor system employing thioglucose and GOx. This can produce H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> simultaneously in a slow and controllable fashion, without generating any bio-unfriendly organic byproducts. We also demonstrated this system can cause efficient S-persulfidation on proteins. Moreover, this system can be expanded to the disulfide of thioglucose and thiolactose. As such, additional factors can be induced to further control the release of H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> dual species.

## RESULTS AND DISCUSSION

Glucose oxidase (GOx) is a flavin containing oxidoreductase which catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone by O<sub>2</sub> gas with the production of H<sub>2</sub>O<sub>2</sub> (Scheme 1a).<sup>23</sup> Because of this property, glucose/GOx system has been used as an alternative for exogenous addition of H<sub>2</sub>O<sub>2</sub> in biological studies.<sup>19</sup> This system can induce hypoxia, oxidation stress, or enhance acidity in tumor microenvironment. These have been strategically utilized to achieve multimodal synergistic cancer therapy by integrating GOx with various therapeutic approaches.<sup>24</sup> From mechanistic point-of-view, if 1-

thio-β-D-glucose (thioglucose)<sup>25</sup> is used as the substrate for GOx, it would produce thio-gluconolactone as the product, which should easily undergo hydrolysis to release H<sub>2</sub>S. Therefore, we envisioned that thioglucose/GOx could be a unique enzyme triggered H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> dual releasing system (Scheme 1b). This is attractive as only non-toxic and biocompatible gluconic acid would accompany with the two species. We also envisioned that this dual release of H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> might be an efficient way to induce protein S-persulfidation. Furthermore, thioglucose moiety can be engineered as stimuli-response prodrugs to deliver H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> upon specific biologically relevant activation.

**Scheme 1.** The idea of using thioglucose/GOx to release H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> and induce protein S-persulfidation

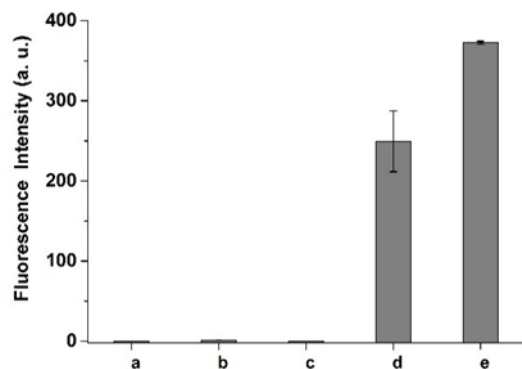


**Figure 2.** H<sub>2</sub>S release profile of thioglucose (100 μM) in the presence and absence of GOx (10 μg/mL) in PBS buffer (50 mM, pH 7.4). The experiments were performed in triplicate, and results are expressed as mean ± SD (*n* = 3).

With this idea in mind, we first evaluated H<sub>2</sub>S release from thioglucose in the presence of GOx. The standard methylene blue (MB) method was used to quantify H<sub>2</sub>S production. As shown in Figure 2, thioglucose itself was found to be stable in buffers and no obvious H<sub>2</sub>S release was detected in the absence of GOx. However, significant H<sub>2</sub>S release was observed when GOx was present. The optimized condition was identified as 100 μM thioglucose in PBS buffer (50 mM, pH 7.4) containing 10 μg/mL GOx.

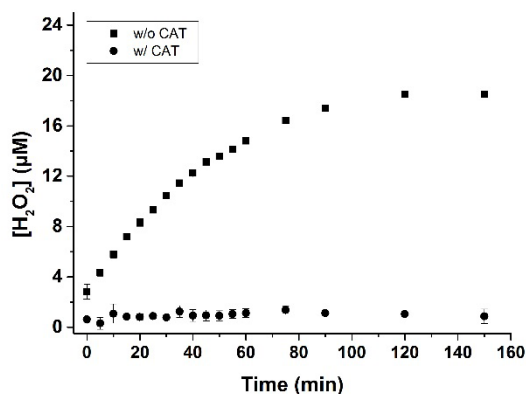
This combination led to slow, continuous, and time-dependent  $\text{H}_2\text{S}$  generation. The concentration of  $\text{H}_2\text{S}$  reached the maximum value of  $\sim 28 \mu\text{M}$  in about 2 h and then slowly decreased presumably due to volatilization of  $\text{H}_2\text{S}$  gas.

