Using resonance synchronous spectroscopy to characterize the reactivity and electrophilicity of biologically relevant sulfane sulfur

Huanjie Li¹, Huaiwei Liu²,³, Zhigang Chen¹, Rui Zhao¹, Qingda Wang³, Mingxue Ran¹, Yongzhen Xia¹, Xin Hu², Jihua Liu², Ming Xian³, Luying Xun¹,⁴,⁺

¹ State Key Laboratory of Microbial Technology, Shandong University, 72 Binhai Road, Qingdao, 266237, People’s Republic of China
² Institute of Marine Science and Technology, Shandong University, 72 Binhai Road, Qingdao, 266237, People’s Republic of China
³ School of Molecular Biosciences, Washington State University, Pullman, WA, 99164-7520, USA
⁴ Department of Chemistry, Washington State University, Pullman, WA, 99164-4630, USA

Abstract

Sulfane sulfur is common inside cells, playing both regulatory and antioxidant roles. However, there are unresolved issues about its chemistry and biochemistry. We report the discovery that reactive sulfane sulfur such as polysulfides and persulfides could be detected by using resonance synchronous spectroscopy (RS₂). With RS₂, we showed that inorganic polysulfides at low concentrations were unstable with a half-life about 1 min under physiological conditions due to reacting with glutathione. The protonated form of glutathione persulfide (GSSH) was electrophilic and had RS₂ signal. GSS⁻ was nucleophilic, prone to oxidation, but had no RS₂ signal. Using this phenomenon, pKa of GSSH was determined as 6.9. GSSH/GSS⁻ was 50-fold more reactive than H₂S/HS⁻ towards H₂O₂ at pH 7.4, supporting reactive sulfane sulfur species like GSSH/GSS⁻ may act as antioxidants inside cells. Further, protein persulfides were shown to be in two forms: at pH 7.4 the deprotonated form (R-S⁻) without RS₂ signal was not reactive toward sulfite, and the protonated form (R-SH) in the active site of a rhodanese had RS₂ signal and readily reacted with sulfite to produce thiosulfate. These data suggest that RS₂ of sulfane sulfur is likely associated with its electrophilicity. Sulfane sulfur showed species-specific RS₂ spectra and intensities at physiological pH, which may reveal the relative abundance of a reactive sulfane sulfur species inside cells.

1. Introduction

Hydrogen sulfide (H₂S) is a new gasotransmitter that serves many important regulatory roles in biological systems [1]. H₂S is involved in vascular homeostasis, neurological function, cytoprotection, anti-inflammation, and revascularization [1–3]. However, accumulating evidences imply that H₂S is converted to reactive sulfane sulfur, which plays the observed roles [4–6]. Reactive sulfane sulfur includes organic persulfides (R-SSSH), organic polysulfides (R-SSnR or R-SSnH, n ≥ 2), and inorganic hydrogen polysulfides (H₂Sn, n ≥ 2) [7]. Reactive sulfane sulfur is different from thiols, as it often possesses both nucelophilic and electrophilic characteristics while thiols mainly function as nucleophiles [8]. The reactive sulfane sulfur can be produced from specific and nonspecific enzymatic oxidations of H₂S [9,10] or from the metabolism of cysteine and N-Acetyl cysteine (NAC) [11–13]. GSSH is a key form of reactive sulfane sulfur in the sulfide oxidation pathway of heterotrophic bacteria and human mitochondria [14,15]. Reactive sulfane sulfur can modify cysteine residues in a large number of proteins by S-persulfidation (R-SSSH), which can alter enzyme activity and influence biological processes via signaling [13,16]. For instance, rhodanese (thiosulfate:cyanide sulfurltransferase) that is present in almost all living organisms catalyzes the transfer of the sulfane sulfur from thiosulfate to cyanide via an intermediate (R-SSSH) at its catalytic Cys residue [17,18]. Collectively, previous reports have revealed the significance of reactive sulfane sulfur in biological processes. Thus, a better understanding of the chemical and biochemical properties of biologically relevant reactive sulfane sulfur will help to advance the field [19,20].

Current methods used for the detection of reactive sulfane sulfur include sulfur chemiluminescence detection, ion chromatography, HPLC analysis of the monobromobimane derivative of H₂Sn, and the use of H₂Sn-sensitive fluorescent dyes in living cells or in vitro [5,7,21]. Gao et al. developed some fluorescent probes that serve as an effective imaging tool for tracing or monitoring concentration changes of endogenous sulfane sulfur [22,23]. All of these methods are reaction-based. A reaction-free method that can real-time probe reactive sulfane sulfur has not been developed. Here, we report the discovery that reactive sulfane sulfur can be detected via resonance synchronous spectroscopy (RS₂) with a conventional spectrorfluometer by simultaneously scanning the excitation and emission (i.e. Δλ = λem–λex) [24]. This method is simple, fast, and nonintrusive for reactive sulfane sulfur analysis, allowing us to distinguish the protonated and unprotonated reactive sulfane sulfur species.

*Corresponding author. State Key Laboratory of Microbial Technology, Shandong University, 72 Binhai Road, Qingdao, 266237, People’s Republic of China.
**Corresponding author.
E-mail addresses: lihuaiwei@email.sdu.edu.cn (H. Liu), luying.xun@vetmed.wsu.edu (L. Xun).

https://doi.org/10.1016/j.redox.2019.101179
Received 18 January 2019; Received in revised form 21 March 2019; Accepted 24 March 2019
Available online 26 March 2019
2213-2317/ © 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/BY/4.0/).
deprotonated forms of persulfides and their reactivity.

2. Materials and methods

2.1. Materials and reactive sulfane sulfur preparations

Sodium hydrosulfide (NaHS), reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine, cystine, thiosulfate, tetrathionate, bis[3-(triethoxysilyl)propyl] tetrasulfide (Tsp-SSSS-Tsp), bis(prop-2-en-1-yl) tetrasulfide (Pey-SSSS-Pey) were purchased from Sigma-Aldrich; di-methyl trisulfide (Me-SSS-Me) was purchased from TCI Company (Shanghai). Preparation of H2Sn, existing as HSnH, HSn−, and Sn2− depending on the pH, was performed as a previous report [10]. GSSG was prepared by following the protocol of Moutiez et al. [25]. Glutathione persulfide (GSSH) was obtained via reacting GSSG with sul- fide, following the protocol of Luebbe et al. [26]. The obtained products were confirmed by HPLC-fluorescence and MS analysis.

