Analysis and Functional Studies of the Gasotransmitters Hydrogen Sulfide and Nitric Oxide

Artur Pawel Jarosz
University of Windsor

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Analysis and Functional Studies of the Gasotransmitters Hydrogen Sulfide and Nitric Oxide

By

Artur Pawel Jarosz

A Dissertation

Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada

2016

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Analysis and Functional Studies of the Gasotransmitters Hydrogen Sulfide and Nitric Oxide

by

Artur Jarosz

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Department of Chemistry & Biochemistry

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May 16, 2016
I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is a result of joint research, as follows:

All chapters of this thesis incorporate research done under the supervision of Dr. Bulent Mutus at the University of Windsor. Experimental design was performed jointly by the author and Dr. Bulent Mutus. Execution of experiments, data analysis, and interpretation was performed by the author.

Chapter 2 includes research in collaboration with Terence Yep from the University of Windsor. Terence Yep was responsible for fabrication of some of the microplate covers used in the author’s experiments. Terence Yep collaborated with the author on some of the early studies trying to adapt silver to detect hydrogen sulfide.

Chapter 3 includes research in collaboration with Wanlei Wei, Dr. James W. Gauld, and Dr. Janeen Auld from the University of Windsor, and Filiz Özcan, and Dr. Mutay Aslan from Akdeniz University. Experimental design for the molecular modeling simulation of GAPDH was performed jointly by the author, Wanlei Wei, Bulent Mutus, and James Gauld. Molecular modeling simulation of GAPDH was performed by Wanlei Wei under the supervision of James Gauld. Interpretation of the results from the molecular modeling simulation was performed jointly by the author, Wanlei Wei, Bulent Mutus, and James Gauld. Experimental design of GAPDH mass spectrometry experiments was performed jointly by the author, and Janeen Auld. Mass spectrometry analysis of GAPDH was performed by Janeen Auld. Interpretation of mass spectrometry results was performed by the author. Filiz Özcan and Mutay Aslan collaborated with the author on some of the early studies of GAPDH using mass spectrometry.

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II. Declaration of Previous Publication

This thesis includes 2 original papers that have been previously published in peer reviewed journals, as follows:

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I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
The discovery of nitric oxide (NO), a toxic gas, as a gasotransmitter produced by mammalian cells has generated interest in other gases that may act as signaling molecules. One of those gases is hydrogen sulfide (H\textsubscript{2}S), produced by the transsulfuration enzymes CBS and CSE, which has been postulated to be a gasotransmitter like NO. We developed a microplate-based colourimetric assay for free H\textsubscript{2}S gas. Solutions of dissolved H\textsubscript{2}S spontaneously release gaseous H\textsubscript{2}S. In our assay gaseous H\textsubscript{2}S is released from solution, and reacts with silver embedded on the underside of a microplate cover, forming Ag\textsubscript{2}S, which can be quantified by absorbance. Using this assay the kinetic parameters of CSE were measured, as was H\textsubscript{2}S production from mouse liver homogenates. H\textsubscript{2}S has been postulated to signal through modification of cysteine residues on proteins (S-sulfuration), and the first protein shown to be S-sulfurated was GAPDH. We reinvestigated the H\textsubscript{2}S-induced S-sulfuration of GAPDH, and the effect of the initial redox state of the enzyme on S-sulfuration. Contrary to the previous literature results, we found that Cys 156 was S-sulfurated, and not the active site Cys 152. S-sulfuration of Cys 156 caused a decrease in enzymatic activity. It was possible to S-sulfurate Cys 152 in a C156S mutant of GAPDH, which caused a decrease in enzymatic activity. We propose a new role for Cys 156 as a protective thiol for Cys 152. Our results also show that polysulfides, which can be generated from H\textsubscript{2}S, are a stronger S-sulfurating agent than NaSH. When analyzing protein modifications by mass spectrometry, the tryptic peptide of interest may be in low stoichiometry compared to the rest of the digest. This may necessitate sample enrichment methods. We tested gold nanoparticles (AuNPs) as an enrichment method for sulfur-containing peptides. AuNPs showed some affinity for sulfur-containing peptides, and may be used in studies of protein thiols by mass spectrometry. NO has been shown to be an important mediator of wound healing, and NO-releasing materials may be used as wound dressings. We started the development of a chitosan-based NO-releasing wound dressing, which releases NO from nitrite (NO\textsubscript{2}\textsuperscript{-}) by copper ions. All major components in our design, chitosan, copper, and NO, have been previously shown to have therapeutic properties.
ACKNOWLEDGEMENTS

A thesis is not just the independent work of a single individual, but is supported by the contributions of many. I would like to thank the following people who have made significant contributions to the completion of this thesis and my graduate studies:

My supervisor Bulent Mutus, for the opportunities I had, and for his support during my studies.

Members of the Mutus lab, Bei Sun, Adam Faccenda, Vasantha Kallakunta, Harmanpreet Kaur, Ryan McLarty, Rebecca Wang, Terence Yep, Yousif Atwan, Hyder Ali Khan, Cody Caba, and Kathleen Fontana, which I had the pleasure of working with over the years.

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Ann English and Marc Ouellet, for proving the GAPDH enzymes used in my experiments.

James Gauld and Wanlei Wei, our collaborators, for computational chemistry calculations.

Last but not least, my family, for their support. Bei Sun, for her love, support, and encouragement.
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<td>--------------</td>
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<tr>
<td>3-MP</td>
<td></td>
<td>3-mercaptopyrurate</td>
</tr>
<tr>
<td>3-MST</td>
<td></td>
<td>3-mercaptopyrurate sulfurtransferase</td>
</tr>
<tr>
<td>AgNP</td>
<td></td>
<td>silver nanoparticle</td>
</tr>
<tr>
<td>AOAA</td>
<td></td>
<td>aminooxyacetic acid</td>
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<tr>
<td>AuNP</td>
<td></td>
<td>gold nanoparticle</td>
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<td>BCA</td>
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<td>β-cyanoalanine</td>
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<td>BSA</td>
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<td>CAM</td>
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<td>CAT</td>
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<td>cGMP</td>
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<td>cyclic guanosine monophosphate</td>
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<td>CSE</td>
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<td>cystathionine γ-lyase</td>
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<tr>
<td>CuNP</td>
<td></td>
<td>copper nanoparticle</td>
</tr>
<tr>
<td>DD</td>
<td></td>
<td>degree of deacetylation</td>
</tr>
<tr>
<td>DTNB</td>
<td></td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
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<td>DTPA</td>
<td></td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td></td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDHF</td>
<td></td>
<td>endothelium-derived hyperpolarizing factor</td>
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<td>EDRF</td>
<td></td>
<td>endothelium-derived relaxing factor</td>
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<td>EGDE</td>
<td></td>
<td>ethylene glycol diglycidyl ether</td>
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<tr>
<td>ESI</td>
<td></td>
<td>electrospray ionization</td>
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<td>G3P</td>
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<td>glyceraldehyde 3-phosphate</td>
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<td>GAPDH</td>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GSH</td>
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<td>reduced glutathione</td>
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<td>GSNO</td>
<td></td>
<td>S-nitrosoglutathione</td>
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<td>GSSG</td>
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<td>oxidized glutathione</td>
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<td>IAM</td>
<td></td>
<td>iodoacetamide</td>
</tr>
<tr>
<td>ISE</td>
<td></td>
<td>ion selective electrode</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
<td></td>
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<td>MMTS</td>
<td>methyl methanethiosulfonate</td>
<td></td>
</tr>
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<td>NAD</td>
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<td></td>
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<td>N-ethylmaleimide</td>
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<td>N-methyl-D-aspartate</td>
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</tr>
<tr>
<td>NO</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>PAG</td>
<td>DL-propargylglycine</td>
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<td>phosphate buffered saline</td>
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<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
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<td>PDMS</td>
<td>polydimethylsiloxane</td>
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<td>PSD</td>
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<tr>
<td>SA</td>
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<td>transient receptor potential cation channel, member A1</td>
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<td>YVH1</td>
<td>dual specificity protein phosphatase 12</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 Hydrogen Sulfide

1.1.1 Introduction and Biological Functions of H$_2$S

Hydrogen sulfide (H$_2$S) has long been known to be a toxic pollutant produced from decomposition of organic matter and from various industrial processes. The toxicity of H$_2$S has been known for over a century, and its toxic effects observed for longer than that. The production of small amounts of H$_2$S in mammalian tissues has been known for decades [1], although the physiological implications of this were not explored until the late 1990’s. The identification of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF) in the late 1980’s likely generated additional interest in other gases produced in the body that may act as signaling molecules [2]. In the late 1990’s and early 2000’s experiments showed alteration in H$_2$S homeostasis affected important physiological processes [3, 4]. This has led to the postulation that H$_2$S is a physiological gasotransmitter [4, 5], like NO and CO, which is synthesized at low levels in tissues to fulfill a signaling function. Since then the interest in H$_2$S research has grown tremendously, and subsequent studies have revealed effects of H$_2$S in almost every organ system.

One of the earliest studies on H$_2$S showed exogenous H$_2$S was, similarly to NO, able to cause vascular smooth muscle relaxation [4]. The mechanism of vascular smooth muscle relaxation was discovered to be through opening of ATP-sensitive potassium channels (K$_{ATP}$) [6]; the mechanism was later hypothesized to be S-sulfuration (sulfhydration) of the K$_{ATP}$ channel SUR subunit [7, 8]. Since then H$_2$S has been shown to act as a vasodilator in many different types of vascular tissues [9-19]. These experiments generally involve the application of exogenous H$_2$S to isolated blood vessels. Although K$_{ATP}$ channels were hypothesized to be the molecular targets of H$_2$S, other pathways have been proposed as well, including voltage-gated Ca$^{2+}$ channels [13], Cl$^-$/HCO$_3^-$ [14, 15], Big Potassium (BK$_{Ca}$) channels [16], potassium voltage-gated channels (KCNQ) [17], L-type Ca$^{2+}$ channels and K channels (but not K$_{ATP}$, K$_{Ca}$, K$_V$, or K$_{ir}$ subtypes) [18], and calcium-activated chloride channels (CaCCs) [19]. The type of blood vessel as well as the species may determine the exact mechanism of vasorelaxation, and both endothelium-dependent and endothelium-independent types of vasorelaxation have
been observed in the literature [18]. Paradoxically it has also been reported that H$_2$S causes vasoconstriction [20-22]; this process has been determined to be O$_2$-dependent. At low O$_2$ concentrations H$_2$S acts as a vasodilator, while at higher than physiological O$_2$ concentrations H$_2$S acts as a vasoconstrictor [20]. It has been hypothesized that H$_2$S acts as an O$_2$ sensor and controls hypoxic pulmonary vasoconstriction [23, 24], although this hypothesis has been recently questioned [25]. One of the most widely-cited lines of evidence is that CSE-knockout mice were found to be hypertensive, displayed a diminished endothelium-dependent vasorelaxation, and had lower tissue levels of H$_2$S [26]. This has not been independently verified since an independent study on CSE knockout mice showed the mice were normotensive [27]. H$_2$S has also been proposed to be endothelium derived hyperpolarizing factor (EDHF) [8, 28-30]. EDHF is a nitric oxide synthase and cyclooxygenase independent vascular relaxation pathway [31]. EDHF is dominant in small blood vessels, while NO is dominant in large ones. Although H$_2$S has been proposed to be EDHF, many chemical species have been proposed to be the EDHF including epoxyeicosatrienoic acids, H$_2$O$_2$, K$^+$, and C-type natriuretic peptide [32]. It is likely EDHF represents a mechanism of smooth muscle hyperpolarization, which can be initiated by many, and not a single, chemical species.

H$_2$S can affect ion channels and numerous functions of H$_2$S have been postulated in the nervous system. The two main proposed roles of H$_2$S in the nervous system are neuromodulation and neuroprotection [33]. One of the first studies on the biological functions of endogenous H$_2$S by Abe and Kimura in 1996 found that H$_2$S enhances N-methyl-D-aspartate (NMDA) receptor mediated current and enhances neuronal long term potentiation [3]. H$_2$S facilitates long term potentiation by increasing Ca$^{2+}$ uptake in astrocytes by activation of transient receptor potential cation member A1 (TRPA1) channels [34, 35]. Astrocytes then release D-serine into the synapse which enhances the activity of NMDA receptors [36]. Polysulfides were ~300 times more effective at increasing Ca$^{2+}$ uptake than H$_2$S [34]. H$_2$S also has the ability to modulate nociception (pain sensing) [37]. H$_2$S causes hyperalgesia (increased sensitivity to pain) in animal models by stimulating Ca$_{v}3.2$ T-type Ca$^{2+}$ channels [38-40] which are present in nerve endings [41]. The mechanism is due to H$_2$S reacting with endogenous Zn$^{2+}$ ions which endogenously inhibit T-type Ca$^{2+}$ channels [42-44]. H$_2$S also acts as a pro-nociceptor by
activating TRPA1, a channel expressed in sensory neurons which responds to temperature and natural chemical irritants, among other stimuli [45-47].

H₂S has been linked to major neurological diseases Alzheimer’s disease, and Parkinson’s disease. Elevated plasma homocysteine level has been associated with Alzheimer’s disease [48], suggesting the H₂S-producing enzyme CBS, which also metabolizes homocysteine, is involved in the pathogenesis of the disease [49]. Decreased levels of H₂S have been detected by GC in brain autopsy samples from Alzheimer’s disease patients [50]. The levels of CBS in Alzheimer’s brain were unchanged, however the levels of CBS activator S-adenosyl-L-methionine (SAM) were lower, and levels of homocysteine were higher. NaSH was able to reduce amyloid beta protein levels in SH-SY5Y cells by inhibiting the γ-secretase protein [51]. In a rat model of Alzheimer’s disease treatment with NaSH or spa-water rich with H₂S (Tabiano water) improved learning and memory [52]. The treatment lowered the size of amyloid beta plaques and increased the amount of healthy neurons. Similarly in a mouse model of Alzheimer’s disease treatment of NaSH improved spatial learning and memory and lessened plaque formation [53].