The  $\text{H}_2\text{S}$  formation was further verified by fluorescence measurements with a  $\text{H}_2\text{S}$ -specific probe WSP5.<sup>26</sup> As shown in Figure 3, the treatment of WSP5 with thioglucose or GOx alone did not give any detectable fluorescence. However, significant fluorescent signals were observed after incubating the probe ( $10 \mu\text{M}$ ) with the mixture of thioglucose ( $100 \mu\text{M}$ ) and GOx ( $10 \mu\text{g}/\text{mL}$ ) at room temperature for 1 h, indicating that thioglucose indeed produced  $\text{H}_2\text{S}$  in the presence of GOx.



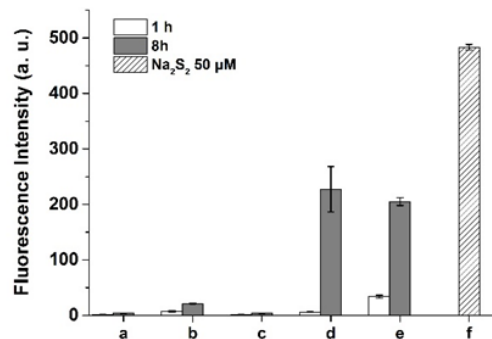
**Figure 3.**  $\text{H}_2\text{S}$  release from thioglucose ( $100 \mu\text{M}$ ) in the presence of GOx ( $10 \mu\text{g}/\text{mL}$ ) detected by WSP5 ( $10 \mu\text{M}$ ) in PBS buffer ( $50 \text{ mM}$ ,  $\text{pH } 7.4$ ). (a) WSP5 only, (b) WSP5 + thioglucose, (c) WSP5 + GOx, (d) WSP5 + thioglucose + GOx, (e) WSP5 +  $100 \mu\text{M}$   $\text{Na}_2\text{S}$ . The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

We next monitored the formation of  $\text{H}_2\text{O}_2$  by the ferrous oxidation–xylenol orange method (FOX 1 assay) under the optimized conditions described above. As shown in Figure 4, a time-dependent  $\text{H}_2\text{O}_2$  formation was observed with the peak concentration of  $\sim 20 \mu\text{M}$  at about 2 h. Additionally, if catalase (CAT) was present in this system (at  $50 \mu\text{g}/\text{mL}$ ), the produced  $\text{H}_2\text{O}_2$  was completely scavenged. The  $\text{H}_2\text{O}_2$  formation was also determined by fluorescence measurements with  $\text{H}_2\text{O}_2$ -sensitive Amplex Red/horseradish peroxidase (AP/HRP) assay (Figure S5). Interestingly, we noticed that the release of  $\text{H}_2\text{S}$  was not affected by the addition of CAT (Figure S2), indicating that thioglucose could be used as a clean  $\text{H}_2\text{S}$  donor if two enzymes (GOx and CAT) were applied.



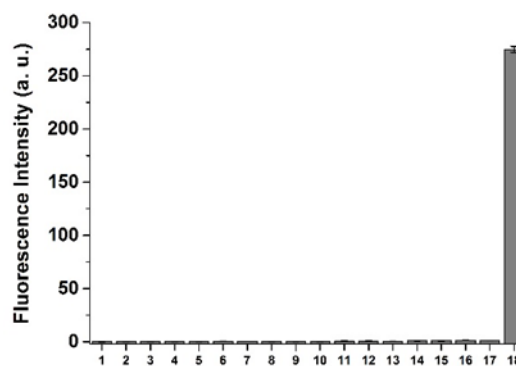
**Figure 4.** Time-dependent generation of  $\text{H}_2\text{O}_2$  from thioglucose ( $100 \mu\text{M}$ ) and GOx ( $10 \mu\text{g}/\text{mL}$ ) in PBS buffer ( $50 \text{ mM}$ ,  $\text{pH } 7.4$ ) in the presence and absence of CAT ( $50 \mu\text{g}/\text{mL}$ ). The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