2.2. RS2 analysis of reactive sulfane sulfur

RF-5301 PC Spectrofluoroumo Photoimeter (SHIMADZU) was used to measure the fluorescence. Sample was diluted into 2ml argon-deox- ygenated buffers (Tris-HCl 50 mM, pH 7.4) in a parafilm-sealed fluo- rometer cell (d = 1 cm). Cluster 5 chemicals were dissolved in acetone to make a 100 mM stock and then diluted into argon-deoxygenated buffer. RS2 was acquired by simultaneously scanning the excitation (λex) and emission (λem) on monochromators setting the offset (Δλ = λem−λex) to a constant [27]. All spectra were acquired with a scan rate of 60 nm/min. The measurement interval was 1.0 nm and slit width was 5 nm. For pH relevant RS2 analysis, the concentrations of reactive sulfane sulfur were carefully selected to let the RS2 intensities fell into the detection range of RF-5301. Known amounts of H2Sn and GSSH were dissolved in 20 ml of 50 mM Tris-HCl solutions (pH 7.4) and 20 mM sodium phosphate solution (pH 6), respectively, and then were titrated with 500 mM NaOH via 10-μl additions. The solution mixture was vortexed, followed with pH measurement and RS2 acquisition. The RS2 intensities were used for determining pKa.

2.3. pKa determination method

The average signal intensities of GSSH (375 nm–384 nm) and DUF442-C344-SH (444 nm–453 nm) were used for determining their pKa values, respectively. The pKa calculation equation is deduced as below:

\[
\text{RS2}_2 (I_{\text{RS2}_2\text{obs}}) = \frac{I_{\text{RS2}_2\text{obs}}}{I_{\text{RS2}_2}} = \frac{\lambda}{\lambda_{\text{RS2}}} = \frac{L_{\text{RS2}}}{L_{\text{RS2}}}.
\]

ForRSSH/RS2− mixture, the R2S2 is equal to that of fully protonated form (RS2+) times the fraction of its protonated form (fR).

\[
I_{\text{RS2}} = I_{\text{RS2}_2\text{obs}} \times f_{R}.
\]

This equation can be rewritten as follow:

\[
I_{\text{RS2}} = I_{\text{RS2}_2\text{obs}} \times \text{RS2}/(\text{RSSH} + \text{RS}^{2−})
\]

According to the Henderson-Hasselbalch equation, p is hill slop; the fill status is succeeded (100).

\[
\text{RSSH}/\text{RS}^{2−} = 10^p (\text{pKa} − \text{pH}) \times p
\]

Substituting the right-hand side of eq. (4) into eq. (3), we obtain:

\[
\text{RS2}_1 = \text{RS2}/(1 + 10^p (\text{pH} - \text{pKa})) \times p
\]

The detected RS2 intensity data of RSSH at different pH values were fitted with eq. (5) to obtain the pKa value.

2.4. 1H NMR and 13C−1H HMQC analysis

The 1H NMR spectra were recorded on a Bruker spectrometer at 600 MHz with a 5-mm probe. 13C−1H HMQC spectra were recorded on the Bruker spectrometer at 600 MHz with a 5-mm-gradient salt-tolerant H/C probe. The pulse sequence was set according to a previous report [28]. Delay = 1.5s, Size of fid = 1024, Number of scans = 64. The NMR data were processed and analyzed with Mestrelab Manova version 10.

2.5. Chemical reactions analysis

For RS2 analysis of GSSH disproportionation, 50 μM of GSSH was transferred into 20 mM sodium phosphate buffer of different pH, and RS2 was measured at selected time points as mentioned in the text. The reaction mixtures were also analyzed by HPLC-fluorescence and MS analysis.

For kinetics analysis, reactions were conducted in a fluorometer cell (d = 1 cm) sealed with parafilm. Reactions of H2Sn with GSSH were performed in deoxygenated HEPES buffer (100 mM, pH 7.4), started by adding 200 μM−5 μM of GSSH to 10 μM of H2Sn. RS2 of 535 nm−545 nm was scanned immediately at 30-s intervals for 3 min. Reactions of RS2 with GSSH were performed in deoxygenated HEPES buffer (100 mM, pH 7.4), started by adding 10 mM−20 mM of GSSH to 500 μM of RS2. RS2 of 535 nm−545 nm was scanned at 1-min intervals for 8 min. The kobs value was calculated by plotting the ln(1−fR) value against the reaction time. The apparent 2nd-order reaction rate constant k was calculated with the formula: kobs = k × [GSSH]. For H2S release detection, these reactions were performed in sealed tubes. Lead acetate papers were fixed in the gas phase of the tubes containing the reaction mixture. Reactions of DTT with H2Sn and RS2 were similar to the GSH reaction above, and the calculations were also similar. Reactions of antioxidants (H2S or GSSH) with H2O2 were conducted in deoxygenated HEPES buffer (100 mM, pH 7.4), started by adding 50 μM−500 μM of the antioxidant to 50 μM of H2O2. H2O2 reacted with GSSH to generate GSSG [29], which had RS2. The RS2 (535−545 nm) intensity of GSSG was obtained and used to calculate the reaction rate. H2O2 reacted with H2S to generate H2S2, which displayed RS2, and the RS2 increase was used to obtain the reaction constant. The kobs value was calculated by plotting the ln(1−fR) value against the reaction time. The apparent 2nd-order reaction rate constant k was calculated using the formula: kobs = k × [antioxidant].

2.6. Protein purification and modification with GSSH

The DUF442 domain of SQD (GenBank accession number: AA262946.1) was cloned from Cupravidus pinatubonensis JMP134. Site-directed mutagenesis was performed according to a revised method [30]. For protein expression, these genes were ligated into the pET30a vector with a His tag at the C-terminus and then expressed in Escherichia coli BL21 (DE3) (Table S1). The recombinant E. coli was grown in LB at 30 °C with shaking until OD600nm Reached about 0.6, and 0.3 mM IPTG was added; the cells were further cultivated at 20 °C for 20 h. Cells were harvested and disrupted with crusher SPCH-18 (STANSTED); protein purification was carried out with nickel-nitrilotriacetic acid agarose resin (Invitrogen). Buffer exchange of the purified proteins was performed via PD-10 desalting column (GE Healthcare). The finally obtained protein was in HEPES buffer (25 mM, pH 8.0) containing 300 mM NaCl.

The purified protein (6.0 mg/ml) was mixed with 200 μM of GSSH in HEPES buffer (100 mM, pH 7.4). After incubated at 25 °C for 20 min, the mixture was loaded onto a PD-10 desalting column to remove small molecules. The re-purified protein was then subjected to LC-MS/MS, RS2 or sulfite reaction analysis. For RS2 analysis, the protein was diluted to 0.1−0.5 mg/ml in the HEPES buffer so that the RS2 intensities were within the detection range of our fluorometer (RF-5301). For protein-
SSH pKa determination, we diluted DUF442-C34-SH (the C94S mutant) and GSSH reacted-DUF442-C34-SH in HEPES buffers of different pH (3, 3.5, 4, 4.5 ... 6.5, 7, 7.4), and then detected their RS2 intensities. The pK\textsubscript{a} was determined using Eq. (5). Titrating HCl solution into a protein solution may cause protein denaturation.