Elevated plasma homocysteine levels have been also associated with severity of Parkinson’s disease [54, 55], a disease characterized by the loss of dopaminergic neurons in the brain. NaSH was able to reduce 1-methyl-4-phenylpyridinium ion (MPP+) induced cytotoxicity and apoptosis in PC12 cells, a cell line commonly used to study Parkinson’s disease [56]. NaSH was also able to reduce cytotoxicity and apoptosis in SH-SY5Y cells (a type of human neuroblastoma cell line) treated with rotenone, a pesticide which can degrade dopaminergic neurons [57], and in SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA), a dopaminergic neurotoxin [58]. Various H₂S-releasing L-DOPA derivatives have been developed and tested in cell [59], and animal models of Parkinson’s disease [60]. Inhaled H₂S has also been shown to be neuroprotective in a mouse model of Parkinson’s disease [61]. H₂S has also been shown to be neuroprotective against lipopolysaccharide (LPS) induced inflammation [62], and glutamate toxicity [63, 64].
Although low concentrations of H$_2$S have been demonstrated to have therapeutic properties in disease models, it is well-known that H$_2$S is a toxic gas which can be lethal at higher concentrations. The concentration of H$_2$S is larger determinant of toxicity rather than the length of exposure [65]. In animal studies using rats, LC$_{50}$ for 2 h of exposure was 587 ppm, while LC$_{50}$ for 6 h of exposure was 335 ppm. The symptoms associated with H$_2$S toxicity are eye irritation, olfactory paralysis, pulmonary edema, and abrupt loss of consciousness [66]. Perhaps the most famous symptom of H$_2$S toxicity is olfactory paralysis. The human nose is extremely sensitive to H$_2$S at low concentrations and can smell 0.01-0.3 ppm H$_2$S, while at higher concentrations of ~100 ppm H$_2$S causes olfactory paralysis and the characteristic H$_2$S smell disappears [66]. Olfactory paralysis occurs due to neurotoxicity of H$_2$S towards olfactory bulb and fibers [67], and a permanent decrease in the sense of smell has been reported in patients who recovered from H$_2$S poisoning [68]. Eye and lung irritation can occur at 20-50 ppm H$_2$S, pulmonary edema at 250-500 ppm, sudden loss of consciousness at 500 ppm and prolonged exposure at 500 ppm may cause death [66]. The mechanism of H$_2$S toxicity is similar to that of cyanide; both cyanide and H$_2$S inhibit cellular respiration by inhibiting mitochondrial cytochrome c oxidase [69]. When exposed to 80 ppm H$_2$S mice exhibit a reduced metabolic rate and enter a state of suspended animation [70]. In this state core body temperature dropped to 2 °C above ambient temperature and breathing rate dropped from 120 breaths per minute to 10. When brought out of suspended animation the mice exhibited no behavioral or functional changes. Similarly oil patch workers who lost consciousness due to high but transient H$_2$S concentrations experienced rapid recovery [66].

### 1.1.2 H$_2$S-Producing Enzymes

H$_2$S may be produced enzymatically by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), or through the collective action of enzymes cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) [1]. As their names suggest the enzymes are not exclusively H$_2$S-producing enzymes, and perform other important functions in addition to H$_2$S production. CBS and CSE are PLP (pyridoxal phosphate) dependent enzymes involved in the reverse transsulfuration pathway [1]. The reverse
transsulfuration pathway is responsible for the conversion of homocysteine, which can be produced from methionine, to cysteine, and as a result cysteine is a non-essential amino acid for humans [71-73]. CBS catalyzes the first step of the transsulfuration pathway, which is the formation of cystathionine by linking homocysteine and serine (Figure 1.1.2-1) [74, 75]. CSE catalyzes the second step of the transsulfuration pathway, which is the cleavage of cystathionine to form α-ketobutyrate, cysteine, and ammonia (Figure 1.1.2-1). Due to low substrate specificity CBS and CSE can also catalyze a number of other reactions where H₂S is formed as a byproduct (Figures 1.1.2-2 & 1.1.2-3) [74]. The second reaction catalyzed by CBS can metabolize homocysteine through a condensation reaction with cysteine, generating cystathionine and H₂S. The third and fourth reactions catalyzed by CBS are the conversion of cysteine to serine and H₂S, and condensation of cysteine to form lanthionine and H₂S. CSE catalyzes the condensation of cysteine to lanthionine and H₂S. CSE can metabolize cysteine to pyruvate, ammonia, and H₂S. CSE catalyzes the condensation of homocysteine to form homolanthionine and H₂S. CSE catalyzes the condensations of cysteine and homocysteine form its transsulfuration substrate cystathionine and H₂S. CSE can also catalyze homocysteine to form α-ketobutyrate, H₂S, and ammonia. The transsulfuration pathway plays an important role in catabolizing homocysteine [76], which is a toxic metabolite, and maintaining glutathione redox status by generating cysteine, which can be incorporated into GSH [76, 77]. Lanthionines are also starting to be considered neuroprotective molecules in the nervous system [76, 78]. CBS and CSE can also use cystine (cysteine disulfide) as a substrate to produce cysteine persulfides (Cys-SSH) [79, 80]. Cysteine persulfides can react with reduced glutathione to form glutathione persulfide, and may S-sulfurate proteins. Cysteine persulfides and glutathione persulfides have also been detected in vivo [79], although their physiological significance has not yet been explored. In H₂S research, the biological effects due to alterations in CBS and CSE homeostasis are generally considered to be solely H₂S-related.

The human CBS \( K_M \) value for condensation of serine and homocysteine to form cystathionine is 2.76 mM [74]. The human CBS \( K_M \) values for H₂S producing reactions; conversion cysteine to serine, condensation of cysteine to lanthionine, and condensation of cysteine and homocysteine to cystathionine are 6.8 mM. The human CSE \( K_M \) value for
conversion of cystathionine to cysteine is 0.5 mM, while the $K_M$ value for H$_2$S generation from cysteine is 2.75 mM [81]. Cytosol cystathionine and cysteine concentrations are estimated at 10 µM and 100 µM respectively [74]. The large $K_M$ values for CBS and CSE, compared to substrate concentrations, indicate activity is highly dependent on substrate concentrations. CSE is more efficient at catalyzing its transsulfuration substrate, cystathionine, than H$_2$S production from cysteine. The catalytic efficiency ($k_{cat}/K_M$) of CSE for cystathionine catabolism is $1.5 \times 10^4$ M$^{-1}$ s$^{-1}$, while the catalytic efficiency of CSE for H$_2$S production from cysteine is $1.5 \times 10^2$ M$^{-1}$ s$^{-1}$ [81]. Cysteine is the most commonly used substrate added to cells or lysates in order to stimulate H$_2$S production.

CBS is a homotetramer, each subunit is 63 kDa [82]. CBS contains a 70 amino acid length N-terminal heme domain, followed by a PLP-binding site and catalytic core, and a C-terminal regulatory domain [83]. The catalytic core includes a CXXC domain. Deletion of the heme domain results in a loss of sensitivity of the enzyme to titanium citrate [84], leading to a hypothesis that CBS heme acts as a redox sensor [84, 85]. CBS activity can also be inhibited by CO or NO binding to CBS heme [86-88]; suggesting CBS is regulated by CO and NO. Deletion of CBS heme domain reduces activity by ~40%; however the enzyme retains the PLP binding ability [89]. The regulatory domain contains a binding site for the allosteric cofactor $S$-adenosylmethionine (AdoMet) [90]. CSE is also a PLP-dependent homotetramer; each subunit is 44.5 kDa [91].

Initially CBS has been described to be localized in the brain and the nervous system, while CSE localized to the circulatory system and peripheral tissue [92]. CBS is highly expressed in the brain [3], while CSE is highly expressed in the liver and kidneys [93]. However a clear tissue division may not exist [92], and most tissues have an intact reverse transsulfuration pathway, meaning both CBS and CSE are present [94]. The relative quantities of CBS and CSE do vary and tissues may have a dominant H$_2$S-producing enzyme; for example CSE protein levels are ~60 fold higher than CBS in mouse liver, and ~20 fold higher in mouse kidney [75]. It has been suggested CSE is absent in the brain since it is undetectable by western blotting [95], however the conversion of $[^{35}S]$-methionine to $[^{35}S]$-cysteine was observed in cultured human neurons, murine neurons, murine astrocytes and mouse brain slices [93, 94], suggesting
an intact transsulfuration pathway. CSE activity was also 100 fold higher in human brain than in mouse brain [96]; animal models may not always be an accurate representation of human physiology.

Mutations in CBS can cause severe hereditary hyperhomocysteinemia [97]. Elevated homocysteine levels in circulation are associated with major diseases including cardiovascular disease [98], and Alzheimer’s disease [48]. Homozygous CBS knockout in mice is lethal; CBS knockout mice suffered from growth retardation, and died within 5 weeks of birth [99]. This is most likely due to inability to metabolize homocysteine rather than H$_2$S-related changes since homozygous CBS knockout mice had ~40 fold the normal plasma homocysteine levels [99]. CSE knockout mice display hypertension [26], although an independent CSE knockout mice study found mice were normotensive [27]. CSE knockout mice also required dietary cysteine to protect against lethal myopathy, and had a higher susceptibility to oxidative injury [27]. Mutations in CSE are very rare, but may cause genetic cystathioninuria, which is characterized by an abnormal level of plasma cystathionine [91, 100, 101].

CBS and CSE activity in cell culture experiments may be modulated by enzyme inhibitors. The most commonly used inhibitors are DL-propargylglycine (PAG), β-cyanoalanine (BCA), and aminooxyacetic acid (AOAA), which target the PLP binding site of CBS and CSE. PAG and BCA have been described in the literature as specific inhibitors of CSE, while AOAA has been described as a specific inhibitor of CBS. Although commonly described as specific inhibitors of CBS and CSE, PAG, BCA, and AOAA display many non-specific effects, as reviewed in detail by Whiteman et al. [92]. Since they target the PLP binding site, CBS and CSE inhibitors inhibit other PLP-dependent enzymes. PAG, BCA, and AOAA are used in high millimolar concentrations and have been used in other research fields as “specific” inhibitors of other physiological processes [92]. It has also been shown that AOAA is a more powerful inhibitor of CSE rather than CBS [102]. The lack of specific inhibitors of CBS and CSE make it difficult to study their functions.

Another pathway of H$_2$S production is through cysteine aminotransferase (CAT) and 3-mercaptoppyruvate sulfurtransferase (3-MST) [1]. 3-MST is also known as tRNA
thiouridine modification protein (TUM1) and two splice variants have been discovered with molecular masses of 35.4 kDa and 33.3 kDa [103]. CAT/3-MST is less widely-studied than CBS and CSE. CAT catabolizes cysteine by reaction with α-ketoglutarate to form 3-mercaptopypyruvate (3-MP) and glutamate (Figure 1.1.2-4) [104]. 3-MST transfers the thiol from 3-mercaptopypyruvate to an acceptor, which can be another thiol, forming a persulfide, or to cyanide, to form the less toxic thiocyanate [105] (Figure 1.1.2-4). This means reducing agents are required to liberate the sulfide from persulfides [1], and 3-MST has been hypothesized to generate H$_2$S for storage [106]. The crystal structure of 3-MST revealed a complex with a Cys 248 persulfide and pyruvate, suggesting 3-MST is the initial thiol acceptor from 3-MP, although the exact reaction mechanism has not yet been elucidated [107]. The maximum enzymatic activity of 3-MST occurs in alkaline conditions [1, 107]. The $K_M$ values for 3-MST using 3-MP as a substrate have been reported as 1.2 mM using 2-mercaptoethanol as a sulfur acceptor [108], and 4.5 mM without a sulfur acceptor [109]. Yadav et al. reported $K_M$ values for 3-MST using 3-MP as a substrate using various sulfur acceptors; $K_M$ values ranged from 20 µM to 350 µM depending on the acceptor [107]. CAT/3-MST pathway has been discovered to produce H$_2$S in the brain [106], and the vascular endothelium [110]. 3-MST has been shown to be localized in the mitochondria and the cytoplasm [103, 111]. In 1992, cysteine concentration in rat liver mitochondria has been determined to be ~0.7-1.0 mM [112]. Based on enzyme $K_M$ values and cellular substrate concentrations the CAT/3-MST pathway in the mitochondria might be the only H$_2$S-producing system where H$_2$S production might be favoured over other reactions [94]. A more recent study found the mitochondrial cysteine concentration in mouse SMCs to be 177.5 ± 5.8 µM [113]. Although high concentrations of H$_2$S can inhibit mitochondrial electron transport by complex IV inhibition, low concentrations have been shown to stimulate mitochondrial electron transport by donating electrons to complex II through sulfide quinone reductase (SQR) [114]. Since 3-MST is present in the mitochondria and 3-MP has been shown to stimulate this process, 3-MST has been hypothesized to be a regulator of mitochondrial bioenergetics. It has been shown 3-MST stimulates angiogenesis and would healing in mice by intraperitoneal injection of 3-MP [115]. It has been hypothesized that endothelial dysfunction due to hyperglycemia occurs partly due to 3-MST pathway impairment. The
combination of 3-MP and lipoic acid was able to restore normal angiogenesis and mitochondrial function in hyperglycemia. Inhibitors of CAT (aspartate) and CSE (PAG) has been found to reduce postischemic cerebral vasodilation, suggesting CAT/3-MST and CSE play a role in blood-brain barrier disruption following a stroke [116]. Recently it was found 3-MST generates \( \text{H}_2\text{S}_3 \) and \( \text{H}_2\text{S}_2 \) polysulfide in addition to \( \text{H}_2\text{S} \) in mouse brain tissues [109]. Red blood cells contain 3-MST and can produce \( \text{H}_2\text{S} \) if provided with 3-MP and DTT [117]. Red blood cells can also oxidize \( \text{H}_2\text{S} \) by hemoglobin to polysulfides and thiosulfate.
Figure 1.1.2-1 Reverse transsulfuration pathway. Reaction I is the first step of the reverse transsulfuration pathway, catalyzed by cystathionine β-synthase (CBS). Reaction II is the second step of the reverse transsulfuration pathway, catalyzed by cystathionine γ-lyase (CSE).
Figure 1.1.2-2 Reactions catalyzed by cystathionine β-synthase (CBS) which produce H₂S or cysteine persulfide (thiocysteine).
Figure 1.1.2-3 Reactions catalyzed by cystathionine γ-lyase (CSE) which produce H₂S or cysteine persulfide (thiocysteine).
Figure 1.1.2-4 Reactions catalyzed by the CAT/3-MST pathway. Reaction I is catalyzed by cysteine aminotransferase (CAT). Reaction II is catalyzed by 3-mercaptopyruvate sulfurtransferase (3-MST). R represents a sulfur acceptor, which can be cyanide (CN\(^{-}\)), organic sulfur (e.g., glutathione), or an inorganic sulfur (HSO\(_{3}^{-}\)).
1.1.3 Chemical Properties of H₂S

When studying H₂S in biological systems it’s important to understand the physical properties of H₂S in order to design accurate experiments and draw appropriate conclusions. H₂S is a colorless, flammable, toxic gas with a characteristic smell of rotten eggs. H₂S gas is lipophilic and has a high solubility in organic solvents, allowing it to cross cellular membranes, although it is not as highly lipophilic as NO and CO [8, 118]. H₂S gas is denser than air with a vapor density of 1.19 and in solution it is a weak acid that dissociates according to the equilibrium:

\[
\text{(1.1.3-1)} \quad \text{H}_2\text{S} \rightleftharpoons \text{H}^+ + \text{HS}^- \rightleftharpoons 2\text{H}^+ + \text{S}^{2-}
\]

pK\text{a1} is around 6.98 at 25 °C, 6.76 at 37 °C, while pK\text{a2} is >17 [119, 120]. As a result H₂S is approximately 20% H₂S and 80% HS\text{−} (hydrosulfide) in solution at physiological conditions (37 °C and pH 7.4), and S\text{2−} (sulfide) is negligible. Unless otherwise specified most researchers refer to all the species in that equilibrium collectively as “hydrogen sulfide”. One consequence of H₂S equilibrium in solution is H₂S solutions release gaseous H₂S, driving equilibrium to the left, until all the hydrogen sulfide is lost from the solution. This has been recognized as a pitfall in H₂S research. It has been speculated that it contributes to the discrepancy between experimental concentrations required to produce biological responses and actual in vivo concentrations. This loss has been shown to be significant in open cell culture plates, bubbling tissue baths, and Langendorff apparatus [121]. The half-life of H₂S in open cell culture plates is only 5 minutes.