The concurrent formation of  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  in this system could lead to a concern that  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  might react with each other so the release of  $\text{H}_2\text{S}$  won't be efficient. However, the results shown in Figures 2 and 4 clearly suggested this should not present a major problem due to the slow reaction between them, especially under biologically relevant concentrations. It is reported that the second-order rate constant of the reaction between  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  is  $0.73 \text{ M}^{-1} \text{ s}^{-1}$  ( $\text{pH } 7.4$ ,  $37 \text{ }^\circ\text{C}$ ), with the formation of a mixture of hydrogen polysulfide ( $\text{H}_2\text{S}_n$ ) as the possible products.<sup>27</sup> We also wondered if this system could produce  $\text{H}_2\text{S}_n$ . To test this, a specific fluorescent probe PSP-3<sup>28</sup> was used to monitor the generation of  $\text{H}_2\text{S}_n$ . As shown in Figure 5, negligible fluorescence signals were observed after 1 h incubation of the probe with thioglucose ( $100 \mu\text{M}$ ) and GOx ( $10 \mu\text{g}/\text{mL}$ ) in PBS buffer ( $50 \text{ mM}$ ,  $\text{pH } 7.4$ ) at rt. However, we did observe some fluorescence increases upon extending the incubation time to 8 h. We also compared this enzyme-generation system with directly mixing  $\text{H}_2\text{O}_2$  and  $\text{Na}_2\text{S}$ . Weak fluorescence signals were observed with the incubation of a mixture of  $\text{Na}_2\text{S}$  ( $100 \mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  ( $100 \mu\text{M}$ ) after 1 h while much stronger fluorescence was noted after 8 h. These results again suggested that  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  could react to form  $\text{H}_2\text{S}_n$  but that requires long reaction time. This should not be a concern when the thioglucose-GOx system was used in our regular conditions (e.g.  $\sim 1 \text{ h}$ ).



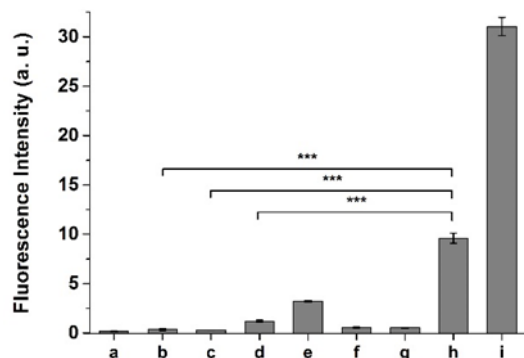
**Figure 5.** Detection of  $\text{H}_2\text{S}_n$  produced from thioglucose-GOx by PSP-3 (10  $\mu\text{M}$ ). Thioglucose (100  $\mu\text{M}$ ) was incubated with GOx (10  $\mu\text{g}/\text{mL}$ ) and PSP-3 in PBS buffer (50 mM, pH 7.4) for 1 h (white) and 8 h (grey), respectively. Fluorescence responses were recorded at 515 nm. (a) PSP-3 only, (b) PSP-3 + thioglucose (c) PSP-3 + GOx, (d) PSP-3 + thioglucose + GOx, (e) PSP-3 +  $\text{Na}_2\text{S}$  (100  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ), (f) PSP-3 +  $\text{Na}_2\text{S}_2$  (50  $\mu\text{M}$ ). The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

Our results thus far have demonstrated the thioglucose-GOx system under the optimized conditions could slowly and consistently produce  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  under biologically relevant concentrations. We next wondered if this system could be used to induce protein S-persulfidation, an important posttranslational modification mediated by  $\text{H}_2\text{S}$ .<sup>9,29</sup> This hypothesis was based on the knowledge that  $\text{H}_2\text{S}$  alone can hardly induce protein S-persulfidation (as the reaction between  $\text{H}_2\text{S}$  and protein disulfides is usually slow and less-productive).  $\text{H}_2\text{O}_2$  can oxidize thiols in human serum albumin (HSA-SH) with a second-order rate constant of 2.3-2.7  $\text{M}^{-1} \text{s}^{-1}$  (pH 7.4, 37  $^\circ\text{C}$ ),<sup>30</sup> leading to the formation of sulfenic acid HSA-SOH that can be easily converted to persulfide upon reacting with  $\text{H}_2\text{S}$ . However, this method needs very high concentrations of  $\text{H}_2\text{O}_2$  (4 mM) and  $\text{H}_2\text{S}$  (2 mM).<sup>31</sup> We expected that our slow and continuous  $\text{H}_2\text{S}/\text{H}_2\text{O}_2$  generation under low concentrations would have some advantages in inducing protein S-persulfidation. Before we tested this in proteins we decided to test if thioglucose-GOx would induce S-persulfidation on low molecular weight (LMW) biothiols (such as Cys and GSH). SSP4, a persulfide sensitive fluorescent probe,<sup>32</sup> was used to measure persulfidation. Fluorescence intensities were measured after incubation of the probe with each analyte in PBS buffer (50 mM, pH 7.4) at rt. As shown in Figure 6, SSP-4 was quite stable upon treatment with a series of substrates alone including thioglucose, GOx, Cys, GSH, as well as the mixtures of thioglucose/Cys, thioglucose/GSH, GOx/Cys, GOx/GSH (columns 1-9). No fluorescence was noted in these studies. In addition, negligible responses were observed in the mixture of thioglucose/GOx (column 10) and the direct mixture of  $\text{Na}_2\text{S}/\text{H}_2\text{O}_2$  under similar concentrations of the enzyme-promoted system (column 11). Furthermore, exposure of biothiols to thioglucose-GOx or the corresponding amounts of  $\text{Na}_2\text{S}/\text{H}_2\text{O}_2$  showed no fluorescence increase (columns 12-17). As a positive control, SSP4 showed high fluorescence response to  $\text{Na}_2\text{S}_2$  (column 18) a persulfide standard. These results clearly demonstrated that the thioglucose-GOx system does not induce effective persulfide formation on small molecular thiols.