2.7. HPLC-fluorescence and MS analysis of persulfides

LC-fluorescence and MS analysis of GSSH and protein-SSH was performed by following a previously reported protocol [10]. Briefly, samples were derivatized with monobromobimane (mBBr) and were injected onto a C18 reverse phase column (VP-ODS, 150 × 4 mm, Shimadzu). The column was maintained at 30 °C and eluted with a gradient of solution A (0.25% acetic acid) and solution B (0.25% acetic acid and 75% methanol) in distilled water from 5% B to 70% B in 8 min, 70% B for 8 min, 100% B for 8 min at a flow rate of 0.8 ml/min. The fluorescence detector (LC-20A) was used for detection with excitation at 370 nm and emission at 485 nm. The ESI mass spectrometer (Ultimate 3000, Burker impact HD) was used with the source temperature at 200 °C and the ion spray voltage at 4.5 kV. Nitrogen was used as the nebulizer and drying gas.

2.8. HPLC analysis of H\textsubscript{2}S\textsubscript{2}

H\textsubscript{2}S\textsubscript{2} (5 mM) was diluted in Tris-HCl buffer at different pH, derivatized with methyl trifluoromethanesulfonate (methyl triflate) and analyzed by reversed-phase liquid chromatography using a C18 reverse phase column (VP-ODS, 150 × 4 mm, Shimadzu) and eluted with pure methanol. HPLC analysis and peak position of dimethylpolysulfides from Me\textsubscript{2}S\textsubscript{2} to Me\textsubscript{2}S\textsubscript{8} and S\textsubscript{8} were found from calibration curves according to a published protocol [31].

2.9. Bioinformatics analysis and protein structure modeling

The three-dimensional structure of DUF442 was generated by SWISS-MODEL (http://swissmodel.expasy.org/) and analyzed by PyMOL-1.5.0.3. Rhodanese from Neisseria meningitidis z2491 (PDB ID: 2F4G) at 1.41 Å resolution was chosen as the template (39% sequence similarity). The global QMEAN score was −0.56 for the DUF442 model. Their catalytic cysteine residues and their nearby residues also showed high reliability scores. The surface electrostatic potentials were analyzed by APBS-1.1.0, and the data and parameters were obtained with the PDB2PQR server (http://nbcr-222.ucsd.edu/pdb2pqr 2.1.1/).

2.10. Detection of sulfane sulfurs by using SSP4

Reactions of GSSH with SSP4 (Sulfane Sulfur Probe 4, Dojindo China Co., Ltd) were conducted by mixing 10 μM of SSP4 with 20 μM of GSSH in 100 μl of HEPES (0.1 M) buffer at different pH. The mixture was incubated at room temperature for 30 min, and then the fluorescence was detected by using Synergy H1 microplate reader. The excitation wavelength was set at 482 nm and the emission wavelength was set at 515 nm.

2.11. Whole cell analysis by RS2

Wild-type E. coli BL21 and recombinant E. coli strain containing pBBR1-CpSQR were used for intracellular polysulfides analysis. The strain was incubated at 37 °C until OD\textsubscript{600nm} reached about 0.6 in LB medium. To induce CpSQR expression, 0.3 mM IPTG was added, and the cells were further cultivated at 30 °C for 5 h. Cells were collected by centrifugation and washed twice with Tris buffer (50 mM, pH 7.4). Different concentrations of NaHS were added to cell suspension of 0.1 OD\textsubscript{600nm}. H\textsubscript{2}S oxidation was performed at 30 °C for 40 min. Then cells were collected and washed with Tris-HCl buffer (50 mM, pH 7.4). For RS2 analysis, cell intensity was adjusted to 0.01 OD\textsubscript{600nm} in Tris-HCl buffer. Wild-type E. coli BL21 was incubated in LB medium at 37 °C. Cells were collected, washed twice with Tris-HCl buffer (50 mM, pH 7.4), and resuspended to 0.01 OD\textsubscript{600nm} before RS2 analysis.

2.12. Whole cell analysis by SSP4

Wild-type E. coli BL21 cells were collected, washed twice with PBS buffer, and resuspended with PBS at 0.1 OD\textsubscript{600nm}. SSP4 (10 μM) and CTAB (0.5 mM) were added to the cell suspension and incubated for 15 min at room temperature. After centrifugation (4000 rpm, 5 min), the supernatant was discarded and remaining cells were washed twice with PBS buffer. The cells were diluted to 0.1 OD\textsubscript{600nm} in PBS buffer. The fluorescence was analyzed by using Synergy H1 microplate reader.

3. Results

3.1. Discovery of strong RS2 in H\textsubscript{2}S\textsubscript{2} and RSnR compounds

When analyzing H\textsubscript{2}S\textsubscript{2} using resonance synchronous spectroscopic (RS2) [21], we found that it had strong fluorescence intensity. Then we set the offset (Δλ = λ\textsubscript{ex}−λ\textsubscript{em}) to a constant between the excitation and detection wavelength, i.e. Δλ = 0, 1, 2 ... 6 nm to scan the sample. The fluorescence intensity was the highest when Δλ = 1 nm and decreased along with Δλ increased. Thus, Δλ = 1 nm was used for all analyses. Distilled water and 50 mM Tris-HCl had low RS2, and we used the Tris-HCl or HEPES buffer (pH 7.4) for most analyses (Fig. 1A). To test whether it is a common property of sulfur-containing compounds, we totally analyzed 14 sulfur-containing chemicals that were sorted into 7 clusters (Table 1). Among them, clusters 2 and 3 are important cellular persulfides and polysulfides; cluster 5 contains diallyl polysulfides (RS\textsubscript{n}R); other clusters are not polysulfides but are all involved in polysulfide metabolism. All chemicals were tested at pH 7.4. In addition to H\textsubscript{2}S\textsubscript{2}, Bis[3-(trithioxylsilyl)]propyl, tetrasulfide (Tsp-SSSS-Tsp) and dimethyl trisulfide (Me-SSS-Me) also showed significant RS2 (Fig. 1A).

We diluted different concentration of H\textsubscript{2}S\textsubscript{2} and cluster 5 compounds to analyze the RS2 intensity. The RS2 detection range was 0.2 μM–20 μM for H\textsubscript{2}S\textsubscript{2} at pH 7.4 and 10 μM–2 mM for cluster 5 compounds (Me-SSS-Me, 100 μM–2 mM; Tsp-SSSS-Tsp, 10 μM–80 μM). To remove the interference of the buffer, the ratiometric resonance synchronous spectroscopy (RS2) value was obtained via dividing the sample RS2 intensity (IRS2\textsubscript{sol}) with the solvent RS2 intensity (IRS2\textsubscript{sol}) (Eq. (1) in methods) (Fig. 1) [27].

The RS2 intensity of H\textsubscript{2}S\textsubscript{2} showed good responses to its concentrations and followed linear dependence at fixed pH values (Fig. 1B, Figs. S1A and S1B). Me-SSS-Me and Tsp-SSSS-Tsp also showed good responses to its concentrations and followed linear dependence (Fig. 1C and D). For RS2 detection of GSSSG, we only showed the range ≤0.025 μM–0.5 μM, but the response is linear up to 5 μM or higher (Fig. S1C). These results indicated RS2 is not a common property of all sulfur-containing chemicals, but a particular property of some chemicals that contain multiple sulfur atoms (n ≥ 2).