Most commonly used forms of H₂S in solution are sodium hydrosulfide hydrate (NaSH•xH₂O) and sodium sulfide (Na₂S•9H₂O). Sodium hydrosulfide is only ~60% pure and is contaminated with elemental sulfur, yet it is the most commonly used H₂S donor. Sodium hydrosulfide crystals have a yellow appearance from elemental sulfur. It has been hypothesized by Toohey that the biological activity attributed to hydrogen sulfide comes from contaminating sulfane sulfur (elemental sulfur), and from oxidation of hydrosulfide to sulfane sulfur and polysulfides [122]. The kinetics of H₂S release from sodium salts may also not be physiologically relevant. Sodium salts are thought to release hydrogen sulfide in a large bolus [123]. Additionally sulfide salts create large local
concentrations of Na⁺ [92], the effect of which in relation to H₂S research has not been studied. An alternative is synthetic H₂S donors, such as GYY4137 [124, 125], which are thought to release H₂S in a slower and more physiologically relevant manner. Many other H₂S releasing molecules were developed recently due to increasing interest in H₂S research [126]. Some H₂S releasing compounds are also naturally occurring, and it has even been hypothesized that the biological activity from garlic (*Allium sativum*) occurs due to H₂S [127, 128]. Interest in H₂S releasing compounds also stems from development of H₂S-based therapeutics [126].

H₂S solutions can oxidize to polysulfides, elemental sulfur, and sulfur oxides in the presence of oxygen. The chemical species and mechanisms involved are surprisingly complex. Oxidation of sulfide has been studied by Chen *et al.* in 1972 [129]. Sulfide is first oxidized by oxygen to elemental sulfur [129, 130]. The oxidation is catalyzed by trace metals, as it can be inhibited by the metal chelator DTPA [130]. Sulfur can then react with sulfide to produce polysulfides. Polysulfides exist in solution as an equilibrium, with different chain lengths present at the same time (Equation 1.1.3-2) [122].

$$\text{HS}^- \rightleftharpoons \text{HSS}^\cdot \rightleftharpoons \text{HSSS}^- \rightarrow \ldots \rightarrow \text{HS}_\ell^- \rightarrow \text{S}_8 + \text{HS}^-$$

Although Chen *et al.* initially reported formation of polysulfides with up to 5 sulfurs [129], improved detection methods allowed the detection and characterization of polysulfides with up to 8 sulfurs [131, 132]. Polysulfide formation is pH dependent, and the distribution of polysulfide species is also pH dependent. In 2009 Stasko *et al.* determined the initial oxidation of H₂S by oxygen occurs through sulphydryl radical (HS•/S•) intermediates [133]. Under this reaction mechanism H₂S could be directly oxidized to polysulfides without forming elemental sulfur intermediates.

H₂S oxidation products also include sulfite (SO₃²⁻), thiosulfate (S₂O₃²⁻), and sulfate (SO₄²⁻) [129, 130]. The oxidation of H₂S combined with the volatility means that H₂S solutions prepared from sulfide salts need to be made fresh before an experiment. Increasing evidence indicates that the H₂S-derived biological activity occurs at least partly due to polysulfides generated from H₂S. Some researchers have used inert gases
and metal ion chelators to prevent oxidation of H$_2$S, although such conditions may not be physiologically relevant.

Given that H$_2$S has been shown to act as an anti-oxidant, and sulfur atom in H$_2$S is in its most reduced -2 state, H$_2$S can react with a number of physiologically relevant redox signaling molecules. H$_2$S can react with superoxide (O$_2^-$), peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), peroxynitrite (ONOO$^-$), nitric oxide (NO), lipid peroxide (LOOH), various forms of biological iron, and protein disulfides. The subject has been reviewed in detail by Li and Landcaster [134]. The mechanisms, kinetics, and biological relevance of many of these reactions are still unclear. Of particular interest to researchers are reactions between H$_2$S and NO as researchers look for evidence of H$_2$S-NO interaction, so called “cross-talk”. Filipovic et al. reported H$_2$S can react with S-nitrosothiols to form thionitrous acid (HSNO) [135]. HSNO is unstable and can decompose into NO$^+$, NO, NO$^-$, and cause protein transnitrosation. It has been hypothesized H$_2$S is able to cause transnitrosation of proteins through HSNO in vivo. Filipovic et al. also reported that H$_2$S can react with the NO donor sodium nitroprusside (SNP) to form nitroxyl (HNO) [136]. It has also been reported that, when catalyzed by heme iron, H$_2$S can react with nitrite (NO$_2^-$) to produce NO, as well as HSNO and HNO [137]. Cortese-Krott et al. reported that the product of NO and H$_2$S is nitrosopersulfide (SSNO$^-$) [138]. Although various S-N hybrid species formed from NO and H$_2$S have been reported in vitro at large concentrations of reactants, the physiological relevance of such reactions has not yet been demonstrated.

Although it has previously been proposed that blood concentrations of H$_2$S are in the 10-100 µM range [139], it is now clear that H$_2$S is not a circulating gasotransmitter [120]. Introduction of H$_2$S in tissues results in rapid H$_2$S catabolism. This is particularly highlighted in a study by Whitfield et al. where an extracorporeal pump was used to draw blood from the dorsal aorta of rainbow trout, and returned across a polarographic H$_2$S sensor into the caudal vein [140]. H$_2$S level was ~0.1 µM at resting state, and when H$_2$S was injected into caudal vein cannula, the blood H$_2$S concentration rose to ~1.4 µM momentarily and rapidly returned to baseline. The amount of H$_2$S injected was theoretically enough to raise the blood H$_2$S concentration to ~30 µM. Half-lives of H$_2$S
were also much lower in whole-blood than in buffer. This study demonstrates the highly reactive nature of H$_2$S in biological systems. The rapid clearance of H$_2$S from blood is likely due to H$_2$S oxidation by hemoglobin [117].

1.1.4 Mechanisms of H$_2$S Signaling: S-Sulfuration

Although H$_2$S can react with other redox signaling molecules and iron, the primary mechanism of H$_2$S biological activity is widely-accepted to be protein post-translational modification [141]. Similar to S-nitrosation by NO, H$_2$S affects cysteine thiols, where H$_2$S can add sulfur to cysteine thiols forming a persulfide thiol (-SSH). Initially this process has been termed sulfhydration, which was later criticized for having incorrect IUPAC nomenclature, and is now commonly referred to as S-sulfuration or persulfuration. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the first protein shown to be S-sulfurated by Mustafa et al. in 2009 [141]. Treatment of purified GAPDH with NaSH caused a ~7 fold increase in enzymatic activity with an EC$_{50}$ of only 15 µM. S-sulfuration of GAPDH was detected using a modified biotin shift assay, and mass spectrometry. Mass spectrometry found the active site Cys 152 (referred to as Cys 150 in the original publication) to be S-sulfurated. The modified biotin shift assay was also used to measure basal S-sulfuration of liver proteins, and it was found that 10% to 25% of all liver proteins were S-sulfurated. The modified biotin shift assay was introduced in the same publication, and it was proposed that the thiol blocking reagent methyl methanethiosulfonate (MMTS) does not react with S-sulfurated (-SSH) thiols, and only reduced thiols (-SH). After MMTS treatment the protein was treated with biotin-HPDP to label S-sulfurated thiols. The nature of the selectivity of MMTS or the product of reaction between biotin-HPDP and persulfide was not explored in the 2009 publication.

Mass spectrometry is generally used to detect S-sulfuration in vitro after treating a purified enzyme with H$_2$S, while variations of the biotin shift protocol have been used combined with western blotting to detect S-sulfuration in cells. This can be problematic if the reagents used for biotin shift labelling are not specific for S-sulfuration. The MMTS selectivity of the modified biotin shift assay was tested by Pan and Carroll in 2013 [142]. MMTS, as well as other commonly thiol-reactive reagents, were reacted with persulfides
(S-sulfurated thiols) and analyzed by LC-MS. It was found that MMTS is not selective to persulfides, and persulfide thiols act as a nucleophile to produce a mixed disulfide product (−SSCH₃). The same reaction occurs between persulfides and other commonly used thiol reactive reagents like iodoacetamide and N-ethylmaleimide. Persulfides may also react with disulfides like DTNB to form a trisulfide linkage. Two possible mechanisms were proposed for the observed signal in the modified biotin shift assay; incomplete labelling of thiols with MMTS will produce a positive signal after labelling with biotin-HPDP; or trace amount of thiols can reduce the dithiometane linkage from MMTS labelling, and biotin-HPDP can label the resulting free thiol. It is possible that numerous publications on the sulfuration of proteins are actually reporting cysteine reduction and not S-sulfuration of cysteines. The increase in modified biotin shift assay signal with increasing H₂S concentration is likely due the second mechanism. Challenges in detecting S-sulfuration using mass spectrometry include the mass increase resulting from S-sulfuration (31.9721 Da) is very close to that to sulfonic acid (−SO₂H) (31.9898 Da). It has also been suggested that unmodified protein persulfides may not be stable under mass spectrometry conditions [143].

In the majority of S-sulfuration studies so far, proteins were shown to be S-sulfurated using the modified biotin shift assay. For example IKCa ion channels and the Kir 6.1 subunit of ATP channels have been shown to be S-sulfurated using the modified biotin switch assay [29, 144]. H₂S has been postulated to act as an endothelium hyperpolarizing factor and fulfill this function through S-sulfuration. Other enzymes that have been reported to be S-sulfurated include PTP1B, a protein tyrosine phosphatase, where S-sulfuration of its active site cysteine inactivated the enzyme [145]. S-sulfuration was detected by mass spectrometry, and a biotinylated iodoacetic acid probe. However, it is unclear how an iodoacetic acid based probe can be selective for S-sulfurated thiols, and not react with free thiols. The p65 subunit of nuclear factor kappa B (NF-κB) has been shown to the S-sulfurated using the modified biotin shift assay, a maleimide assay for S-sulfuration and S-nitrosation, and mass spectrometry to pinpoint the modification [146]. The maleimide assay involves reaction of protein thiols with two versions of fluorophore-conjugate maleimide. First free thiols and S-sulfurated thiols are reacted with red maleimide (Alexa Fluor® 680 C2 maleimide), ascorbate is added to denitrosate S-
nitrosothiols, and S-nitrosothiols are labelled with green maleimide (Alexa Fluor® 488 C5 maleimide or IRDye 800CW). Finally DTT is added to cleave the protein-maleimide disulfides formed from labeling persulfides with red maleimide, and loss of red fluorescence corresponds to S-sulfuration. H₂S has been hypothesized to be an in vivo modulator of NF-κB activity. S-sulfuration of NF-κB p65 was also studied using the modified biotin shift assay [147].

H₂S has been linked to Parkinson’s disease since human brain samples from Parkinson’s disease patients showed reduced S-sulfuration of parkin [148]. In this study endogenous S-sulfuration in cells and tissues was detected using a maleimide assay for S-sulfuration and S-nitrosation. The modified biotin switch assay was used to detect S-sulfuration in Hek293 cells transfected with parkin. Sites of S-sulfuration were determined using purified protein and mass spectrometry. Liu et al. studied the effect of H₂S on bone marrow mesenchymal stem cells (BMMSCs) [149]. It was determined H₂S is required for BBMSC self-renewal and differentiation. The effect was attributed to Ca²⁺ channel S-sulfuration. S-sulfuration of ion channels was determined using multiple methods including Ellman’s reagent (DTNB); although not specific to S-sulfuration, live cells were added to a solution of DTNB to measure S-sulfuration on cell surfaces. TRPV6 calcium channel was immunoprecipitated and analyzed for S-sulfuration by a maleimide assay where only one fluorophore-conjugated maleimide was used (Alexa Fluor® 488 C5 maleimide) to test for S-sulfuration but not S-nitrosation. After treatment with maleimide, the protein was treated with DTT to reverse protein-maleimide disulfides formed from reaction with persulfides. The loss of fluorescence after DTT treatment has been correlated to S-sulfuration. Mass spectrometry was used to find S-sulfurated cysteine residues on TRPV6 channel from cells treated with NaSH. S-sulfuration of GAPDH was also studied using this type of assay, as well as modified biotin shift, by Mir et al. in 2014 [150]. Other proteins shown to be S-sulfurated using the modified biotin shift assay include Keap [151, 152], MEK1 [153], PLN [154], androgen receptor (AR) [155], eNOS [156], Kv4.3 [157], PTEN [158], and pyruvate carboxylase [159].

Zhang et al. developed a tag-switch technique for detecting S-sulfuration [160]. The technique involves reacting free thiols (-SH) and persulfides (-SSH) with a thiol
blocking reagent. After the reaction thioether linkages are formed from free thiols, and disulfides are formed from persulfides. After addition of a nucleophile, the nucleophile will react with disulfides, and not thioethers, selectively labeling the persulfides. One important consideration for the design of the assay is the first blocking reagent must give a chemical linkage with properties different from native protein disulfides. Commonly-used thiol blocking reagents like iodoacetamide and maleimides produce linkages which are chemically similar to native protein disulfides. This would result in native protein disulfides being labelled with the second reagent. To generate a linkage different from native protein disulfides, the authors used methylsulfonyl benzothiazole (MSBT) to label free thiols and persulfides. Derivatizing with MSBT generates an activated disulfide linkage which has high reactivity to nucleophiles. The nucleophile chosen was methyl cyanoacetate (MCA), which was biotin-linked. Using the technique the investigators detected $S$-sulfuration of GAPDH after NaSH treatment. GAPDH was $S$-sulfurated at basal conditions as well, although the signal was very weak. The technique was also used to visualize $S$-sulfuration in cells by fluorescent microscopy when exposed to fluorescein-labeled streptavidin.

$H_2S$ cannot react with free cysteine thiols directly and needs additional oxidants to $S$-sulfurate proteins (Equations 1.1.4-1 & 1.1.4-2). Thiols in $H_2S$ and free cysteines (-SH) are both in their most reduced oxidation states (-2) [161]. It may be possible for $H_2S$, in the form of sulfide ($HS^-$), to react with disulfides resulting in an $S$-sulfurated thiol and a free thiol (Equation 1.1.4-3).

$$\text{(1.1.4-1)} \quad H_2S + \text{protein-SH} \rightarrow \text{no reaction}$$

$$\text{(1.1.4-2)} \quad HS^- + \text{protein-SH} \rightarrow \text{no reaction}$$

$$\text{(1.1.4-3)} \quad HS^- + \text{protein-S-S-protein} \rightarrow \text{protein-SSH + protein-S}^-$$

Francoleon et al. investigated the reaction of disulfides with $H_2S$ by reacting oxidized glutathione (GSSG) and oxidized papain with $H_2S$ [162]. Papain is a cysteine protease and a reduced cysteine is required for catalytic activity. It was found $H_2S$ can reduce GSSG to form a glutathione persulfide (GSSH) and reduced glutathione (GSH). When
treated with low concentrations of H$_2$S, the enzymatic activity of papain decreased, while S-sulfuration increased. At higher concentrations of H$_2$S the S-sulfuration of papain decreased, while the activity increased. It was theorized that at low H$_2$S concentrations; H$_2$S acts as an S-sulfurating agent, while at higher H$_2$S concentrations; excess H$_2$S can act as a reducing agent for protein persulfides and regenerate the free thiol. It has been reported that the H$_2$S can activate vascular endothelial growth factor receptor 2 (VEGFR2) by cleaving a disulfide bond between Cys 1045 and Cys 1024 [163]. Greiner et al. reported that H$_2$S can cause protein oxidation through polysulfides [143]. This is an interesting finding since the work by Francoleon et al. suggests H$_2$S acts as a reducing agent. It was found different H$_2$S donors can reduce the enzymatic activity of PTEN by thiol oxidation through polysulfides. H$_2$S donor GYY4137 was also able to produce polysulfides, and the effect is not only attributed to sulfide salts. The mechanism of oxidation was determined to be S-sulfuration of active site Cys 124, followed by formation of an internal disulfide between Cys 124 and nearby Cys 71.