**Figure 6.** Fluorescence responses of SSP4 toward various low molecular weight biothiols in the presence of thioglucose-GOx. SSP4 (10  $\mu\text{M}$ ) was incubated with different substrates in PBS buffer (50 mM, pH 7.4) for 1h, then the fluorescence responses were recorded at 515 nm. (1) SSP4 only, (2) SSP4 + thioglucose (100  $\mu\text{M}$ ), (3) SSP4 + GOx (10  $\mu\text{g}/\text{mL}$ ), (4) SSP4 + Cys (100  $\mu\text{M}$ ), (5) SSP4 + GSH (1 mM), (6) SSP4 + thioglucose (100  $\mu\text{M}$ ) + Cys(100  $\mu\text{M}$ ), (7) SSP4 + thioglucose (100  $\mu\text{M}$ ) + GSH (1 mM), (8) SSP4 + GOx (10  $\mu\text{g}/\text{mL}$ ) + Cys(100  $\mu\text{M}$ ), (9) SSP4 + GOx (10  $\mu\text{g}/\text{mL}$ ) + GSH(1 mM), (10) SSP4+ thioglucose (100  $\mu\text{M}$ ) + GOx (10  $\mu\text{g}/\text{mL}$ ), (11) SSP4 +  $\text{Na}_2\text{S}$  (30  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), (12) SSP4+ thioglucose (100  $\mu\text{M}$ ) + GOx (10  $\mu\text{g}/\text{mL}$ ) + Cys(100  $\mu\text{M}$ ), (13) SSP4+ thioglucose (100  $\mu\text{M}$ ) + GOx (10  $\mu\text{g}/\text{mL}$ ) + GSH(1 mM), (14) SSP4+ thioglucose (100  $\mu\text{M}$ ) +  $\text{Na}_2\text{S}$  (30  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), (15) SSP4 + GOx (10  $\mu\text{g}/\text{mL}$ ) +  $\text{Na}_2\text{S}$  (30  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), (16) SSP4+ Cys(100  $\mu\text{M}$ ) +  $\text{Na}_2\text{S}$  (30  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), (17) SSP4 + GSH (1 mM) +  $\text{Na}_2\text{S}$  (30  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (20  $\mu\text{M}$ ), (18) SSP4 +  $\text{Na}_2\text{S}_2$  (50  $\mu\text{M}$ ). The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