3.2. The pH effect on RS2 of reactive sulfane sulfur and its applications

Since GSSH is a pivotal intermediate in cellular reactive sulfane sulfur metabolism [29], it was a surprise that RS2 of GSSH was hardly detectable at pH 7.4 (Fig. 1A). When we analyzed GSSH at different pH, it showed clear RS2 at lower pH, especially at pH ≤ 6.0 (Fig. 2A). The highest peak was around 300 nm, which is consistent with its absorbance peak at 300 nm [10].

We then used RS2 to determine pK\textsubscript{a} of GSSH/GSSG. GSSH was dissolved in 20 ml aliquots of 50 mM Tris-HCl solution (pH 6). The solution was titrated with NaOH, followed with pH measurement and RS2 acquisition (375 nm–384 nm). The pK\textsubscript{a} value was determined as 6.9 via data fitting by using the Henderson-Hasselbach derived equation (Fig. 3B). GSSH can react with non-fluorescent SSP4 to release
When GSSH and SSP4 were mixed at different pH, the reaction was rapid at pH 6 but not at pH 9.5 (Fig. S2). The results were logical from a chemical perspective, as GSSH is the electrophile and SSP4 is the nucleophile in the reaction. GSS$^-$ should be more nucleophilic, while GSSH should be more electrophilic. Therefore, the reaction of SSP4 with GSSH should be faster than that with GSS$^-$.

To determine the reaction rates at different pH, the data were fitted with the Henderson-Hasselbach derived equation to obtain the estimated pK$_a$ of GSSH. The value was 6.9, the same as that determined via R$_2$S$_2$ (Fig. 3C). Interestingly, the SSP4 reaction rates and R$_2$S$_2$ intensities at different pH were highly correlated (Fig. 3D), indicating that RS$_2$ correlates with the electrophilicity of GSSH.

The pH change did not show apparent effect to RS$_2$ of class 5 chemicals (RS$_n$R) (data not shown). This is expected as R$_n$R compounds have no conditional protonation issues. When H$_2$Sn in Tris buffer at pH 7.4 was titrated with 500 mM NaOH, the RS$_2$ intensity decreased and reached the lowest level at pH around 8.5. However, RS$_2$ increased again when more NaOH was added into the H$_2$Sn solution. To confirm those results, we diluted H$_2$Sn in Tris buffers of different pH (7.4, 8.0, 8.5, 9.0, and 10). The RS$_2$ intensity was high at pH 7.4 and low at pH 8.5 (Fig. 2B); RS$_2$ increased again at pH 9.0 and pH 10, but the spectrum changed similarly to that of Tsp-S$_4$-Tsp (Fig. 1D). When H$_2$Sn...
Fig. 2. The pH effect on RS₂ analysis of GSSH and H₂Sn. (A) GSSH showed strong RS₂ at low pH. (B) The RS₂ intensity of H₂Sn could be affected by different pH range. GSSH (20 μM) and H₂Sn (5 μM) were diluted in 20 mM phosphate buffer (pH 5.0, 6.0, 7.0, 8.0) or 50 mM Tris buffer (7.4, 8.0, 8.5, 9.0, 10.0). The buffers had the same low RS₂ intensity as that of water (data not shown). The RF-5301 PC spectrofluorophotometer was used for all analyses.

Fig. 3. pKₐ determination of GSSH by using RS₂ and reaction with SSP4. Deduction of the equation is shown in Method 2.3. (A) Solution pH as a function of titrant volume (μl) for 20 ml of 20 μM GSSH. (B) The RS₂ values of the GSSH solution at different pH. The data were used to calculate pKₐ of GSSH. (C) The fluorescence of SSP4 after reacting with GSSH at different pH. The data also used to calculate pKₐ of GSSH. (D) Electrophilicity shown as relative SSP₄-induced fluorescence (R.S.F) after 30 min of reaction and RS₂ values of GSSH are well correlated.
was derivatized and analyzed by HPLC, the chain length distribution at various pH corresponded well to the calculated equilibrium distribution of polysulfide ions in aqueous solutions of different pH [31]. At pH 7.4, most H2Sn was detected as Sn, and a small peak of S2− was also detectable (Fig. S3A). At low concentrations such as 10 μM, H2S2/HS2− has been detected as the dominant species [10,32]. With pH increased to 8.0 and 8.5, Sn gradually decreased, and S2−, S−, S2−, S4−, S7−, and S8− were all detectable with S2− being the main species (Fig. S3B). At pH 9.0 and 10.0, Sn became a minor species, and Sα−, Sβ−, Sγ−, and Sδ− were the dominant species (Fig. S3A). Large portions of Sn were detected in most samples except at pH 10 (Fig. S3), and we believe that this is likely due to the high concentration (5 mM) of H2Sn used in the test for UV detection. Nonetheless, the variations in chain lengths associated with pH changes prevent us using RnSnR to determine the pKa value of H2Sn. The data also suggest that the RnSnR spectra of H2Sn depend on protonation as well as on the chain lengths (Fig. 2B). The chain length of H2Sn detected here should reflect the length in the solutions, as the method is optimized to ensure the derivatization reaction was fast enough to minimize chain elongation reactions [31]. However, if the derivatization step is low and if the alkylation agent reacts with the sulfane sulfur in the middle of polysulfides, some conversion reactions could occur, which interferes with the chain length detection [32].

3.3. RS2 of RnSnR may correlate with the presence of thiosulfoxide

RSnR contains sulfane sulfur that may tautomerize to a thiosulfoxide bond (sulfur-sulfur double bond, e.g., R2S = S) [33]. We hypothesized that RS2 of RnSnR may correlate with the presence of thiosulfoxide. So we analyzed the structures of Pey-SSSS-Pey and Tsp-SSSS-Tsp by using 1H NMR and 13C−1H HMQC spectroscopy [13C−1H HMQC]. In 13C−1H HMQC spectra, the two −CH2− groups connecting to sulfur atoms in Pey-SSSS-Pey (Cn and Cn+1) showed two distinguishable peaks, while those of Tsp-SSSS-Tsp showed three (Fig. 4, Fig. S4 and Fig. S5), suggesting Cn and Cn+1 are not symmetrical. In 1H NMR spectroscopy, protons linked to Cn and Cn+1 had two or more groups of peaks, while those linked to other Cs did not (Fig. S6 and Fig. S7). These results indicated the four sulfur atoms in these compounds are not linear and isomers containing the branched thiosulfoxide bond (> S=S) should exist, which causes the asymmetric configuration of Cn and Cn+1. The branched thiosulfoxide bond might lead to the generation of sulfane sulfur, and the RS2 observed from cluster 5 chemicals might be caused by the presence of thiosulfoxide.

Table 2

| Reactions | 2nd-order rate constant \((M^{−1}s^{−1})\) |
|----------------|------------------|
| RS2 + DTT → DTT-red + RS2− + H2S | 0.52 |
| H2Sn + GSH → GSSG + H2S | 0.89 |
| H2Sn + DTT → oxidized DTT + H2S | 1.16 |
| GSSG + H2O2 → GSSG + 2H2O | 23.76 |
| 2H2S + H2O2 → 2H2O + 2OH− | 0.46 |

*The reactions were conducted at 25°C and pH 7.4. The GSSH preparation contains equal molar GSH; the reaction products are primarily GSSG and GSSG [29].