Other mechanisms of S-sulfuration have been postulated to be reaction of H$_2$S with protein sulfenic acids (-SOH), S-nitrosothiols (-SNO), trans-sulfuration between proteins, and reaction of reduced protein thiols with polysulfides generated from H$_2$S [94]. Although H$_2$S cannot react with reduced cysteines directly, polysulfides generated from H$_2$S oxidation may. Equation 1.1.4-4 shows the proposed reaction of a reduced protein thiol with a polysulfide resulting in S-sulfuration.

$$\text{HSSH} + \text{protein-S}^- \rightarrow \text{HS}^- + \text{protein-S-SH}$$

In addition to S-sulfurating protein thiols H$_2$S has been shown to react with and sulfurate 8-nitro cGMP (8-nitroguanosine 3',5'-cyclic monophosphate) to form a novel secondary messenger 8-SH cGMP [164]. The reaction of H$_2$S and 8-nitro cGMP was later shown to be mediated by polysulfides [165]. Increasing evidence shows that polysulfides are important H$_2$S derived molecules, and protein S-sulfuration may occur through polysulfides. Although protein polysulfides (e.g. -SSSH) have been predicted, they have not been reported in the literature, and S-sulfuration appears to involve the addition of only one sulfur to cysteines (-SSH).
1.1.5 Tissue Levels of H$_2$S

A very wide range of H$_2$S concentrations have been reported in blood and tissues ranging from undetectable to as high as 600 µM [166]. This issue has been reviewed several times and stems from the use of unreliable detection methods for H$_2$S in biological systems (vide infra) [92, 120, 130, 166]. Sometimes working experimental concentrations, i.e. the concentration of exogenous H$_2$S required to produce a biological effect, or H$_2$S production stimulated by the addition of cysteine, may be reported as a tissue concentration. Most commonly reported blood H$_2$S concentrations are in the 10-100 µM range [166]. The human nose is very sensitive to H$_2$S; Furne et al. reported a 1 µM solution in pH 7.4 buffer produces a “detectable, but weak” odour, while a 5 µM solution is “unpleasant” [167]. This observation and other experiments indicate many reported H$_2$S tissue concentrations are not accurate. Furthermore, calculations by researchers suggest that the human body does not contain enough cysteine and sulfur to maintain H$_2$S production at the commonly reported micromolar levels [166, 167]. Micromolar H$_2$S concentrations are also high enough to be toxic; in cell homogenates NaSH inhibited cytochrome c oxidase with an IC$_{50}$ of only 0.32 µM [168].

When measured using detection methods that are considered more reliable, endogenous H$_2$S concentrations are in the submicromolar range. Furne et al. reported brain and liver H$_2$S concentrations following 30 s homogenation as 14 ± 3 nM and 17 ± 3 nM using gas chromatography [167]. Whitfield et al. was unable to detect H$_2$S in trout blood using real time measurement with a polarographic H$_2$S sensor [140]. Wintner et al. found rat blood H$_2$S levels were in the 0.4–0.9 µM range using an HPLC method [169]. Experiments by Whitfield et al. also showed H$_2$S is rapidly catabolized in the blood (vide supra) [140]. Experiments where H$_2$S was intravenously administered to rats while exhaled H$_2$S was simultaneously measured showed H$_2$S was rapidly cleared when intravenous administration was stopped [170]. It is now clear H$_2$S is not a circulating gasotransmitter and reacts rapidly in the presence of tissues [120]. Due to the reactivity of H$_2$S with a large number of different compounds, a clear and convenient biomarker of H$_2$S catabolism or turn-over does not exist [92]. While H$_2$S tissue concentrations are low and production by CBS and CSE is relatively low, usually relatively high H$_2$S
concentrations required to produce biological effects. For instance high micromolar concentrations are required to produce vasorelaxant effects of H$_2$S [92]. Production by CBS and CSE may be insufficient to explain the biological activities attributed to endogenous H$_2$S [171].

In addition to free H$_2$S, two other sulfide sources are present in biological systems, acid-labile sulfide, and sulfane sulfur, also known as reductant labile sulfur or bound sulfur [172, 173]. Acid-labile sulfide is sulfide contained in iron-sulfur clusters contained in non-heme iron-containing proteins, which upon treatment with acid release sulfide. Sulfane sulfur refers to a divalent sulfur atom which is bonded to another sulfur atom. Sulfane sulfur species include persulfides (R-S-S'), polysulfides (RS$_n$SR), thiosulfate ($S_2O_3^{2-}$), thiosulfonates (R-S$_2$O$_2^-$), polythionates (SO$_3$-$S_n$-SO$_3$), and elemental sulfur (S$_8$). Treatment with a reducing agent such as DTT may release sulfide from sulfane sulfur species [173]. Depending on the sample handling and analytical method, sulfide may be released from these two sources during experiments leading to an over-estimation of free H$_2$S [120].

1.1.6 H$_2$S Detection Methods

The most commonly used and the oldest method of H$_2$S detection is the methylene blue method [1, 174]. The methylene blue method is a spectrophotometric method based on the reaction of iron (III) chloride (FeCl$_3$) and N,N-dimethyl-p-phenylenediamine (NDPA) in the presence of HCl. The method involves trapping of H$_2$S from biological samples through the use of zinc acetate, and variations of the H$_2$S extraction and trapping procedure has led to two versions of the method, the direct, and indirect [166]. Zinc reacts with H$_2$S to form zinc sulfide (ZnS). In the direct method zinc acetate is added directly to the H$_2$S-containing sample, proteins are removed from the sample by precipitation with trichloroacetic acid (TCA), and the zinc sulfide is reacted with NDPA in the presence of FeCl$_3$ and HCl. In the indirect method a piece of filter paper is soaked in zinc acetate and placed in a sealed flask with the sample. Due to its volatility, H$_2$S is slowly driven from the sample into the gas phase and reacts with the zinc acetate on filter paper. The filter paper is then removed and reacted with NDPA in the presence of FeCl$_3$ and HCl. The final reaction with NDPA is usually incubated for 30 minutes to allow the
formation of methylene blue. The solution is measured spectrophotometrically at 670 nm, and is stable for hours.

The methylene blue method has the advantages of being inexpensive, easy to use, and requires no specialized equipment. The major flaw with the methylene blue method is the use of acidic conditions in the direct methylene blue method may cause protein desulfuration and release acid labile sulfur from biomolecules [120, 166, 172, 175, 176]. Effectively, the methylene blue method is measuring bound sulfur in addition to free H$_2$S and vastly overestimating the H$_2$S concentration. When using the indirect methylene blue method acids such as TCA may be added to tissues [177], which results in the same issues as with the direct method. The detection limit for the methylene blue method is 2 µM [176], making it unsuitable for detection of submicromolar concentrations of H$_2$S. Methylene blue has also been reported to form dimers and trimers, and as a result may not follow Beer’s law [178].

Another commonly used H$_2$S detection method is the sulfur specific ion selective electrode (ISE) [140, 179]. This method is actually selective towards S$_2^-$ and requires highly alkaline conditions to convert H$_2$S to S$_2^-$. Similarly to the acid conditions in the methylene blue method, the alkaline conditions employed using the ISE cause drastic alterations in biological samples [120, 166]. The standard buffers used with the ISE are known to cause cysteine desulfuration, for example the alkaline conditions can release sulfide from bovine serum albumin [140]. Like the methylene blue method, the ISE is not specific to free H$_2$S, and use of this method for measuring free H$_2$S leads to overestimation. Like the methylene blue method, it is also unsuitable for real-time measurements.

H$_2$S can be detected by high-performance liquid chromatography (HPLC) or gas chromatography (GC). Detection with HPLC involves derivatization with the thiol-reactive probe monobromobimane [169, 176, 180]. Two equivalents of monobromobimane react with sulfide to form a fluorescent sulfide dibimane molecule which can be separated and detected by HPLC with a fluorescence detector. This method is also an endpoint method. The optimum conditions for the derivatization reaction were found to be pH 9.5, 1% O$_2$ atmosphere, and the presence of the metal chelator DTPA
The limit of detection was 5 nM. The method has also been adapted to measure acid labile sulfide and reductant labile sulfide in addition to free H$_2$S [173]. Although the monobromobimane level is vastly more accurate and sensitive than the methylene blue method, Wintner et al. reported discrepancies between monobromobimane detection and the polarographic H$_2$S sensor when measuring Na$_2$S spiked human blood [169]. While the polarographic H$_2$S sensor showed rapid tissue catabolism of H$_2$S, monobromobimane did not, and showed a gradual decrease in H$_2$S level over time. The authors suggest monobromobimane may be reacting with the sulfide-containing products of H$_2$S catabolism in tissues. H$_2$S can also be detected by headspace analysis using GC when coupled to a sulfur chemiluminescence detector [167, 175, 181, 182]. Since gaseous headspace is being analyzed this method is specific to free H$_2$S gas. Like HPLC, GC is an end-point measurement.

The polarographic (amperometric) H$_2$S sensor is a modification of the Clark-type oxygen microsensor [140, 183, 184]. This sensor is the only commonly used real-time method of H$_2$S measurement. The specificity to free H$_2$S is due to the use of a gas-permeable polymer membrane that separates the anode, cathode, and the electrolyte solution from the sample being measured [184]. The electrolyte solution contains ferricyanide ([Fe(CN)$_6$]$^{3-}$), which is reduced at the anode to ferrocyanide ([Fe(CN)$_6$]$^{4-}$) and re-oxidized at the cathode, which generates a background current. After it diffuses through the gas-permeable membrane, H$_2$S reduces ferricyanide to ferrocyanide, generating a current which is subtracted from the background current. Detection limits of 10 nM [184] and 14 nM [140] have been reported. Since the sensor consumes H$_2$S it might not be appropriate for measurement of low volume samples [166]. The use of a gas permeable membrane has also been used to detect free H$_2$S using the thiol-reactive probe 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by Faccenda et al. [185].
1.2 Nitric Oxide

1.2.1 Introduction and Biological Functions of NO

Nitric oxide (NO) is an important gasotransmitter synthesized in mammalian tissues. A number of studies by multiple research groups in the late 1970’s and 1980’s led to the identification of NO as the endothelium derived relaxing factor (EDRF), and later the arginine-NO pathway which synthesizes NO. In 1977 Murad et al. discovered NO could activate guanylate cyclase and generate cyclic GMP [186]. However, at the time NO was not known to be produced in the body, and was most well known as a toxic pollutant. In 1980 Furchgott and Zawadzki observed endothelium-dependent vasorelaxation, where acetylcholine relaxed isolated blood vessels only when the endothelium was intact [187]. It was later discovered acetylcholine stimulated the release of an unknown compound dubbed the endothelium derived relaxing factor (EDRF), which can be inhibited by methylene blue and hemoglobin, suggesting EDRF was a free radical [188]. In 1986 Gryglewski et al. discovered EDRF can be inhibited by superoxide anion (O$_2^-$) and protected by superoxide dismutase (SOD) [189]. In 1987, based on the accumulating evidence, Furchgott, Ignarro, and Moncada each identified EDRF as nitric oxide [2, 190, 191]. In 1988 Moncada discovered NO is synthesized from L-arginine [192, 193]. Thus the field of NO biochemistry was born. The 1998 Nobel Prize in medicine was awarded to Furchgott, Ignarro, and Murad for their discoveries concerning NO. Moncada was somewhat controversially excluded from the prize due to the policy of awarding the prize to only three individuals.

While a vast number of physiological processes are affected by NO the major role of NO is control of vascular tone [194]. NO is synthesized in vascular endothelium, after which it diffuses to the surrounding vascular smooth muscle, and activates soluble guanylate cyclase causing an increase in intracellular cGMP. The increase in cGMP triggers a signal cascade, which causes a decrease in intracellular Ca$^{2+}$ and results in vasorelaxation [195]. NO is a major determinant of basal vascular tone, and pharmacological inhibition of NOS raises systolic blood pressure [196]. NO production in endothelial cells is controlled by a number of different stimuli [194], the major being endothelial shear stress [197]. Control of vascular tone and blood pressure by NO is
ubiquitous to all mammals, and some, but not all, non-mammalian vertebrates [198]. Penile erection is controlled by NO, and drugs used to treat erectile dysfunction such as sildenafil (Viagra®) alter NO homeostasis [199-201]. NO synthesized in endothelial cells also prevents platelet aggregation [202]; this protects blood vessels against thrombosis, and atherosclerosis [203]. NO mediates angiogenesis and wound healing [204-206]. NO produced by iNOS in macrophages is responsible for antimicrobial activity of macrophages [207]. In the nervous system NO controls vascular tone, and acts as a neuromodulator; affecting long-term potentiation, learning, and memory [208].

1.2.2 NO-Producing Enzymes

Nitric oxide is synthesized by three isoforms of nitric oxide synthases (NOS), named either after their localization (endothelial NOS, neuronal NOS), or by their basal level of NO production (inducible NOS). All nitric oxide synthases are homodimers, and require flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH₄) as co-factors [201]. Two isoforms of NOS; neuronal NOS (nNOS, NOS1), and endothelial NOS (eNOS, NOS3) are regulated by intracellular Ca²⁺ through calmodulin [201]. Inducible NOS (iNOS, NOS2) also binds calmodulin, however the binding is not affected by intracellular Ca²⁺ concentration, and instead the enzyme is constitutively active. Nitric oxide synthases convert L-arginine and O₂ to L-citrulline and NO, using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate. For every mol NO formed NOS requires 2 mol O₂ and 1.5 mol NADPH [209].

nNOS is constitutively expressed in the brain, although it is not exclusively localized to the brain [210]. nNOS maintains synaptic plasticity and blood pressure in the nervous system [201]. nNOS is also present in the peripheral nervous system, and has been detected in epithelial cells of lung, uterus, stomach, in kidney macula densa cells, pancreatic islet cells, and human skeletal muscle cells [209, 210]. Although eNOS is largely responsible for vasodilation outside of the nervous system, accumulating evidence shows nNOS also contributes [211]. eNOS is expressed mostly in endothelial cells and is responsible for endothelium-dependent vasorelaxation and vasoprotective effects of NO [201]. iNOS is expressed in macrophages and is responsible for the cytotoxic effects of NO.
1.2.3 Mechanisms of NO Signaling: cGMP and S-Nitrosation

The major mechanisms of NO action are through the secondary messenger cGMP and protein post-translational modification (S-nitrosation). The vasodilation effects of NO are mediated through the cGMP dependent pathway. NO activates soluble guanylate cyclase (sGC) by binding to sGC heme iron in an ATP and GTP dependent manner [212]. Activation of sGC causes an increased production of the secondary messenger cGMP, which activates cyclic GMP-dependent protein kinase (also known as Protein Kinase G,PKG, cGK) [213]. The number of targets for PKG is vast [214]. In the case of vascular smooth muscle relaxation, the activation of PKGI causes a decrease in intracellular Ca\(^{2+}\), which decreases the activity of myosin light kinase (MLCK), decreases myosin light chain phosphorylation and leads to vasorelaxation. Decrease in intracellular Ca\(^{2+}\) is caused by multiple molecular targets of PKGI, and not a single factor. cGMP-dependent NO signaling has been described as the “classic” NO signaling pathway [195].