Next, we tested if the combination of thioglucose and GOx could lead to the formation of protein persulfides. In this study, reduced BSA (30  $\mu\text{M}$ ) was incubated with thioglucose (200  $\mu\text{M}$ ) and GOx (10  $\mu\text{g}/\text{mL}$ ) in PBS at room temperature for 1 h. CAT (25  $\mu\text{g}/\text{mL}$ ) was then added to remove excess  $\text{H}_2\text{O}_2$ . SSP4 (10  $\mu\text{M}$ ) was next applied to measure persulfide formation.<sup>33</sup> As demonstrated in Figure 7, this treatment (column h) led to an obvious increase in fluorescence, indicating the desired persulfide formation on BSA. Control experiments, e.g. BSA treated with each individual reagent used in the study (columns a-d, f, g), did not give significant fluorescence. We also tested a known protein persulfidation method using  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{S}$ . When the concentrations of  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{S}$  were similar to those of thioglucose-GOx system (e.g. 60  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 60  $\mu\text{M}$   $\text{Na}_2\text{S}$ , shown in column e), we only observe weak fluorescence. When much higher concentrations of the reagents were used (600  $\mu\text{M}$  BSA with 4 mM  $\text{H}_2\text{O}_2$  and 2 mM  $\text{Na}_2\text{S}$ ) we were able to observe strong fluorescent signals (column i). It should be noted that these high concentrations are unrealistic for real biological applications. Therefore, our results indicated that thioglucose-GOx is an efficient method to cause protein persulfidation under physiologically relevant  $\text{H}_2\text{O}_2/\text{H}_2\text{S}$  concentrations and SSP4 is suitable for the detection of protein S-persulfidation.