3.4. Analysis of reaction kinetics by using RS2

We used RS2 as a real-time probe in assays of reactive sulfane sulfur-involved reactions (Table 2). First, we tested the stability of RSnR chemicals (Me-SSS-Me and Tsp-SSSS-Tsp) in the presence of 100 mM GSH at pH 7.4 (100 mM HEPES). The RS2 spectra of RSnR were unchanged, and there was no H2S released from the solution. Thus, RS2 is rather stable. Second, we tested the reaction of H2Sn with GSH in deoxygenated HEPES buffer (100 mM, pH 7.4). After adding GSH (200 μM–5 mM) to 10 μM of H2Sn, the RS2 spectra of H2Sn quickly decreased, and H2S was released. At low H2Sn concentrations and at pH 7.4, H2S2 is the dominant species [10]. By recording the RS2 decreases, we determined the 2nd-order rate constant of the reaction between H2Sn and GSH as 0.89 M−1 s−1 (Table 2). Because GSH is at least two-orders-of-magnitude higher than H2Sn [14,29,34], the reaction between H2Sn and GSH should occur in the pseudo-first-order manner (e.g., t1/2 = 78 s) at the physiological pH and GSH concentration. Third, because RS2 has limitations, we could not use it to determine GSSH reduction at pH 7.4 due to its low RS2 signal. We used RS2 as a real-time probe in assays of reactive sulfane sulfur-involved reactions (Table 2). First, we tested the stability of RSnR chemicals (Me-SSS-Me and Tsp-SSSS-Tsp) in the presence of 100 mM GSH at pH 7.4 (100 mM HEPES). The RS2 spectra of RSnR were unchanged, and there was no H2S released from the solution. Thus, RS2 is rather stable. Second, we tested the reaction of H2Sn with GSH in deoxygenated HEPES buffer (100 mM, pH 7.4). After adding GSH (200 μM–5 mM) to 10 μM of H2Sn, the RS2 spectra of H2Sn quickly decreased, and H2S was released. At low H2Sn concentrations and at pH 7.4, H2S2 is the dominant species [10]. By recording the RS2 decreases, we determined the 2nd-order rate constant of the reaction between H2Sn and GSH as 0.89 M−1 s−1 (Table 2). Because GSH is at least two-orders-of-magnitude higher than H2Sn [14,29,34], the reaction between H2Sn and GSH should occur in the pseudo-first-order manner (e.g., t1/2 = 78 s) at the physiological pH and GSH concentration. Third, because RS2 has limitations, we could not use it to determine GSSH reduction at pH 7.4 due to its low RS2 signal. We used RS2 as a real-time probe in assays of reactive sulfane sulfur-involved reactions (Table 2). First, we tested the stability of RSnR chemicals (Me-SSS-Me and Tsp-SSSS-Tsp) in the presence of 100 mM GSH at pH 7.4 (100 mM HEPES). The RS2 spectra of RSnR were unchanged, and there was no H2S released from the solution. Thus, RS2 is rather stable. Second, we tested the reaction of H2Sn with GSH in deoxygenated HEPES buffer (100 mM, pH 7.4). After adding GSH (200 μM–5 mM) to 10 μM of H2Sn, the RS2 spectra of H2Sn quickly decreased, and H2S was released. At low H2Sn concentrations and at pH 7.4, H2S2 is the dominant species [10]. By recording the RS2 decreases, we determined the 2nd-order rate constant of the reaction between H2Sn and GSH as 0.89 M−1 s−1 (Table 2). Because GSH is at least two-orders-of-magnitude higher than H2Sn [14,29,34], the reaction between H2Sn and GSH should occur in the pseudo-first-order manner (e.g., t1/2 = 78 s) at the physiological pH and GSH concentration. Third, because RS2 has limitations, we could not use it to determine GSSH reduction at pH 7.4 due to its low RS2 signal. We used RS2 as a real-time probe in assays of reactive sulfane sulfur-involved reactions (Table 2). First, we tested the stability of RSnR chemicals (Me-SSS-Me and Tsp-SSSS-Tsp) in the presence of 100 mM GSH at pH 7.4 (100 mM HEPES). The RS2 spectra of RSnR were unchanged, and there was no H2S released from the solution. Thus, RS2 is rather stable. Second, we tested the reaction of H2Sn with GSH in deoxygenated HEPES buffer (100 mM, pH 7.4). After adding GSH (200 μM–5 mM) to 10 μM of H2Sn, the RS2 spectra of H2Sn quickly decreased, and H2S was released. At low H2Sn concentrations and at pH 7.4, H2S2 is the dominant species [10]. By recording the RS2 decreases, we determined the 2nd-order rate constant of the reaction between H2Sn and GSH as 0.89 M−1 s−1 (Table 2). Because GSH is at least two-orders-of-magnitude higher than H2Sn [14,29,34], the reaction between H2Sn and GSH should occur in the pseudo-first-order manner (e.g., t1/2 = 78 s) at the physiological pH and GSH concentration. Third, because RS2 has limitations, we could not use it to determine GSSH reduction at pH 7.4 due to its low RS2 signal.
Both H$_2$S and GSSH were reported to have antioxidant functions, as their reactivity towards H$_2$O$_2$. Using RS$_2$ intensity curves of H$_2$S and GSSSG concentration, we analyzed the kinetics of H$_2$O$_2$ with H$_2$S or GSSH at pH 7.4 and 25 °C (Table 2). At pH 7.4 and 25 °C, H$_2$S reacted with H$_2$O$_2$ slowly. The 2nd-order rate constant was determined to be 0.46 M$^{-1}$s$^{-1}$, close to a previously reported value (0.73 M$^{-1}$s$^{-1}$) determined at pH 7.4 and 37 °C [14]. On the other hand, GSSH rapidly reacted with H$_2$O$_2$ to produce GSSSG [29]; the 2nd-order rate constant was 23.8 M$^{-1}$s$^{-1}$ as determined with the RS$_2$ increase of GSSSG, 50-fold higher than that between H$_2$S and H$_2$O$_2$. The rate constant is likely an underestimate, as the GSSH preparation contains abundant than H$_2$S inside cells, it has been proposed that GSSH is a major reactive oxygen species (ROS) scavenger other than H$_2$S [29]; our finding proves the kinetic support for the hypothesis.

We also analyzed the reaction kinetics of GSSH with SSP4 at pH 7.4 and 25 °C by recording the fluorescence increase of the released chromophore from SSP4; the rate constant of this reaction was 9.53 M$^{-1}$s$^{-1}$.