NO may modify cysteine residues by S-nitrosation, which is the attachment of NO-derived nitrosonium (NO\(^{+}\)) to cysteine, to form S-nitrosocysteine (R-SNO). The modification is also commonly called S-nitrosylation. S-nitrosation of a protein is dependent on the chemical microenvironment; the presence of S-nitrosating species, cellular compartmentalization, and the degree of cysteine reactivity [195]. S-nitrosation is particularly gaining interest in pathophysiological states where hyper- or hypo- S-nitrosation of proteins contribute to pathophysiology of various diseases [215, 216]. Rather than general hyper-S-nitrosation from increased nitrosative and oxidative stress, changes in S-nitrosation status of specific proteins are changed in disease states. Changes in protein S-nitrosation has been implicated in the pathology of cardiovascular disease, sickle cell anemia, cancer, pulmonary arterial hypertension, diabetes, malignant hyperthermia, and neurodegenerative diseases like Parkinson’s disease and Alzheimer’s disease [215, 216].

NO cannot react directly with cysteine thiols to form S-nitrosothiols. For S-nitrosation to occur, NO first needs to be oxidized. The mechanisms of S-nitrosothiol formation in vivo have been reviewed by Broniowska and Hogg [217]. Two possible S-nitrosating agents are dinitrogen trioxide (N\(_2\)O\(_3\)) and nitrogen dioxide (NO\(_2\)) which can
be formed by the oxidation of NO by O$_2$. N$_2$O$_3$ may react with thiols directly to form an S-nitrosothiol (Equation 1.2.3-1).

\[
(1.2.3-1) \quad \text{RSH} + \text{N}_2\text{O}_3 \longrightarrow \text{RSNO} + \text{NO}_2^-
\]

Another pathway for S-nitrosothiol formation is NO$_2$ reacting with thiols to form thiol radicals (RS$^\bullet$). Thiol radicals may then react with NO directly with to form S-nitrosothiols (Equations 1.2.3-2 & 1.2.3-3).

\[
(1.2.3-2) \quad \text{RS}^\bullet + \text{NO} \longrightarrow \text{RS}^\bullet + \text{NO}_2^-
\]

\[
(1.2.3-3) \quad \text{RS}^\bullet + \text{NO} \longrightarrow \text{RSNO}
\]

Although N$_2$O$_3$ and NO$_2$ provide a pathway for S-nitrosothiol formation, some questions remain about the kinetics of the initial oxidation of NO to NO$_2$ and N$_2$O$_3$, which are slow under physiological NO and O$_2$ concentrations [217]. S-nitrosation of proteins may also occur through transnitrosation reactions with small molecular weight S-nitrosothiols like S-nitrosoglutathione (GSNO) (Equation 1.2.3-4).

\[
(1.2.3-4) \quad \text{RS}^\bullet + \text{GSNO} \longrightarrow \text{RSNO} + \text{GS}^-
\]

Interestingly cytochrome c can promote the formation of GSNO through its heme iron [218, 219]. The increased GSNO formation in-turn promotes protein S-nitrosation. Another possible pathway for S-nitrosation is through dinitrosyl iron complexes (DNIC) which can S-nitrosate proteins by transnitrosation [220-222]. DNIC are complexes with NO, iron, and either small molecular weight thiols or protein thiols. The relative contribution of various S-nitrosation mechanisms in vivo is still unknown.

### 1.2.4 Role of NO in Wound Healing

Wound healing is an incredibly complex and coordinated process which can be divided into three overlapping phases; inflammation, tissue formation, and tissue remodeling [223]. This process is controlled by numerous cytokines that act on many different cell types. NO has been shown to be important factor in wound healing, and affect the activity of these cytokines [206]. Even before the discovery of NO as a gasotransmitter it has
been shown arginine-deficient animals showed impaired wound healing [224]. Increased arginine metabolism and conversion to citrulline has been observed in wounds [225]. Urinary NO\(_2^-\), a biomarker for NO, increases dramatically after injury, and remains elevated during healing [226]. Both eNOS [227], and iNOS [228] knockout mice have exhibited impaired wound healing. Although iNOS is highly upregulated during inflammation, NO plays a role in all stages of wound healing [229]. The production and action of many growth factors necessary for wound healing is NO-dependent. For example the production of vascular endothelial growth factor (VEGF) is NO-dependent [230], as well as the downstream effects of VEGF [231]. VEGF is one of many cytokines responsible for angiogenesis during wound healing [223]. In addition to cytokines, numerous enzymes and transcription factors involved in wound healing are affected by NO. Numerous animal studies have been conducted on the efficacy of NO-releasing materials on cutaneous wound healing [232-241]. The development of NO-releasing wound dressings is however hampered by the inconvenient chemistry of commonly-used NO-releasing groups. The most commonly used NO-releasing groups, diazeniumdiolates (NONOates) and S-nitrosothiols, are simple to prepare, however exhibit short or very short half-lives [242]. S-nitrosothiols are also temperature sensitive. The second challenge is, NO-releasing materials cannot merely release NO to be beneficial, and must have other properties which make it appropriate to use as a wound dressing [233].
CHAPTER 2

MICROPLATE-BASED COLOURIMETRIC DETECTION OF FREE H$_2$S
2.1 Introduction

Driven by the need for sensitive and specific detection methods in H$_2$S research we sought to develop a new method for H$_2$S detection. This method was inspired by lead acetate test strips for H$_2$S, and the use of zinc acetate in the methylene blue method. In the lead acetate test, lead reacts with H$_2$S to form a black PbS precipitate, which serves as a quick chemical test for H$_2$S [243]. Similarly, in the methylene blue method H$_2$S reacts with zinc acetate to form ZnS, which is further reacted to form methylene blue [174]. The chemical basis for our assay is the reaction of Ag$^+$ with H$_2$S to form Ag$_2$S. This reaction has been used to synthesize Ag$_2$S nanoparticles, which have a high absorbance in the low wavelength visible and UV range [244]. The assay has been adapted to the 96-well plate microplate format, and takes advantage of the volatility of H$_2$S. The underside of a microplate cover, the circular area directly over the wells, was coated with a solution containing Nafion, glycerol, and AgNO$_3$. Nafion is a tetrafluoroethylene based polymer with regularly spaced perfluorovinyl side chains terminated with a sulfonate group. The combination of a hydrophobic backbone and hydrophilic side chains results in a polymer which forms a reverse micelle nanostructure [245]. The reverse micelle pores are 4-5 nm in diameter and has been used for templated nanoparticle synthesis [246, 247]. Solutions of sulfide spontaneously release gaseous H$_2$S according to Equation 2.1-1 [121]. When open to the atmosphere the equilibrium is driven to the left until virtually all the sulfide is lost as H$_2$S gas. The released H$_2$S will interact with the bottom of the microplate cover and react with Ag$^+$ to produce Ag$_2$S nanoparticles (Equation 2.1-2). The formation of Ag$_2$S can be monitored in real time using spectroscopy and is proportional to the H$_2$S concentration of the solution.

\[
(2.1-1) \quad 2\text{H}^+(\text{aq}) + \text{S}^2-(\text{aq}) \rightleftharpoons \text{H}^+(\text{aq}) + \text{HS}^-\text{(aq)} \rightleftharpoons \text{H}_2\text{S(}\text{aq}) \rightleftharpoons \text{H}_2\text{S(g)}
\]

\[
(2.1-2) \quad \text{H}_2\text{S} + 2\text{Ag}^+ \rightarrow \text{Ag}_2\text{S} + 2\text{H}^+
\]
2.2 Materials

2.2.1 Reagents

Costar 96-well plates (3370) were purchased from Corning Life Sciences. Nafion LIQUION™ (LQ-1115) dispersion was purchased from Ion Power Inc. (www.nafionstore.com). Glycerol (G3700) was purchased from ACP Chemicals. AgNO₃ was purchased from Fisher Scientific. Na₂S•9H₂O (02152580) was purchased from MP Biomedicals.

2.2.2 CSE Purification

Recombinant CSE was purified according to the detailed protocol described by Faccenda et al. [185].

2.2.3 Animals and Tissue Collection

Mouse livers were provided by Dr. Lisa Porter (University of Windsor). All experimental techniques complied with the guidelines set forth by the University of Windsor animal care committee. BALB-c mice were purchased from The Jackson Laboratory (Bar Harbor, USA). The mice were housed at the University of Windsor animal care facility. Mice were euthanized by CO₂ inhalation. The liver was removed and placed on ice.
2.3 Methods

2.3.1 Nafion-Coated Microplate Cover Fabrication

Nafion dispersion (LQ-1115) was mixed with glycerol in a 4:1 ratio. AgNO₃ was added from a 100 mM solution in Milli-Q H₂O to a final concentration of 3 mM. The solution was mixed well by vortexing. 15 µL of the solution was carefully pipetted (in order not to introduce bubbles) onto each well on the underside of a microplate cover. The cover was dried at room temperature for at least 1 hour before use.

2.3.2 Microplate Measurements

For all H₂S measurements using the assay system, the absorbance of the entire microplate along with the Nafion-coated microplate cover was measured. All solutions were spaced-out in the microplate wells. In a microplate row, a well adjacent to a sample was left empty, and the next sample was loaded into the well following the empty well. An entire row below or above solution-containing samples was also left empty. As a result a maximum of 24 samples were measured per 96-well microplate. All measurements were done using a Spectramax 384 plate reader at 310 nm.

2.3.3 Standard Curve and Specificity Test

Na₂S standard solutions were diluted in 100 mM phosphate buffer (pH 7.0). 300 µL of each dilution was pipetted into the 96-well plate and the plate was covered immediately with the fabricated Nafion-coated cover. H₂S was allowed to form from Na₂S for 1 h. The absorbance of the microplate and cover was then measured to generate a standard curve. To test specificity of the assay system, cysteine (Sigma), reduced glutathione (Sigma), and DTT (MP Biomedicals) were compared to Na₂S using the same methodology.

2.3.4 LoD Determination

The limit of detection (LoD) for the assay was determined according to the EP17 guideline published by the National Committee for Clinical Laboratory Standards (NCCLS) [248, 249]. The LoD (limit of the detection) is defined as the lowest analyte concentration that can be reliably differentiated from the LoB (limit of blank). The LoB
is the highest apparent concentration that can be produced by a blank sample. 72 replicates of a blank sample (300 µL 100 mM pH 7.0 phosphate buffer), and 72 replicates of a low concentration sample (300 µL 10 µM Na₂S in 100 mM pH 7.0 phosphate buffer) were used for the calculations.

### 2.3.5 CSE Assay

CSE was diluted to a final concentration of 0.29 µg/µL in pH 8.0 100 mM phosphate buffer with 2 mM EDTA. PLP was added to the enzyme to a final concentration of 25 µM. 295 µL of cysteine dilutions in the same buffer (without PLP) were added to the 96-well plate, and the plate including the cover was pre-incubated at 37°C for 15 minutes in the plate reader. 5 µL of the enzyme mixture was added to each cysteine dilution and the assay was run for 40 minutes at 37°C. Absorbance at 310 nm was measured every 20 s. Data was fit into a Michaelis-Menten equation using the Solver function in Microsoft Excel to calculate kinetic parameters. For determination of the kinetic parameters of CSE the tail of each real time curve (1900-2400 s) was chosen to calculate the enzymatic rate.

### 2.3.6 Mouse Liver Homogenate H₂S Production

An endpoint assay was conducted since homogenate solutions absorb UV light. To accomplish this, two different microplates were used, and the Nafion-coated cover was switched between them during the experiment. One microplate was used for absorbance readings, and one for incubating the homogenate. Prior to the trial, the Nafion-coated microplate cover was read on the first microplate to establish baseline absorbance. Mouse liver was homogenized in PBS using a glass dounce homogenizer. Commercial protease inhibitor cocktail (Sigma P8340) was added in a 1:100 ratio to the homogenate. 200 µL of the homogenate was pipetted into the second microplate. L-cysteine was added to the homogenate to a concentration of 5 mM. The Nafion-coated cover was placed immediately on the second microplate with the homogenate and cysteine mixture. The microplate was incubated at 37 ºC for 40 min. Afterwards, the Nafion-coated cover was transferred from the second microplate (with homogenate), to the first one (blank) to measure the change in absorbance.
2.4 Results

**Figure 2.4-1** Schematic of the H$_2$S assay. H$_2$S is volatilized in the microplate well, and reacts with the silver ions on the underside of the Nafion/Ag-coated microplate cover to produce silver sulfide, which is quantified spectrophotometrically. Adapted from [250] with permission.
Figure 2.4-2 A photograph of the Nafion/Ag-coated 96-well plate cover after exposure to 100 mM pH 7.0 phosphate buffer with various concentrations of Na$_2$S, dithiothreitol (DTT), reduced glutathione (GSH), and L-cysteine (Cys). Adapted from [250] with permission.
Figure 2.4-3 Corrected absorbance of the Nafion/Ag-coated 96-well plate cover after exposure to 100 mM pH 7.0 phosphate buffer with various concentrations of Na$_2$S, dithiothreitol (DTT), reduced glutathione (GSH), and L-cysteine (Cys). Adapted from [250] with permission.
Figure 2.4-4 UV–vis spectrum of a blank microplate and cover (black, dashed), the microplate with cover coated with Nafion containing Ag\(^+\) ions (blue, solid), and the same microplate and cover after exposure to 300 µL of 100 µM Na\(_2\)S solution in pH 7.0 100 mM phosphate buffer for 1 h (red, solid). The resulting UV–vis spectrum (red, solid) is characteristic of Ag\(_2\)S nanoparticles. Adapted from [250] with permission.
Figure 2.4-5 Standard curve for the colourimetric microplate H₂S assay. Na₂S was used as a H₂S donor in 300 μL pH 7.0 100 mM phosphate buffer. Absorbance was measured after 1 h. Error bars represent the standard deviation (n=3). Adapted from [250] with permission.
Figure 2.4-6 H₂S generation by CSE, with various concentrations of L-cysteine, measured by the colourimetric microplate assay. Adapted from [250] with permission.
Figure 2.4-7 Single Michaelis-Menten plot of CSE activity using L-cysteine as a substrate. Three plots were generated to calculate the $K_M$ and $V_{max}$ of the enzyme. Adapted from [250] with permission.