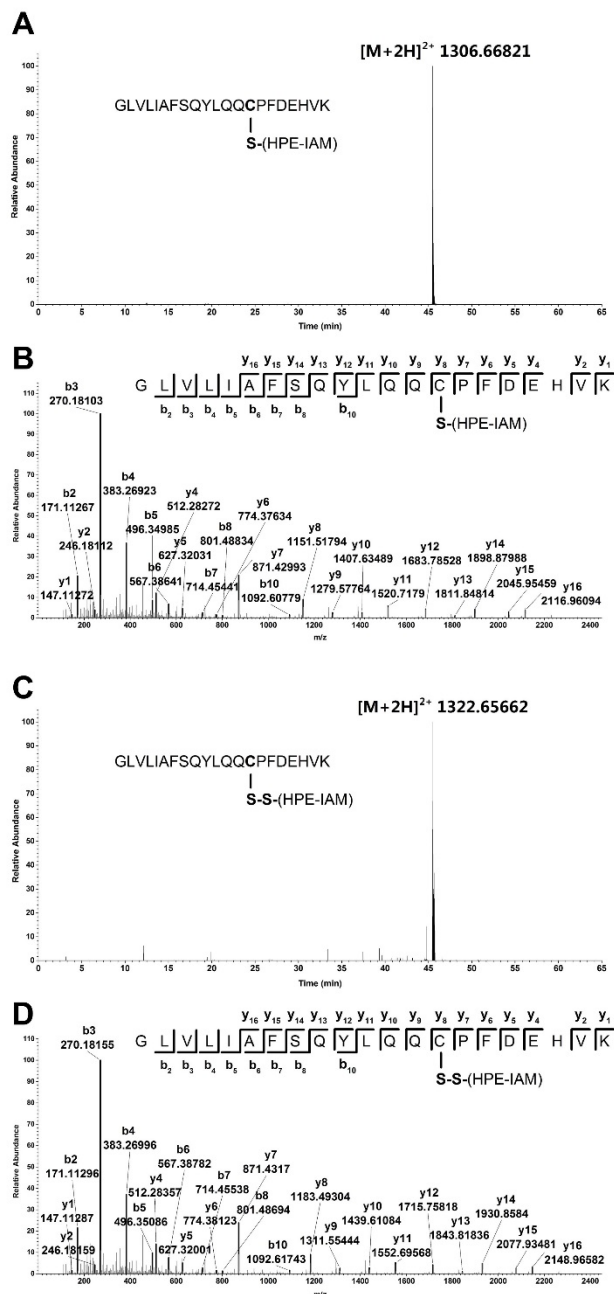


**Figure 7.** Thioglucose-GOx induced BSA S-persulfidation detected by SSP4. In these studies, 30  $\mu\text{M}$  reduced BSA and 10  $\mu\text{M}$  SSP4 were used. Fluorescence response was recorded at 515 nm. (a) SSP4 only, (b) SSP4 + BSA, (c) SSP4 + BSA +  $\text{Na}_2\text{S}$  (60  $\mu\text{M}$ ), (d) SSP4 + BSA +  $\text{H}_2\text{O}_2$  (60  $\mu\text{M}$ ) + CAT (25  $\mu\text{g}/\text{mL}$ ), (e) SSP4 + BSA +  $\text{Na}_2\text{S}$  (60  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (60  $\mu\text{M}$ ) + CAT (25  $\mu\text{g}/\text{mL}$ ) (f) SSP4 + BSA + thioglucose (200  $\mu\text{M}$ ), (g) SSP4 + BSA + GOx (10  $\mu\text{g}/\text{mL}$ ), (h) SSP4 + BSA + thioglucose (200  $\mu\text{M}$ ) + GOx (10  $\mu\text{g}/\text{mL}$ ) + CAT (25  $\mu\text{g}/\text{mL}$ ), (i) BSA (600  $\mu\text{M}$ ) +  $\text{H}_2\text{O}_2$  (4 mM) + CAT (25  $\mu\text{g}/\text{mL}$ ) +  $\text{Na}_2\text{S}$  (2 mM) + SSP4. The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ). Statistical analysis was performed using one-way ANOVA. \*\*\* $P < 0.001$ .

The formation of BSA persulfide was further confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Briefly, BSA was treated with thioglucose-GOx as described above. The resulted protein was then incubated with  $\beta$ -(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM, 10 mM) to block the -SH and -SSH. Controls using untreated BSA were also performed. Proteins were then digested with trypsin, and subjected to LC-MS/MS. As shown in Figure 8, in thioglucose/GOx treated samples the extracted ion chromatogram (XIC) signals clearly showed a substantial level of persulfide (-SSH) adduct on peptide GLVLIAFSQYLQQCPFDEHVK (Figure 8C). The site of persulfidation (Cys34) was confirmed by higher-energy collision dissociation (HCD) MS/MS (Figure 8D). Instead, the main observed peptide species containing Cys34 in untreated BSA was the Cys34-thiol (-SH) alkylated form (Figure 8A/B). In addition to BSA we also tested this thioglucose/GOx method with two other proteins-papain and GAPDH. Effective persulfidation was obtained on both proteins (Figure S8 and Figure S9 in the Supporting Information).

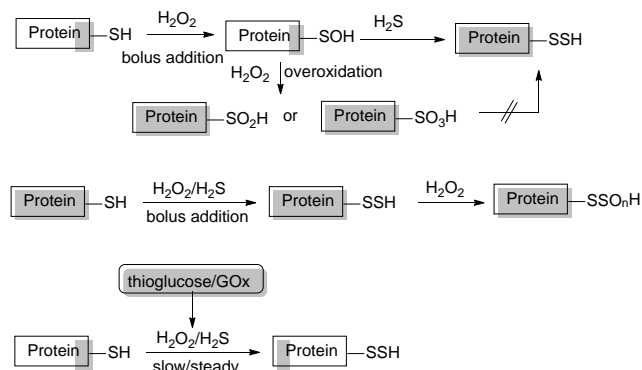
It was interesting to discover that the slow and steady production of  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  from thioglucose/GOx acted as a more efficient protein persulfidation system than direct addition of  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  even at similar concentrations. While the detailed mechanism is still unclear, we propose the following explanations (Scheme 2): In previous studies protein persulfidation was normally achieved by sequential treatments with high concentrations of  $\text{H}_2\text{O}_2$  and then  $\text{H}_2\text{S}$ . A problem with this method is overoxidation in the first step, which produces protein sulfinic acid (P-SO<sub>2</sub>H) or sulfonic acid (P-SO<sub>3</sub>H). These species cannot be converted to P-SSH by  $\text{H}_2\text{S}$ . This overoxidation may exist when BSA is treated with bolus

additions of  $\text{H}_2\text{O}_2/\text{H}_2\text{S}$  pair, which diminishes the efficiency of persulfidation. Another possibility is that excess  $\text{H}_2\text{O}_2$  would always exist in bolus additions and it could rapidly react with the newly formed protein persulfide and therefore, decreasing persulfidation. It is known that persulfides (RSSH) are highly reactive to  $\text{H}_2\text{O}_2$  to form RSSO<sub>n</sub>H.<sup>34,35</sup> On the other hand, the slow and steady  $\text{H}_2\text{O}_2/\text{H}_2\text{S}$  production from thioglucose/GOx may be able to avoid the presence of excess  $\text{H}_2\text{O}_2$  in the system, thus preventing the overoxidation reactions.