3.5. Detection of GSSH disproportionation reactions

Trace amounts of GSSSH and GSSSG have been found in cancer cells, whether they are from GSSH disproportionation reactions (Fig. 5A) are still inconclusive [29,35,36]. We studied these reactions using the RS$_2$ method. When GSSH was incubated at pH 9.5, no appearance of RS$_2$ was detected. At pH 6.9, RS$_2$ spectra of protonated GSSH was initially observed, then it gradually changed to a spectrum overlapping those of GSSH and GSSSG (Fig. 5B). At pH 6.0, the RS$_2$ spectral change was also observed with a slower increase of the GSSSG peak. In consistent, LC-ESI-MS analysis (Fig. 8) indicated the amount of unreacted GSSH (remaining in solution) was the highest at pH 9.5 and the lowest at pH 6.9 (Fig. 5C). GSSSG was produced the most at pH 6.9 with less at pH 6.0 and the lowest at pH 9.5 (Fig. 5D). GSSH was also produced, but at about one order of magnitude lower than that of GSSSG, following the same trend at various pH values (Fig. 5E; pH 6.9 > pH 6.0 > pH 9.5). At pH 9.5, a small amount of GSSS(S) was also detected, which should be produced from GSS$^-$ oxidation (Fig. 5F). These results indicated that GSSH disproportionation occurred most efficiently at its pH$_a$. Considering GSSH can be as high as 100 μM in cancer cell and its pH$_a$ is close to the intracellular pH [29], it is highly possible that the intracellular GSSH and GSSSG are produced from these reactions.

3.6. RS$_2$ separates protein S-persulfidation into active and inactive forms

DUF442, a domain of Cupriavidus pinatubonensis JMP134 sulfide:quinoone oxidoreductase (GeneBank: AAZ62946.1), has rhodanese activity and catalyzes the reaction of GSSH with sulfite to produce thiosulfate [10]. The DUF442 domain consists of 128 amino acid residues with two cysteine residues, C34 and C94, and only C94 is conserved and functionally essential [10]. We used GSSH to react with DUF442 and LC-MS/MS to analyze the modification. Both C34-SSH and C94-SSH modifications were detected (Fig. S9 and Fig. S10). The modified DUF442 displayed significant RS$_2$, which was not observed from unmodified protein at pH 7.4 (Fig. 6A). In addition, the C34S/C94S double-mutant DUF442 showed no RS$_2$ after reacting with GSSH (Fig. 6B). Next, we reacted GSSH with the two single-mutants of DUF442 (C34S and C94S) at pH 7.4. C34S mutant showed significant RS$_2$, while C94S mutant did not, although the individual Cys residues were modified by GSSH treatment to form persulfides (confirmed via LC-MS/MS analysis). These results indicated that C94-SSH is likely in the protonated form (C94-SSH) and C34-SSH is in the deprotonated form (C34-SS-) at pH 7.4. When reacted sulfite, the RS$_2$ intensity of C94-SSH (C34S mutant) significantly decreased with the production of thiosulfate (C94-SSH + SO$_3$$^-$$^-$ →C94-SS + S$_2$O$_3$$^-$$^-$) [10]; whereas, C34-SSH (C94S mutant) without RS$_2$ did not produce thiosulfate when reacted with sulfite. When C34-SSH was titrated with HCl, the RS$_2$ intensity was increased at low pH. In the control containing C34-SSH (GSSH unreacted), RS$_2$ was not detectable at all the tested pH. Considering HCl titration may cause aggregation of protein, which disturbs...
RS2 detecting, we used different pH buffer for the titration. We diluted C34-SH or C34-SSH protein in HEPES buffers of different pH. The pKa was determined by using R2S2 method to be 6.29 (Fig. 6C). Thus, only C94-SSH in the DUF442 wild type or the DUF442C34S mutant is protonated at pH 7.4 and the sulfane sulfur can be transferred to sulfite to produce thiosulfate.

To inspect what makes DUF442-C94-SSH in the protonated form (C94-SSH) at pH 7.4, we modeled 3D structures of DUF442 with a putative rhodanese from Neisseria meningitides z2491 (PDB ID: 2F46) as the template (39% sequence similarity). The C94 sulfur was located at the bottom of a positively electrostatic pocket and is surrounded by –NH2– and –NH3+ groups (D and E). The red arrow points to the sulfur atom of C34, which is > 7.8 Å away from the nearest –NH3+ group (F). The distances (Å) from the sulfur atom to the circumjacent nitrogen atoms of the peptide backbone (red dotted line) and the side chain of R99 (yellow dotted line) were shown.

3.7. RS2 method application in whole cells

We also used the RS2 method to analyze intracellular changes of reactive sulfane sulfur in wild-type E. coli. E. coli contained more reactive sulfane sulfur at the stationary phase of growth (12 h) than at the log phase (6 h), as revealed by RS2 intensity (Fig. 7A) and SSP4 analysis (Fig. 7B). The sulfane sulfur species have different RS2 spectra at pH 7.4 (Figs. 1A, 5B and 6A). The RS2 peak of whole cells around 450 nm suggest the possible presence of H2Sn. Further, the RS2 peak of whole cells at 450 nm suggest the possible presence of H2Sn.

Previously, we reported that recombinant E. coli strain expressing a sulfide:quinone oxidoreductase of Cupriavidus pinatubonensis JMP134 (CpSQR) can oxidize H2S to H2Sn and the produced H2Sn is associated with the cell [10]. Herein, we used the recombinant E. coli to oxidize H2S. After the cells oxidized H2S, the cells were harvested, washed, and diluted for RS2 measurement. The RS2 peak at 450 nm increased, suggesting the production of H2Sn; however, the increase slowed down with increased H2S oxidation (Fig. 7C). On the basis of the RS2 spectra, other reactive sulfane sulfur species inside E. coli also increased after H2S oxidation, possibly including protein-SSH.

4. Discussion

Individual fluorophores are often considered as simultaneous photon absorbers and emitters with no significant light scattering due to small sizes [37]. Consequently, RS2 is often observed from the aggregated fluorophores, which usually are simultaneous photon absorbers, scatterers, and fluorescence emitters [21]. However, RS2 of reactive sulfane sulfur is most likely from soluble, individual molecules, since GSSH at pH 6 and DUF442-C94S-SSH at pH 7.4 are soluble and have RS2 (Figs. 2 and 6). At pH 8 to 8.5, H2Sn is mainly present as S2− (Fig. S3) and the solution has low RS2 (Fig. 2B). At pH > 9, long chain S2− species are dominant and RS2 was increased (Fig. 2B and Fig. S3). Our data showed that GSSH in the protonated form has RS2 and is electroneutral, and the deprotonated form does not have RS2 and is not electrophilic (Fig. 3A&B). The ratio of GSSH/GSS− at various pH determines the electrophilicity and RS2 intensity (Fig. 3D). Apparently, the electrophilicity of the sulfane sulfur in GSSH is strongly affected by deprotonation because the negatively charged terminal sulfur (S−) affects its adjacent sulfur through the α-effect, making both sulfur atoms with negative charge. The same logic may also apply to long chain S2− (n > 4) with the terminal sulfur (S−) having minimal effects on distant...
S in the middle (Fig. S3). The electrophilicity of sulfane sulfur can be explained in the form of thiosulfoxide (R′RSS′ = S) [33,38]. Although our NMR analysis showed evidence to support the thiols of thioisulfate in R-SSSSS-R (Fig. 4), for GSSH and H₂S₈, whether the sulfane sulfur is in present as thiosulfoxide or in a linear form is still unsettled [39,40]. Thus, our results associate RS₂ with the electrophilicity of sulfane sulfur; the deprotonated persulfides (R-SS⁻) are nucleophilic and prone to oxidation but does not react with SSP₄ [41].