Table 2.4-1 Kinetic parameters of recombinant CSE enzyme, and mouse liver homogenate H$_2$S production determined by the colourimetric microplate assay. The values are averages of three trials (n=3), with standard deviations. L-cysteine was used as a substrate for CSE. Homogenates were aerobically incubated with 5 mM L-cysteine. Adapted from [250] with permission.

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE $K_M$</td>
<td>11.13 ± 0.57 mM</td>
</tr>
<tr>
<td>CSE $V_{max}$</td>
<td>0.45 ± 0.01 nmol min$^{-1}$</td>
</tr>
<tr>
<td>Mouse liver H$_2$S production</td>
<td>4.89 ± 0.19 nmol min$^{-1}$ mL$^{-1}$ homogenate</td>
</tr>
</tbody>
</table>
2.5 Discussion

2.5.1 Assay Design and Microplate Cover Preparation

A Nafion dispersion (Ion Power LQ-1115) was used to fabricate Nafion films. LQ-1115 is a 15% wt. Nafion dispersion in alcohols and H₂O. Casting of the dispersion and evaporation of alcohols generates a Nafion film. Addition of AgNO₃ to the mixture resulted in cracking of the Nafion film after drying. Glycerol was added to the mixture to prevent film cracking. With glycerol it was determined empirically that 3 mM was the highest AgNO₃ concentration that could be used without the resulting film cracking after drying. After drying the resultant film is soft due to the glycerol and should not be mechanically disturbed. After the addition of glycerol it is unknown if the reverse micelle nanostructure of Nafion is maintained. Although the resultant Nafion film is stable for at least a day, extended storage resulted in spontaneous colour change likely due to the photoreactivity of silver. The measured wells were spaced out leaving an empty well surrounding every well being measured (eg. Figure 2.4L2). This was done to minimize the chance of interference from H₂S diffusion from an adjacent well. As a result a 96-well plate could only measure a maximum of 24 different wells using our procedure.

2.5.2 Optical Properties

After casting, the resulting Ag-containing Nafion film is transparent and does not alter the absorbance properties of the microplate cover (Figure 2.4-4). After exposure to H₂S a brown colour develops. Due to the design of the assay only gaseous H₂S will come in contact and react with the silver to produce a colour change. As expected other commonly used thiols in biochemistry do not produce a reaction and colour change (Figure 2.4-3). Ag₂S nanoparticles produce very strong absorbance in the 200-300 nm range and broad absorbance tail [244]. The spectra produced after exposure to 100 µM Na₂S is consistent with Ag₂S nanoparticle formation. Although absorbance at 200-300 nm would allow the highest sensitivity to Ag₂S formation, the polystyrene microplate used absorbs light in this spectral range (Figure 2.4-4). Measurement at 310 nm was chosen as a compromise between maximizing detection, and minimizing interference from the microplate cover, and possibly biomolecules, for example amino acids and
nucleic acids absorb light in the sub-300 nm range. Due to measurement at 310 nm real-
time measurements are also limited by the turbidity of the sample. Turbid samples cannot
be measured in real-time using our assay system since the absorbance of the solution is
measured simultaneously, however they can be measured in an end-point manner.

2.5.3 Limit of Detection and Linear Range

The limit of detection (LOD) of the assay was determined according to the EP17
guideline published by the National Committee for Clinical Laboratory Standards
(NCCLS) [248, 249]. The LOD was calculated to be 2.61 nmol (8.70 µM), and the linear
range of the assay was from 2.61 nmol (8.70 µM) to 30 nmol (100 µM) (Figure 2.4-5).
Larger concentration samples may produce lower absorbances (Figure 2.4-3) than 100
µM. When this occurred the colour of the reacted Nafion was not uniform and a dark ring
was produced (Figure 2.4-2). Although the spots at concentrations greater than 100 µM
appeared darker, a lower absorbance was measured. The LOD of 8.7 µM does not allow
measurement of H$_2$S in biological samples without adding substrate to stimulate H$_2$S
production.

2.5.4 CSE Enzyme Assay

To test the assay, the kinetic parameters of the enzyme CSE were determined using L-
cysteine as a substrate, and compared to the literature values. CSE activity was measured
in real-time, the $V_0$ was calculated from the slopes, and fit into the Michaelis-Menten
equation using the Solver function in Microsoft Excel. A $K_M$ of 11.13 ± 0.57 mM was
calculated and a $V_{\text{max}}$ of 0.45 ± 0.01 nmol min$^{-1}$. Although the $V_{\text{max}}$ is very close to the
$V_{\text{max}}$ determined by Faccenda et al. (0.37 ± 0.02 nmol min$^{-1}$) [185], the $K_M$ differs from
previously determined values. Previously obtained $K_M$ values are 3.79 ± 2.07 mM using
the PDMS-based DTNB method used by Faccenda et al. [185], and 2.75 mM, using the
methylene blue method [81]. This is somewhat different from the $K_M$ value determined
using our colourimetric assay. The discrepancy could be due to our rates being a
combination of enzymatic H$_2$S production, and H$_2$S volatilization. The enzymatic curves
generated do not represent normal enzymatic activity where after steady-state kinetics the
enzymatic activity decreases over time, and instead in our curves the enzyme activity
appears to increase over time until after ~1700 s (Figure 2.4-6). The tail end of each curve, from 1900 s to 2400 s, was used for $V_0$ calculations, in order to differentiate between the different substrate concentrations. The initial apparent increase in enzymatic activity is likely due to the establishment of equilibrium between H$_2$S production and volatilization. Although described as “real time” [250], the assay could perhaps be more accurately described as “near real-time”.

The $k_{\text{cat}}$ for CSE catalysis of cysteine was been reported as 0.4 s$^{-1}$ by Sun et al. [81], and 3.26 s$^{-1}$ by Faccenda et al. [185]. Based on the enzyme concentration used in our assay, volume of solution used in our assay, and the $k_{\text{cat}}$ determined by Sun et al. it was calculated that, after 1900 s, a total of 5.8 nmol (19 µM) H$_2$S has formed. Using the $k_{\text{cat}}$ value determined by Faccenda et al. the total H$_2$S formed after 1900 s was calculated to be 47 nmol (1.6 x 10^2 µM). These values are 1-2 orders of magnitude lower than the lowest cysteine concentration tested (4.0 mM). The steady-state assumption, that the concentration of the enzyme-substrate complex ([ES]) does not change, is likely true even after 1900 s (~31 min) from the start of the enzyme assay.

2.5.5 Mouse Liver Homogenate H$_2$S Production

Mouse liver homogenate H$_2$S production was measured using an endpoint variation of the microplate assay. As commonly done in the literature, H$_2$S production was stimulated by the addition of L-cysteine under aerobic conditions. Using 5 mM cysteine, mouse liver homogenate H$_2$S production was determined to be 4.89 ± 0.19 nmol min$^{-1}$ mL$^{-1}$ homogenate. Furne et al. measured H$_2$S production from mouse liver homogenate at 3.210 ± 0.162 nmol min$^{-1}$ mL$^{-1}$ using 10 mM L-cysteine and the polarographic H$_2$S sensor, a method specific to H$_2$S gas [167]. Using our assay system we were able to obtain similar values.
2.5.6 Possible Improvements in Assay Design

The design of the assay may be improved by allowing the Ag-containing Nafion to be in contact with the solution, but separated by a gas-permeable membrane. The Nafion and gas-permeable membrane could be moved to the bottom of the well plate. This might eliminate some of the lag during real-time measurements. The use of Ag could also be eliminated altogether, and replaced with a fluorescent probe. Fluorescence measurements are generally more sensitive than absorbance, and a significant decrease in detection limit would be expected if this could be accomplished.
2.6 Conclusions

In this chapter we developed a method for measuring H\textsubscript{2}S in biological samples. By coating the bottom of a microplate cover with Nafion polymer mixed with AgNO\textsubscript{3} a simple colourimetric assay for measuring H\textsubscript{2}S in 96-well plates was developed. The assay has a limit of detection of 8.7 µM, which is similar to the methylene blue method, and is linear up to 100 µM. As a demonstration of the assay, the kinetic parameters of CSE enzyme were measured, as well as H\textsubscript{2}S production from mouse tissue homogenate in the presence of cysteine. Our assay is simple, inexpensive, and specific to H\textsubscript{2}S gas. Although some limitations of the assay exist, they offer opportunity for improvement.
CHAPTER 3

REACTIONS OF H₂S WITH PROTEIN THIOLS AND S-SULFURATION OF GAPDH
3.1 Introduction

In this chapter we investigated the reactivity of H$_2$S with protein thiols. H$_2$S was previously discovered to mediate biological activity by post-translational modification of cysteine residues (S-sulfuration). The first protein discovered to be modified by H$_2$S was GAPDH [141]. In the original publication, purified GAPDH was S-sulfurated at Cys 152. The site of S-sulfuration was detected by mass spectrometry. When analyzed in cells, ~30% of GAPDH was S-sulfurated under basal conditions, determined by the modified biotin shift assay. Many other proteins, including actin and tubulin, have been shown to be S-sulfurated at high proportions. Massive basal S-sulfuration of proteins like actin and tubulin does not support H$_2$S’s role as a gasotransmitter, since a gasotransmitter by definition requires specific molecular targets [5]. Given that the levels of H$_2$S in tissues are in the nM range [167], it also raises the question of whether H$_2$S production is high enough to cause mass S-sulfuration of proteins. The modified biotin shift assay has recently been shown to be non-specific to S-sulfuration [142], and it is possible the high basal S-sulfuration of proteins is an artifact.

GAPDH is an important glycolysis enzyme with additional functions continually being discovered [251-253]. GAPDH contains three cysteine residues, Cys 152, Cys 156, and Cys 247. Some authors use a numbering system that maximizes the sequence homology between organisms where N-terminal residues are ignored, and the conserved residues are aligned [252]. Under this numbering system Cys 152 would be labeled as Cys 149. Mustafa et al. refer to this cysteine as Cys 150 [141]. Cys 152 is the active site catalytic cysteine residue, which attacks the substrate glyceraldehyde 3-phosphate [252]. A reduced Cys 152 is required for GAPDH activity, and oxidation of Cys 152 can abolish catalytic activity [253]. This raises the question of whether H$_2$S can react with the free Cys 152 on GAPDH and S-sulfurate it. The sulfurs in a free cysteine and in H$_2$S are in their most reduced states (-2), and a direct reaction with no additional oxidants is impossible [161].

The initial redox state of GAPDH was not explored in the initial publication; however it is an important consideration for the redox modification of biomolecules. We tested the possibility whether H$_2$S can react with reduced cysteines (-SH), cysteine
disulfides (-S-S-), or perhaps another oxidized form of cysteine (-SOH, -SNO). Work by Francoleon et al. has shown evidence that at low H$_2$S concentrations S-sulfuration can occur, while at higher H$_2$S concentrations, the excess H$_2$S can reduce the S-sulfurated protein thiols [162]. This is an interesting theory that has been considered in this work. Polysulfides are gaining recognition in recent years as important H$_2$S-derived biomolecules [34]. It may be possible for polysulfides to react with reduced cysteine thiols to S-sulfurate proteins. We have explored the S-sulfurating ability of polysulfides, as well as NaSH.

In this chapter, when analyzed by mass spectrometry, GAPDH was derivatized with excess iodoacetamide after treatments to yield carbamidomethylated persulfide derivatives (R-S-S-CH$_2$-CO-NH$_2$). This was done in order to help differentiate S-sulfuration from sulfinic acid formation. Depending on mass accuracy of an instrument, unmodified R-S-SH may be erroneously assigned to R-S-O$_2$H due to very similar masses.
3.2 Materials

3.2.1 Reagents

Sodium sulfide (161527), potassium polysulfide (12665), oxidized glutathione (G4376), BSA (A9647), NAD$^+$ (N7004), sodium arsenate (A6756), glyceraldehyde 3-phosphate barium salt (G5376), iodoacetamide (I1149), sinapinic acid (D7927), N-ethylnmaleimide (E3876), α-cyano-4-hydroxycinnamic acid (476870), vasopressin (V0377) were purchased from Sigma-Aldrich. NaCl (S2830) was purchased from ACP Chemicals. Dithiothreitol (DTT) (100597) was purchased from MP Biomedicals. Zeba™ desalting columns (89882) and tris base (BP152-1) were purchased from Fisher Scientific. C18 Zip Tips® (ZTC18S096) were purchased from Millipore. Sequencing grade trypsin (V511A) was purchased from Promega.

3.2.2 H$_2$O Purity

Terrific broth for growing *E. coli* was made with distilled H$_2$O. All other aqueous solutions were made using Milli-Q H$_2$O.
3.3 Methods

3.3.1 Vasopressin Treatments and Mass Spectrometry

1 µM vasopressin was diluted to 200 nM with pH 7.4 100 mM sodium phosphate buffer. Polysulfides in buffered solution were added to vasopressin in a 1:1 ratio. The solutions were incubated 30 min at 37 °C, and purified with Millipore Zip Tips® according to manufacturer protocol. Samples were diluted 1 part sample with 3 parts Milli-Q H₂O. 1 µL of sample was spotted with 1 µL of 10 mg/mL matrix solution (α-cyano-4-hydroxycinnamic acid in 60 % acetonitrile, 0.1 % trifluoroacetic acid) on a MALDI plate and analyzed by an Applied Biosystems Voyager DE-Pro mass spectrometer in reflector mode.

3.3.2 Polysulfide Concentration

Since polysulfides exist in equilibrium as a mixture of species, the concentration of polysulfides used was estimated. Since potassium polysulfide (12665) is ≥42% K₂S, polysulfide concentrations were calculated based on the assumption of 100% K₂S. This over-estimates polysulfide concentrations as the presence of larger molecular weight polysulfides will reduce the molarity of the solution.

3.3.3 GAPDH Assay Buffer Handling

GAPDH assay buffer (20 mM Tris pH 7.8, 100 mM NaCl, 1 mM NAD⁺, 10 mM sodium pyrophosphate, 20 mM sodium arsenate, and 0.1 mg/mL BSA) was made fresh weekly. Unused buffer was stored in at 4 °C in the dark. When performing the GAPDH assay, an aliquot of the buffer were taken out to be pre-warmed at 37 °C. After completion of an enzymatic assay any leftover pre-warmed buffer was discarded and not re-used. Milli-Q H₂O was used for buffer preparation.

3.3.4 Glyceraldehyde 3-Phosphate Preparation

Glyceraldehyde 3-phosphate (G3P) which was prepared from the barium salt according to the manufacturer protocol (Sigma G5376). After preparation the concentration of G3P was quantified using a standard colourimetric phosphate assay using molybdate. Aliquots
of G3P were stored at -20 °C. After thawing of an aliquot of G3P for use in the GAPDH enzyme assay any remaining G3P in the aliquot was not re-used and discarded.