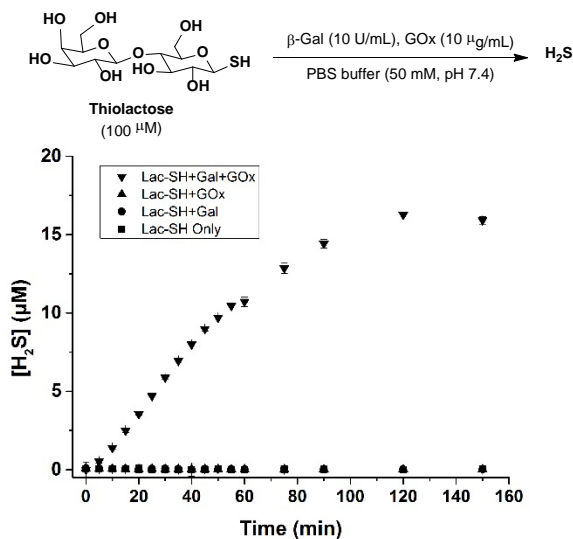
**Figure 8.** Extracted ion chromatograms (XIC) and MS/MS spectra of the HPE-IAM labeled Cys34 containing peptide (GLVLIAFSQYLQQCPFDEHVK) from BSA, with or without the treatment of thioglucose/GOx. (A) XIC of the -SH peptide, (B) MS/MS spectrum of the -SH peptide, (C) XIC of the -SSH peptide, (D) MS/MS spectrum of the -SSH peptide.

**Scheme 2.** Proposed explanations on the high efficiency of protein persulfidation by the thioglucose/GOx system



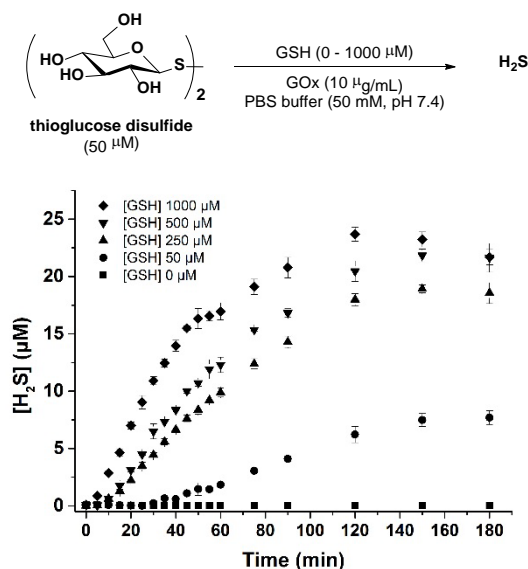
It was also interesting to see that thioglucose/GOx induced persulfidation worked effectively on proteins but not on small molecules. This could be attributed to two reasons: 1)  $\text{H}_2\text{O}_2$ -oxidation works less effectively on small molecule thiols as compared to proteins. We analyzed the total -SH contents in the reaction between Cys and thioglucose/GOx. As shown in Figure S7 in the SI, only minor thiol concentration decrease in this reaction was observed, suggesting Cys oxidation in this system was slow. 2) Even small molecule thiols react with  $\text{H}_2\text{O}_2$ , the resulted sulfenic acids (RSOH) are much more unstable than protein-derived sulfenic acids. They should further react with another molecule of thiol to form disulfide. As such, persulfide formation on small molecules in this system won't be feasible.

Having demonstrated that thioglucose-GOx is a controllable platform for  $\text{H}_2\text{S}/\text{H}_2\text{O}_2$  dual release under biologically friendly environments, we wondered if we could further tune the release ability with a dual enzyme system or multi-stimuli response. In this strategy, thioglucose could be considered as a caged  $\text{H}_2\text{S}/\text{H}_2\text{O}_2$  vehicle. Engineering this motif with another triggering group would enable their generation in response to specific biological stimuli. To test this hypothesis, we prepared 1-thio- $\beta$ -D-lactose (thiolactose) which could be hydrolyzed by  $\beta$ -galactosidase ( $\beta$ -Gal) to form galactose and thioglucose. As such, only under the dual-enzyme catalysis ( $\beta$ -Gal and GOx) it will produce  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$ . As shown in Figure 9, thiolactose was stable in PBS buffers. It did not release  $\text{H}_2\text{S}$  under the treatment of either  $\beta$ -Gal or GOx. However, in the presence of both  $\beta$ -Gal and GOx, a time-dependent  $\text{H}_2\text{S}$  formation was observed with the peak concentration of  $\sim 16 \mu\text{M}$  in about 2 h. The capability of  $\text{H}_2\text{O}_2$  delivery from this thiolactose-dual enzyme system was also confirmed by AP/HRP assay under the same conditions (Figure S10). It is worth noting that elevated lysosomal  $\beta$ -Gal activity has been well known as an important biomarker for senescent cells and primary ovarian cancers.<sup>36,37</sup> Also  $\text{H}_2\text{S}$  exhibits protective effects against cellular senescence and certain cancers.<sup>38,39</sup> Thus,  $\beta$ -Gal activated  $\text{H}_2\text{S}$  donors like thiolactose might be explored as potential anti-aging or anti-cancer agents.