The pKₐ values of thiols are critical to their reactivity at physiological pH. The pKₐ of Cys thiol at active center of enzyme may be lowered so that the thiol is deprotonated at neutral pH, which are strongly nucleophilic and are prone to oxidation by ROS. The pKₐ values of R-SSH are also likely important and have previously been reported within the range of 4.3–6.23 [16,42], implying that the persulfides should be mostly in the deprotonated form (RSS⁻) at pH 7 and displaying nucleophilic properties. The pKₐ value of cumyl-SSH has recently been determined as 7.0 [43], close to the value of GSSH (6.9) that we determined with two different approaches (Fig. 3). According to this value, the ratio of deprotonated form and protonated form of GSSH is within the range of 2–9 at physiological pH range (7.2–7.8). Disproportionation of GSSH requires both the deprotonated and protonated forms with one playing an electrophile and another acting as a nucleophile, which is consistent with our observation that the disproportionation was the most efficient at pH closed to its pKₐ (Fig. 3). These chemical reactions might be the origin of intracellular GSSH and GSSSG. Sulfane sulfur prefers to move from a high reactive polysulfur to form a lower one [10,44]. Thus, in the cell the flow of sulfane sulfur is likely from H₂S₈ to GSSH and then to GSSSG.

Protein S-persulfidation is common inside cells [39]. Here we showed that like cystinyl thiols at the active site, the pKₐ values of protein persulfides can also be affected by its location. Most protein persulfides are likely deprotonated at physiological pH because they have no apparent RS₂ and cannot react with sulfite (Fig. 6), but the sulfane sulfur at the active site of rhodanese is not deprotonated, due to its location in a positive electrostatic field. Rhodanese can then transfer the sulfane sulfur to small nucleophiles, such as cyanide and sulfite, which act as sulfane sulfur acceptors. Our finding implies that the catalysis of rhodanese is likely to generate an electrophilic sulfane sulfur that is easily transferred between two nucleophilic substrates, such as from GSS⁻ to SO₄²⁻, producing S₂O₄²⁻.

5. Conclusions

We discovered reactive sulfane sulfur species have RS₂ properties only when the molecules contain an electrophilic sulfane sulfur. It can be applied to reactive sulfane sulfur analyses, such as pKₐ determination, reaction kinetics, pH-dependent sulfane reactivity of small and protein persulfides, etc. For whole cell analysis, it may reveal the relative abundance of a reactive sulfane sulfur species. The RS₂ method is rapid, sensitive and convenient, allowing us to reveal several new chemical and biochemical properties of biologically relevant reactive sulfane sulfur. The results that were reported here, such as the pKₐ of GSSH, the reaction parameters, the distribution of H₂S₈ species at different pH, may fill some gaps in the field.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

The work was financially supported by grants from the National Natural Science Foundation of China (91751207, 31770093), the National Key Research and Development Program of China (2016YFA0601103), and the Natural Science Foundation of Shandong Province, China (ZR2016CM03, ZR2017BZ0210).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101179.

References

[1] G.K. Kolluru, X. Shen, C.G. Kevi, A tale of two gases: NO and H₂S, foes or friends for life? Redox Biol. 1 (2013) 313–318.
[2] K.R. Olson, Vascular actions of hydrogen sulfide in nonmammalian vertebrates, Antioxidants Redox Signal. 7 (2005) 804–812.
[3] S. Rajpal, P. Katiikaneni, M. Deshotels, S. Paradue, J. Gawe, et al., Total sulfane sulfur bioavailability reflects ethnic and gender disparities in cardiovascular disease, Redox Biol. 15 (2018) 480–489.
[4] R. Greiner, P. Pálinkás, K. Bässel, D. Becher, H. Antelmann, et al., Polysulfides link H₂S to protein thiol oxidation, Antioxidants Redox Signal. 19 (2013) 1749–1765.
[5] H. Liu, M.N. Radford, C.T. Yang, W. Chen, M. Xian, Inorganic hydrogen poly-sulfides: chemistry, chemical biology and detection, Br. J. Pharmacol. 176 (2019) 616–627.
[6] Y. Huang, F.B. Yu, J.C. Wang, L.X. Chen, Near-Infrared fluorescence probe for in situ detection of superoxide anion and hydrogen polysulfides in mitochondrial oxidative stress, Anal. Chem. 88 (2016) 4122–4129.
[7] S.I. Bibli, B. Luck, S. Zukunft, J. Wittig, W. Chen, et al., A selective and sensitive method for quantification of endogenous polysulfide production in biological samples, Redox Biol. 18 (2018) 295–304.
[8] K. Ono, T. Akaike, T. Sawa, Y. Kumagai, D.A. Wink, et al., The redox chemistry and chemical biology of H₂S, hydropersulfides and derived species: implications to their possible biological activity and utility, Free Radical Biol. Med. 77 (2014) 82–94.
[9] K.R. Olson, Y. Gao, E.R. DeLeon, M. Arrif, F. Arrif, et al., Catalase as a sulfide-sulfur oxide-reductase: an ancient (and modern?) regulator of reactive sulfur species (RSS), Redox Biol. 12 (2017) 325–339.
[10] Y. Xin, H. Liu, F. Cai, L. Xun, Recombinant Escherichia coli with sulfide: quinone oxidoreductase and persulfide dioxygenase rapidly oxidizes sulfide to sulfitole and thiosulfate via a new pathway, Environ. Microbiol. 18 (2016) 5123–5136.
[11] T. Akaike, T. Ida, F.Y. Wei, M. Nishida, Y. Kumagai, et al., Cysteinyl-tRNA synthetase link hydrogen sulfide to sulfane sulfur.
synthetase governs cysteine polysulfidation and mitochondrial bioenergetics, Nat. Commun. 8 (2017) 1–15.

[12] J.I. Toohey, Sulfur signaling: is the agent sulfide or sulfane? Anal. Biochem. 413 (2011) 1–7.

[13] M.I. Gerda, M.D. Pluth, S marks the spot: linking the antioxidant activity of N-acetylcysteine to H₂S and sulfane sulfur species, Cell Chem. Biol. 25 (2018) 353–355.

[14] T.V. Mishanina, M. Lubiad, R. Banerjee, Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways, Nat. Chem. Biol. 11 (2015) 457–464.

[15] M. Lubiad, P.K. Yadav, V. Vitvitsky, M. Martinov, R. Banerjee, Organization of the human mitochondrial hydrogen sulfide oxidation pathway, J. Biol. Chem. 289 (2014) 30901–30910.