3.3.5 GAPDH Purification

BL21(DE3) E. coli containing a pET15b vector expressing wild-type His-tagged human GAPDH, as well as a second cell stock expressing the C156S mutant, was kindly provided by Dr. Ann English (Concordia University). 1 L of Terrific broth containing 0.1 mg/mL ampicillin was inoculated with cells grown from an overnight pre-culture. Cells were grown at 37 °C until OD_{600} reached 0.4 to 0.6, at which point expression was induced by addition of 0.4 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were grown for a further 4 h, pelleted by centrifugation (3950 g, 30 min, 4 °C), supernatant was discarded and cell pellets were frozen overnight at -84 °C. Cells were resuspended in lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM PMSF, 1 mM DTT, 100 µg/mL lysozyme, 50 µg/mL DNase I, 0.2% Triton X100), incubated on ice for 30 min, lysed by sonication, and centrifuged (11300 g, 30 min, 4 °C). The supernatant was purified using a nickel affinity column (Sigma P6611) strictly following the manufacturer protocol published by Sigma-Aldrich. The protein was buffer exchanged into storage buffer (50 mM Tris 1 mM DTT pH 7.4) using a centrifugal filter (Millipore UFC903008), following manufacturer protocol. 2 M sorbitol (cryoprotectant) was added, and aliquots were stored at -84 °C.

3.3.6 Reduced GAPDH Enzyme Assay

GAPDH was buffer exchanged using desalting columns (Fisher 89882) into 20 mM Tris pH 7.8 before each trial. NaSH and PS stock solutions were diluted in 20 mM Tris pH 7.8 immediately before addition to GAPDH (0.5 µg/µL), and were added at a 1:1 volume ratio. GAPDH was incubated with NaSH or PS for 30 min at 37 °C. For the control untreated enzyme, buffer was used instead of NaSH or PS. 0.5 µg of the enzyme was added to 500 µL of assay buffer (20 mM Tris pH 7.8, 100 mM NaCl, 1 mM NAD^{+}, 10 mM sodium pyrophosphate, 20 mM sodium arsenate, and 0.1 mg/mL BSA). Buffer was pre-warmed at 37 °C before measurements. Reaction was initiated by the addition of
glyceraldehyde 3-phosphate (G3P), which was previously prepared from the barium salt according to manufacturer protocol (Sigma G5376). The conversion of NAD$^+$ to NADH was quantified by measuring absorbance at 340 nm with an Agilent 8453 UV/vis spectrophotometer equipped with a temperature controller set at 37 °C. Measurements were started immediately after the addition of G3P, and each sample was measured for 120 s. The treated GAPDH enzyme samples were stored on ice while waiting to be measured. A single set of different concentration samples was measured first, following a replicate set of the same samples, and a second replicate of the same samples, ie. the same concentration sample was not measured three times back-to-back.

3.3.7 Oxidized GAPDH Enzyme Assay

In the GSSG-oxidized GAPDH assay, GAPDH, in 20 mM Tris pH 7.8 was treated with equal-volume GSSG (10 mM in 20 mM Tris pH 7.8) for 1 h at room temp. The GAPDH assay was continued with buffer exchange, NaSH, PS, or DTT treatments, same as reduced GAPDH assay above. In the H$_2$O$_2$-oxidized GAPDH assay, GAPDH, in 20 mM Tris pH 7.8 was treated with equal-volume H$_2$O$_2$ (200 µM) for 30 min at room temp. The GAPDH assay was continued with NaSH, PS, or DTT treatments, same as reduced GAPDH assay above. In the GSNO-oxidized GAPDH assay, GAPDH, in 20 mM Tris pH 7.8 was treated with equal volume GSNO (1 mM in 20 mM Tris pH 7.8) for 30 min at 37 °C in the dark. The GAPDH assay was continued with buffer exchange, NaSH or PS treatments, same as reduced GAPDH assay above, except NaSH and PS treatments were done in the dark.

3.3.8 GAPDH Preparation and Digestion for Mass Spectrometry

Reduced wild-type GAPDH, or reduced C156S mutant GAPDH, or GSSG-treated GAPDH was prepared and treated with NaSH or PS using the same conditions as per enzyme assay. For the control untreated enzyme, buffer was used instead of NaSH or PS. GAPDH was then treated with equal volume 40 mM iodoacetamide in 20 mM Tris pH 7.8 for 2 h at room temperature in the dark. Enzyme was buffer exchanged using desalting columns into 50 mM ammonium bicarbonate (pH 8.0). Trypsin was added in a 10:1 protein to protease ratio, and GAPDH was digested overnight at 37 °C. GAPDH
tryptic digest was desalted using Millipore Zip Tips® according to manufacturer protocol.

To analyze GSNO-treated GAPDH a differential labeling protocol was used [24]. First GAPDH was treated with GSNO, then NaSH or PS, as per the oxidized enzyme assay. For the untreated control enzyme, buffer was used instead of NaSH or PS. After NaSH or PS treatment, GAPDH was treated with 20 mM iodoacetamide (from 200 mM stock in 20 mM Tris pH 7.8) for 2 h at room temperature in the dark. GAPDH was buffer exchanged into 100 mM sodium phosphate pH 7.0 using desalting columns. 10 mM sinapinic acid (from 200 mM stock in DMSO), and 20 mM N-ethylmaleimide (from 320 mM stock in Milli-Q H₂O) was added to GAPDH and the enzyme was incubated for 3 h at room temperature in the dark. GAPDH was buffer exchanged using desalting columns into 50 mM ammonium bicarbonate (pH 8.0) and trypsin digested overnight at 37 °C using a 10:1 protein to protease ratio. GAPDH tryptic digest was desalted using Millipore Zip Tips® according to manufacturer protocol.

### 3.3.9 Mass Spectrometry

Samples were analyzed using a Waters SYNAPT G2-Si mass spectrometer configured for nano-Electrospray ionization operated in positive mode coupled to a Waters nanoACQUITY UPLC system. The UPLC was configured for 1D single pump trapping with a Waters ACQUITY UPLC Symmetry C18 2G V/M trap column coupled with a Waters ACQUITY Peptide BEH C18. Mobile phase buffer A was 0.1% formic acid. Mobile phase buffer B was acetonitrile 0.1% formic acid. Samples were loaded on the trap column for 3 min at a flow rate of 5 µL/min in 97% buffer A. 60 min gradient was run at a flow rate of 0.5 µL/min where buffer A concentration was 97% at 0 min to 65% at 30 min, 40% at 35 min, 15% at 36 min, 15% at 41 min, and 97% at 42 min. All samples were first analyzed in MSe mode, followed by MS/MS sequencing. Reduced and GSSG-oxidized samples were analyzed using DDA MS/MS mode. The DDA acquisition method for MS/MS used an include list of theoretical m/z values for precursor ions matching the possible modifications of cys-containing peptides based on sulfide, iodoacetamide, and glutathione treatments. GSNO-oxidized and C156S mutant samples were analyzed by targeted MS/MS of precursor ions from MSe acquisitions.
3.3.10 Molecular Dynamics (MD) Simulations

All structure preparation and MD calculations were performed using the Molecular Operating Environment 2013 (MOE) program [25]. A high-resolution (1.75 Å) X-ray crystal structure of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) complexed with NAD+, from Homo sapiens, was obtained from the Protein Data Bank (PDB ID: 1U8F) [26]. The structure was then protonated using the default propKa [25, 27] method in MOE, and all water molecules were removed except those in or near the catalytic and ligand binding sites. The resulting complex was then solvated with water to four layers deep. Exploiting the fact that GAPDH is a homo-tetramer, Cys156 of chain B (Cys156B) was mutated to include a persulfide (-SSH) moiety, while Cys156 of chain A was maintained in its wild-type thiol (-SH) state. The entire solvated complex was then optimized (energy minimized) using the molecular mechanics AMBER12EHT force field until the root mean square gradient of the total energy fell below 0.21 kJ mol⁻¹ Å⁻¹. The MM optimized structure was then used as a starting structure for subsequent MD simulations in which a standard protocol was used. More specifically, the complex underwent an equilibration period in which temperature was gradually raised from 150 K to 300 K over a period of 100 ps. This was then followed by a 10 ns production MD in which the temperature was held at 300 K. A cluster analysis was then performed, and the median of the most populated cluster was chosen as the average structure. These analyses were performed separately on both chain A (-SH) and B (-SSH). The average structures of these two chains were superimposed and analyzed to find the effect of the mutation on GAPDH.
3.4 Results

3.4.1 S-sulfuration of Vasopressin

Vasopressin was used as a model peptide for studying S-sulfuration. Vasopressin ionizes well in MALDI-MS. Vasopressin has an amino sequence of GRPCNQFYC and forms an internal disulfide between the cysteines. Vasopressin was reacted with varying concentrations of polysulfides and NaSH. Figure 3.4.1-1 shows the spectra of vasopressin after reaction with increasing amounts of polysulfides relative to vasopressin. S-sulfuration of vasopressin is first observed at ~1,000 fold polysulfides where a singly S-sulfurated vasopressin peptide (m/z 1117) is visible. Relative intensity of the S-sulfurated peptide is ~10% of the unmodified (m/z 1084). At ~10,000 fold polysulfides the singly S-sulfurated vasopressin (m/z 1117) is now more intense at ~18% of the unmodified (m/z 1084). Doubly S-sulfurated (m/z 1150) vasopressin appears at ~10,000 fold polysulfide concentration and the relative intensity compared to the unmodified is ~8%. Higher concentrations of polysulfides produced more S-sulfuration; however the modification is in low stoichiometry compared to the unmodified peptide. NaSH produced the same results as polysulfides, requiring ~1,000 fold NaSH to observe S-sulfuration. To test the possibility that the disulfide in vasopressin is reforming, vasopressin was reacted with iodoacetamide to trap the cysteine persulfide. Blocking with iodoacetamide did not increase the relative amount of S-sulfuration detected. An example spectrum is included in Appendix A. m/z 1107 and m/z 1123 are unknown derivatives of vasopressin present in the purchased product.
Figure 3.4.1-1 MALDI-MS spectra of vasopressin treated with A) 10 fold (10 µM) polysulfides B) 100 fold (100 µM) polysulfides C) 1,000 fold (1 mM) polysulfides D) 10,000 fold polysulfides (10 mM). m/z 1084 represents unmodified vasopressin, m/z 1117 represents vasopressin with a single S-sulfurated cysteine, m/z 1150 represents vasopressin with two S-sulfurated cysteines.
3.4.2 Reduced GAPDH Activity and Analysis by Mass Spectrometry

The activity of the reduced GAPDH enzyme was measured with and without treatment with 50 µM NaSH. 50 µM NaSH had no effect on the kinetic parameters of GAPDH (Figure 3.4.2-1). The control enzyme had a $K_M$ of $0.35 \pm 0.03$ mM and a $V_{max}$ of $51 \pm 5$ µM/min. After NaSH treatment the $K_M$ was $0.30 \pm 0.03$ mM, and the $V_{max}$ was $52 \pm 6$ µM/min. Reduced GAPDH enzyme activity was tested with increasing concentrations of NaSH and polysulfides. NaSH produced a small, however statistically insignificant, change in enzyme activity while polysulfides produced a decrease in enzymatic activity to a minimum of ~42% of untreated enzyme at 200 µM polysulfides (Figure 3.4.2-2).

50 µM NaSH or polysulfides was used to treat the enzyme before mass spectrometry. Peptides were labeled with iodoacetamide to trap the persulfide. It was found that after treatment with NaSH Cys 247 was $S$-sulfurated, however Cys 152 and Cys 156 were not (Table 3.4.2-1). After treatment with polysulfides Cys 247 and Cys 156 were $S$-sulfurated, however the active site residue Cys 152 was not (Table 3.4.2-1) (Figure 3.4.2-3). $S$-sulfuration of Cys 156 correlates with a decrease in enzyme activity, while $S$-sulfuration of Cys 247 appears to have no change in enzymatic activity.
Figure 3.4.2-1 (A) Michaelis-Menten kinetics of purified GAPDH enzyme ($K_M = 0.35 \pm 0.03$ mM, $V_{\text{max}} = 51 \pm 5$ µM/min). (B) Michaelis-Menten kinetics of purified GAPDH enzyme treated with 50 µM NaSH ($K_M = 0.30 \pm 0.03$ mM, $V_{\text{max}} = 52 \pm 6$ µM/min). The error bars represent standard error (n=3). Adapted from [254] with permission.
Figure 3.4.2-2 (A) Enzymatic activity of purified GAPDH (1 µg/mL) as a function of increasing sodium hydrosulfide concentration. (B) Enzymatic activity of purified GAPDH (1 µg/mL) as a function of increasing polysulfide concentration. The error bars represent standard error (n=3). Adapted from [254] with permission.
Figure 3.4.2-3 (A) MS/MS spectrum of active site peptide (m/z 918.00) from control GAPDH enzyme. (B) MS/MS spectrum of active site peptide (m/z 933.43) from polysulfide treated GAPDH enzyme. The mass of y ions after $y_7$ increases by 32 Da indicating additional sulfur on Cys 156. The mass difference between $y_{10}$ and $y_{11}$ does not change indicating Cys 152 is unchanged. CAM denotes carbamidomethyl group after iodoacetamide treatment. Adapted from [254] with permission.
Table 3.4.2-1 Cysteine-containing tryptic peptides sequenced by ESI-MS/MS analysis of purified GAPDH enzyme: reduced control, treated with sulfide (NaSH), treated with polysulfides (PS). Adapted from [254] with permission.

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<tr>
<th>Treatments</th>
<th>Sequence</th>
<th>Modification</th>
<th>Cysteine</th>
<th>Precursor ion m/z&lt;sup&gt;a&lt;/sup&gt;</th>
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</table>

<sup>a</sup> All precursor ions reported are +2 ions unless otherwise indicated in () beside the m/z.

<sup>b</sup> <sup>13</sup>C isotope ion.

Abbreviations: CAM, carbamidomethyl; IAM, iodoacetamide; PS, polysulfide.
3.4.3 GSSG-Oxidized GAPDH Activity and Mass Spectrometry Analysis

GAPDH was oxidized with GSSG to form glutathionylated GAPDH. The amount of GSSG was chosen with intent to produce approximately 50% reduction in enzyme activity, in order to observe a possible decrease or increase in enzymatic activity. A plot of enzyme activity with increasing GSSG concentration is included in Appendix A. During enzyme activity experiments 5 mM GSSG produced a larger decrease in enzymatic activity than anticipated; the enzyme retained approximately 25% activity compared to the untreated control (Figure 3.4.3-1). After treatment with DTT, the GAPDH enzymatic activity increased to ~70% of untreated control at 100 µM DTT (Figure 3.4.3-1A). Increasing the DTT concentration past 100 µM produced no further increase in enzymatic activity. NaSH treatment produced very similar results; activity increased to approximately ~60% of untreated control at 100 µM NaSH, and to ~70% of untreated control at 500 µM (Figure 3.4.3-1B). After polysulfide treatment, activity increased to approximately ~55% of untreated control at 100 µM and 65% of untreated control at 500 µM (Figure 3.4.3-1C). After mass spectrometry analysis glutathionylated Cys 247 was detected (m/z 889.92) (Table 3.4.3-1). NaSH treatment caused S-sulfuration of Cys 247 (m/z 781.92), while polysulfide treatment caused S-sulfuration of Cys 247 (m/z 781.88) and Cys 156 (m/z 933.46).
Figure 3.4.3-1 (A) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), GSSG-treated, as a function of increasing DTT concentration. (B) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), GSSG-treated, as a function of increasing NaSH concentration. (C) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), GSSG-treated, as a function of increasing polysulfide (PS) concentration. The error bars represent standard error (n=3). Rate is expressed as percentage of untreated control. Adapted from [254] with permission.
Table 3.4.3-1 Cysteine-containing tryptic peptides sequenced by ESI-MS/MS analysis of purified GAPDH enzyme: GSSG-treated control, GSSG-treated and treated with sulfide (NaSH), GSSG-treated and treated with polysulfides (PS). Adapted from [254] with permission.