**Figure 9.**  $\text{H}_2\text{S}$  release profile of thiolactose ( $100 \mu\text{M}$ ) in the presence of  $\beta$ -Gal ( $10 \text{ U/mL}$ ) and GOx ( $10 \mu\text{g/mL}$ ) in PBS buffer ( $50 \text{ mM}$ ,  $\text{pH } 7.4$ ). The experiments were performed in triplicate, and results are expressed as mean  $\pm$  SD ( $n = 3$ ).

To further demonstrate the tunability of the thioglucose-GOx platform, we next synthesized thioglucose disulfide. This symmetrical glucosyl disulfide is expected to undergo disulfide-bond cleavage induced by biothiols to form thioglucose, which could subsequently release  $\text{H}_2\text{S}$  and  $\text{H}_2\text{O}_2$  in the presence of GOx. To test this idea, thioglucose disulfide ( $50 \mu\text{M}$ ) was incubated with GSH ( $0$ - $1000 \mu\text{M}$ ) in PBS ( $50 \text{ mM}$ ,  $\text{pH } 7.4$ ) containing GOx ( $10 \mu\text{g/mL}$ ) and its  $\text{H}_2\text{S}$  production was monitored by MB assay. As shown in Figure 10, thioglucose disulfide did not produce detectable  $\text{H}_2\text{S}$  in the absence of GSH. However, in the presence of GSH, thioglucose disulfide exhibited dose- and time-dependent  $\text{H}_2\text{S}$ -releasing. The formation of  $\text{H}_2\text{O}_2$  was also confirmed using AP/HRP assay (Figure S11). These results indicate that thioglucose disulfide is an efficient thiol-activated  $\text{H}_2\text{S}/\text{H}_2\text{O}_2$  donor catalyzed by GOx.



**Figure 10.** H<sub>2</sub>S release profile of thioglucose disulfide (50 μM) in the presence of GSH (0 μM, 50 μM, 250 μM, 500 μM and 1000 μM) and GOx (10 μg/mL) in PBS buffer (50 mM, pH 7.4). The experiments were performed in triplicate, and results are expressed as mean ± SD (*n* = 3).

## CONCLUSIONS

Compounds that can release redox regulating molecules such as H<sub>2</sub>S and H<sub>2</sub>O<sub>2</sub> are not only useful research tools, but also potential therapeutic agents. While many such compounds have been reported, a common problem is that they also produce organic byproducts that could cause unwanted side effects. This problem should be considered in the development of next generation donor templates. In addition, current efforts in this field tend to focus on individual regulating species, which could miss the important crosstalk and synergistic effects between multiple species. Nevertheless, the co-existence of these species is real in nature but donors that can release multiple ROS/RSS are still unavailable. In this work we attempted to solve the aforementioned challenges by developing novel dual-releasing donor templates. We have demonstrated that thioglucose combined with GOx can serve as a new platform for controlled H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> dual release with no bio-unfriendly byproducts. The generation of H<sub>2</sub>O<sub>2</sub> in this system did not affect H<sub>2</sub>S release. This thioglucose-GOx system was used to effectively induce protein S-persulfidation. Moreover, thioglucose is a highly tunable motif and can be readily modified to induce additional release-control factors so other biologically relevant stimuli can be used to regulate the delivery of H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub>. We expect this thiosugar-GOx platform will be a useful tool for elucidating the mechanisms of H<sub>2</sub>S/H<sub>2</sub>O<sub>2</sub> signaling and promoting H<sub>2</sub>S based therapeutic applications.

## ASSOCIATED CONTENT

### Supporting Information.

Compound characterizations, experimental protocols, additional experimental data. The supporting information is free of charge via the Internet at <http://pubs.acs.org>.

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