[16] A.K. Mustafa, M.M. Gadalla, N. Sen, S. Kim, W. Mu, et al., H₂S signals through protein s-sulfhydration, Sci. Signal. 2 (ra72) (2009) 1–15.

[17] T. Tang, X. Li, X. Liu, Y.L. Wang, C.C. Ji, et al., A single-domain rhodanese homologue MnDH3 helps to maintain redox balance in \( \text{IT Macron Bacillus nipponensis} \), Dev. Comp. Immunol. 78 (2018) 160–168.

[18] B. Bordo, P. Bork, The rhodanese/Gdc 25 phosphatase superfamily-Sequence-structure-function relations, EMBO Rep. 3 (2002) 741–746.

[19] M. Gao, F.B. Yu, H. Chen, L.X. Chen, Near-infrared fluorescent probe for imaging mitochondrial hydrogen polysulfides in living cells and in vivo, Anal. Chem. 87 (2015) 3631–3638.

[20] T. Kakeo, K. Hanasek, K. Shimamoto, R. Miyamoto, T. Komatsu, et al., Development of a reversible fluorescent probe for reactive sulfur species, sulfane sulfur, and its biological application, Chem. Commun. 53 (2017) 1064–1067.

[21] M. Gao, R. Wang, F.B. Yu, L.X. Chen, Evaluation of sulfane sulfur bioeffects via a mitochondria-targeting selenium-containing near-infrared fluorescent probe, Biomaterials 160 (2018) 1–14.

[22] M. Gao, R. Wang, F.B. Yu, B.W. Li, L.X. Chen, Imaging of intracellular sulfane sulfur expression changes under hypoxic stress via a selenium-containing near-infrared fluorescent probe, J. Mater. Chem. B 6 (2018) 6637–6645.

[23] M. Gao, R. Wang, F.B. Yu, J.M. Youc, L.X. Chen, Imaging and evaluation of sulfane sulfur in acute brain ischemia using a mitochondria-targeted near-infrared fluorescent probe, J. Mater. Chem. B 6 (2018) 2608–2619.

[24] J.B.E. Lloyd, I.W. Evet, Prediction of peak wavelengths and intensities in synchronously excited fluorescence emission-spectra, Anal. Chem. 49 (1977) 1710–1715.

[25] M. Musteau, M. Aumercier, E. Teissier, B. Parmentier, A. Tartar, et al., Reduction of a thiolate derivative of glutathione by glutathione reductase, Biochem. Biophys. Res. Commun. 202 (1994) 1380–1386.

[26] J.L. Luebke, J. Shen, K.E. Bruce, T.E. Kehl-Fie, H. Peng, et al., The Ca⁺⁺-like sulfuryltransferase repressor (CaR) is a persulfide sensor in Staphylococcus aureus, Mol. Microbiol. 94 (2014) 1343–1360.

[27] C.B. Nettles, Y.D. Zhou, S.L. Zou, D.M. Zhang, UV-Vis ratiometric resonance synchronous spectroscopy for determination of nanoparticle and molecular optical cross sections, Anal. Chem. 88 (2016) 2891–2898.

[28] A. Bax, R.H. Griffey, B.L. Hawkins, Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR, J. Magn. Reson. 55 (1983) 301–315.

[29] T. Ida, T. Sawa, H. Ijara, Y. Tsuchiya, Y. Watanabe, et al., Reactive cysteine per-sulfides and S-polythiolation regulate oxidative stress and redox signaling, Proc. Natl. Acad. Sci. U. S. A 111 (2014) 7606–7611.

[30] Y. Xia, W. Chu, Q. Qi, L. Xun, New insights into the QuikChange™ process guide the use of Phusion DNA polymerase for site-directed mutagenesis, Nucleic Acids Res. 43 (e12) (2015) 1–19.

[31] J. Alexey Kamoshaya, A. Goisman, J. Gun, Dan Rizkov, O. Lev, Equilibrium distribution of polysulfide ions in aqueous solutions at 25°C: A new approach for the study of polysulfides’ equilibria, Environ. Sci. Technol. 38 (2004) 6633–6644.

[32] V. Bogdandi, T. Ida, T.R. Sutton, C. Bianco, T. Ditroi, et al., Speciation of reactive sulfur species and their reactions with alkylating agents: do we have any clue about what is present inside the cell? Br. J. Pharmacol. 176 (2019) 646–670.

[33] G.W. Kotney, K. Turnbull, Compounds containing the sulfur-sulfur double bond, Chem. Rev. 82 (1982) 333–357.

[34] H. Kimura, Signaling molecules: hydrogen sulfide and polysulfide, Antioxidants Redox Signal. 22 (2015) 362–376.

[35] J.I. Toohey, A.J.L. Cooper, Thiosulfate (Sulfane) sulfur: new chemistry and new regulatory roles in biology, Molecules 19 (2014) 12789–12813.

[36] W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco, et al., New fluorescent probes for sulfane sulfur and the application in bioimaging, Chem. Sci. 4 (2013) 2892–2896.

[37] B.C.N. Vithanage, J.N.X.Z. Xu, D.M. Zhang, Optical properties and kinetics: new insights to the porphyrin assembly and disassembly by polarized resonance synchronous spectroscopy, J. Phys. Chem. B 122 (2018) 8429–8438.

[38] J.I. Toohey, A.J.L. Cooper, Thiosulfate (Sulfane) sulfur: new chemistry and new regulatory roles in biology, Molecules 19 (2014) 12789–12813.

[39] C.M. Park, L. Weersinghe, J.J. Day, J.M. Fukuto, M. Xian, Persulfides: current knowledge and challenges in chemistry and chemical biology, Mol. Biosyst. 11 (2015) 1775–1785.

[40] R. Steudel, V. Druszkova, K. Minkiewicz, R.H. Hertwig, W. Koch, How unstable are thiosulfides? An ab initio MO study of various disulphanes R₂SR (R=H, Me, Pr, All), their branched isomers R₂S₂S, and the related transition states, J. Am. Chem. Soc. 119 (1997) 1990–1996.

[41] E. Cuesavasta, M. Lange, J. Bonanata, E.L. Cotino, G. Ferrer-Sueta, et al., Reaction of hydrogen sulfide with disulfide and sulfenic acid to form the strongly nucleophilic persulfide, J. Biol. Chem. 290 (2015) 26866–26880.

[42] S.A. Everett, L.K. Folkes, P. Wardman, K.D. Aumu, Free-radical repair by a novel perhydrotransfer and perthyl radical formation, Free Radic. Res. 20 (1994) 387–400.

[43] J.P.R. Chatvin, M. Grieser, D.A. Pratt, Hydropersulfides: H-atom transfer agents par excellence, J. Am. Chem. Soc. 139 (2017) 6484–6493.

[44] S.I. Melideo, M.R. Jackson, M.S. Jorns, Biosynthesis of a central intermediate in hydrogen sulfide metabolism by a novel human sulfurtransferase and its yeast ortholog, Biochemistry 53 (2014) 4739–4753.