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<sup>a</sup> All precursor ions reported are +2 ions unless otherwise indicated in () beside the m/z. Abbreviations: CAM, carbamidomethyl; Glu, glutathione; GSSG, glutathione disulfide; IAM, iodoacetamide; PS, polysulfide.
**3.4.4 $H_2O_2$-Oxidized GAPDH Activity**

GAPDH was oxidized with $H_2O_2$ to form sulfinic acid (-SOH) derivatives of cysteine, and perhaps higher oxidation state sulfinic acid (-SO$_2$H), and sulfonic acid (-SO$_3$H). $H_2O_2$ concentration was chosen to produce ~50% inactivation. After treatment with DTT an increase from ~35% of untreated control to ~60% of untreated control was observed with the lowest concentration tested of 5 µM (Figure 3.4.4-1A). Additional concentration of DTT did not increase activity past ~60% of control. Treatment with 5 µM NaSH increased GAPDH activity from ~45% to ~60% of untreated control with no further increase observed (Figure 3.4.4-1B). Treatment with polysulfides did not significantly alter the activity of $H_2O_2$-oxidized GAPDH (Figure 3.4.4-1C).
Figure 3.4.4-1 (A) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), H$_2$O$_2$-treated, as a function of increasing DTT concentration. (B) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), H$_2$O$_2$-treated, as a function of increasing NaSH concentration. (C) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), H$_2$O$_2$-treated, as a function of increasing polysulfide (PS) concentration. The error bars represent standard error (n=3). Rate is expressed as percentage of untreated control. Adapted from [254] with permission.
**3.4.5 GSNO-Oxidized GAPDH Activity and Mass Spectrometry Analysis**

GAPDH was pre-treated with GSNO to test whether S-nitrosation may affect the S-sulfuration of GAPDH. Both NaSH and polysulfides produced a biphasic response where activity initially decreased with increasing sulfide concentration, but later increased as sulfide concentration was increased past 50 µM (Figure 3.4.5-1). NaSH treatment decreased GAPDH activity from ~55% of untreated control to ~40% of untreated control at 50 µM NaSH (Figure 3.4.5-1A). At 200 µM NaSH activity increased to ~80% of untreated control. After 50 µM polysulfide treatment, GAPDH activity decreased from ~70% of untreated control to ~20% of untreated control (Figure 3.4.5-1B). Increasing polysulfide concentration to 500 µM produced ~60% activity relative to untreated control.

Peptides were labeled for mass spectrometry using a differential labeling protocol. Free thiols were first reacted with iodoacetamide, sinapinic acid was used to de-nitrosate S-nitrosothiols and N-ethylmaleimide was used to label de-nitrosated cysteine residues. Using this protocol, S-sulfurated cysteines will be trapped with iodoacetamide, while S-nitrosated cysteines will be labeled with N-ethylmaleimide. After GSNO treatment Cys 247, Cys 152, and Cys 156 were found labeled with N-ethylmaleimide, indicating S-nitrosation (Table 3.4.5-1). Cys 152 reacted with carboxymethyl was also sequenced (m/z 951.48), indicating a free thiol, while Cys 156 was only detected labeled with N-ethylmaleimide. After NaSH and polysulfide treatments the same set of peptides were sequenced as the control (Table 3.4.5-1). No S-sulfuration was detected.
Figure 3.4.5-1 (A) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), GSNO-treated, as a function of increasing NaSH concentration. (B) Enzymatic rate of purified wild-type GAPDH (1 µg/mL), GSNO-treated, as a function of increasing polysulfide (PS) concentration. The error bars represent standard error (n=3). Rate is expressed as percentage of untreated control. Adapted from [254] with permission.
Table 3.4.5-1 Cysteine-containing tryptic peptides sequenced by ESI-MS/MS analysis of purified GAPDH enzyme, control GSNO, GSNO-treated and treated with sulfide (NaSH), GSNO-treated and treated with polysulfide (PS). Adapted from [254] with permission.

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<th>Precursor ion m/z&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> All precursor ions reported are +2 ions unless otherwise indicated in () beside the m/z. Abbreviations: CAM, carbamidomethyl; GSNO, S-nitrosoglutathione; IAM, iodoacetamide; NEM, N-ethylmaleimide; SA, sinapinic acid; PS, polysulfide.
3.4.6 C156S Mutant GAPDH Activity and Mass Spectrometry Analysis

A C156S mutant of GAPDH was used to test whether it was possible to S-sulfurate Cys 152, and what effect the modification has on enzymatic activity. It was found the C156S mutant of GAPDH had the same kinetic parameters, within experimental error, as the wild-type enzyme ($K_M = 0.23 \pm 0.08 \text{ mM}, V_{\text{max}} = 49 \pm 8 \text{ } \mu\text{M/min}$) (Figure 3.4.6-1). After NaSH treatment the activity of C156S GAPDH gradually decreased to 70% of untreated enzyme at 500 µM NaSH (Figure 3.4.6-2A). Treatment with 10 µM polysulfides caused a drop in C156S GAPDH activity to 25% of untreated control (Figure 3.4.6-2B). Increasing polysulfide concentration past 100 µM caused an increase in enzymatic activity.

Mass spectrometry confirmed the mutation of cysteine 156 to a serine. After NaSH or polysulfide treatments S-sulfuration of active site Cys 152 was detected (m/z 896.95) (Table 3.4.6-1). S-sulfuration of Cys 247 was also detected after NaSH, or polysulfide treatments (m/z 781.89).
Figure 3.4.6-1 Michaelis-Menten kinetics of purified C156S mutant of GAPDH enzyme ($K_M = 0.23 \pm 0.08 \text{ mM}$, $V_{\text{max}} = 49 \pm 8 \mu\text{M/min}$). The error bars represent standard error (n=3). Adapted from [254] with permission.
Figure 3.4.6-2 (A) Enzymatic activity of purified C156S GAPDH (1 µg/mL) as a function of increasing NaSH concentration. (B) Enzymatic activity of purified C156S GAPDH (1 µg/mL) as a function of increasing PS concentration. The error bars represent standard error (n=3). Adapted from [254] with permission.
Table 3.4.6-1 Cysteine-containing tryptic peptides sequenced by ESI-MS/MS analysis of purified C156S GAPDH enzyme; control, treated with sulfide (NaSH), treated with polysulfides (PS). Adapted from [254] with permission.

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<th>Modification</th>
<th>Cysteine</th>
<th>Precursor ion m/z&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>All precursor ions reported are +2 ions unless otherwise indicated in ( ) beside the m/z.

Abbreviations: CAM, carbamidomethyl; IAM, iodoacetamide; PS, polysulfide.
3.4.7 Additional MS/MS Spectra

Individual MS/MS spectra have been included in Appendix A. Identical precursor ions sequenced in multiple samples are only shown once.

3.4.8 Molecular Dynamics Simulations of GAPDH

GAPDH S-sulfuration was studied computationally using a molecular dynamics simulation to examine the effect of S-sulfuration of Cys 156 on the structure of GAPDH. Two important areas of the enzyme were examined; the NAD$^+$ binding site and the catalytic site. Three important residues in the NAD$^+$ binding were examined; Arg 13, Asp 35, and Asn 316 [255]. After S-sulfuration of Cys 156, the residues changed position <0.5 Å, including the Cys 152 to Cys 156 distance (Figure 3.4.8-1). The Cys 152 to Asn 316 distance did increase by 0.7 Å. From these results the NAD$^+$ binding site did not change significantly after Cys 156 S-sulfuration. Critical catalytic residues Cys 152 and His 179, as well as residues Ser 151, Cys 152, Thr 153, Thr 211 responsible for inorganic phosphate binding (Pi site) were examined [256]. After S-sulfuration of Cys 156, the Cys 152 to His 179 distance increased by 0.5 Å (Figure 3.4.8-2). Residues in the GAPDH Pi site Ser 151, Cys 152, and Thr 153 do not change significantly relative to each other (<0.5 Å) after S-sulfuration of Cys 156, with the exception of the distance from Thr 153 to Thr 211, which does increase by 0.78 Å. The pK$_a$ of Cys 152 was studied computationally using the propKa algorithm. The pK$_a$ of Cys 152 was 5.81 in the wild-type GAPDH enzyme, while the pK$_a$ of Cys 152 was 6.31 in the GAPDH enzyme S-sulfurated at Cys 156. The pK$_a$ of the catalytic cysteine was observed across all time scales and was found to be 0.5 higher on average when Cys 156 was S-sulfurated.
Figure 3.4.8-1 Representative structure taken from Molecular Dynamics (MD) simulation of GAPDH showing residues from two NAD\(^+\) binding sites overlaid, gray carbons represent the structure with free (-SH) Cys 156, while brown carbons represent the structure with S-sulfurated (SSH) Cys 156. Distances in Å were measured between critical NAD\(^+\) binding residues (Arg 13, Asp 35, Asn 316) as well as Cys 152 and Cys 156. Only one protein backbone is shown. Reprinted from [254] with permission.
**Figure 3.4.8-2** Representative structure taken from Molecular Dynamics (MD) simulation of GAPDH showing residues from two active sites overlaid, gray carbons represent the structure with free (-SH) Cys 156, while brown carbons represent the structure with S-sulfurated (-SSH) Cys 156. Distances in Å between critical catalytic residues Cys 152 and His 179 were measured. Distances between residues in the Pi site (Ser 151, Cys 152, Thr 153, Thr 211) were also measured. Only one protein backbone is shown. Reprinted from [254] with permission.
3.5 Discussion

3.5.1 S-Sulfuration of Vasopressin

Vasopressin was used as a model peptide for the S-sulfuration of oxidized thiols. In our experiments vasopressin was S-sulfurated by NaSH and polysulfides, however high concentrations of NaSH or polysulfides were required to produce vasopressin S-sulfuration (Figure 3.4.1-1). At least 1000 fold NaSH or polysulfide was required to detect vasopressin S-sulfuration by MALDI-MS. These results show that S-sulfuration of oxidized protein thiols is possible by NaSH and polysulfides. S-sulfuration at low concentration of sulfide, and protein thiol reduction at high concentration, as described by Francoleon et al. [162] was not detected with vasopressin.

3.5.2 S-Sulfuration of Reduced GAPDH

In our experiments with reduced GAPDH, it was found that NaSH had no significant effect on reduced GAPDH activity, even with 500 µM NaSH (Figure 3.4.2-2A). 50 µM NaSH also had no effect on the kinetic parameters of GAPDH (Figure 3.4.2-1). Since polysulfides have been postulated to be important H₂S-derived signaling molecules, we tested the effect of polysulfides on GAPDH activity. It was found polysulfides significantly decreased the activity of GAPDH (Figure 3.4.2-2B). When analyzed by ESI-MS/MS it was found active site Cys 152 was not S-sulfurated by either NaSH or polysulfides (Table 3.4.2-1). Cys 247 was S-sulfurated in the NaSH treated sample, while Cys 247 and Cys 156 were S-sulfurated after polysulfide treatment. Cys 156 S-sulfuration correlated with a decrease in enzymatic activity. These results indicate that polysulfides are a stronger S-sulfurating agent than NaSH.

Since S-sulfuration of reduced GAPDH at Cys 247 was observed this raises the question of how NaSH can react with cysteine thiols, since both NaSH and cysteine thiols are in fully reduced states [161]. NaSH is only 60% pure and is contaminated with elemental sulfur; it is highly likely polysulfides are formed in solution when NaSH is dissolved. We therefore propose that NaSH mediated S-sulfuration of free cysteine occurs from NaSH-derived polysulfides. This idea is consistent with our results where polysulfides produce more S-sulfuration (both Cys 156 and Cys 247) than NaSH (only
Cys 247). It is however possible that S-sulfuration of Cys 156 is also occurring in the NaSH-treated sample, since the parent peptide (m/z 933) was detected in MS measurements, but was below intensity threshold to trigger MS/MS sequencing (data not shown). The stoichiometry of the modification may be too low to affect enzyme activity.

3.5.3 S-Sulfuration of Glutathionylated GAPDH

After oxidation of GAPDH with GSSG to form glutathionylated cysteines it was found NaSH and polysulfides had near equal ability to restore GAPDH activity as DTT (Figure 3.4.3L1). Activity increased proportionally with the addition of NaSH or polysulfides from ~25% of control untreated enzyme to a maximum of ~70%. This represents ~3 fold increase in enzymatic activity from the GSSG-treated enzyme. We speculate that the fold increase in enzymatic activity may be increased if the initial oxidation of GSSG is stronger. It is unknown why 100% restoration of enzymatic activity with DTT was not observed. During treatments and buffer exchanges the control samples were subject to the same sample handling steps to minimize error.

Mass spectrometry analysis revealed glutathionylation of Cys 247 (Table 3.4.3L1) after GSSG treatment. After NaSH or polysulfide treatment glutathionylation of Cys 247 was still detected, meaning NaSH and polysulfides did not reduce all protein-glutathione disulfides. Similarly to the reduced GAPDH results, NaSH treatment produced S-sulfuration of Cys 247, while polysulfides S-sulfurated Cys 247 and Cys 156. When treated with NaSH or polysulfides, oxidized protein thiols therefore have the potential to be both reduced and S-sulfurated. A complex mixture of oxidized, reduced, and S-sulfurated thiols is present (Table 3.4.3-1). The resultant enzymatic activity is therefore expected to be determined by the relative contribution of each modification. In addition, free unmodified thiols (-SH) were detected. These could be from incomplete labeling with iodoacetamide or de-glutathionylation of the enzyme after iodoacetamide treatment.

3.5.4 H₂O₂-Oxidized GAPDH Activity

After H₂O₂ treatment DTT was partially able to restore H₂O₂-oxidized GAPDH activity (Figure 3.4.4-1). NaSH was able to restore GAPDH activity to a much smaller extent than DTT. Polysulfides did not affect H₂O₂-oxidized GAPDH activity. Large reactivation
was not observed, likely due to a mixture of sulfenic, sulfinic, and sulfonic acid derivatives being formed. Sulfinic and sulfonic acid derivatives are considered chemically irreversible, while sulfenic acids may be reduced by reducing agents [257]. The results suggest NaSH is a stronger reducing agent than polysulfides for sulfenic acids, although additional experiments would be required to prove this. H$_2$O$_2$ oxidized GAPDH was not studied using mass spectrometry.

### 3.5.5 Reaction of NaSH or Polysulfides with S-Nitrosated GAPDH

GSNO pre-treated GAPDH showed a biphasic response to increasing NaSH and polysulfide concentrations (Figure 3.4.5-1). Initially, activity decreased with increasing NaSH or polysulfide concentration until 50 µM, after which, activity increased with concentration. This may be due to $S$-nitrosothiols promoting $S$-sulfuration at low sulfide concentrations, and sulfide acting as a reducing agent at high concentrations. However, when analyzed by mass spectrometry no $S$-sulfuration was detected (Table 3.4.5-1). For mass spectrometry analysis 50 µM NaSH or polysulfides was used, which is the point of lowest activity, and presumably highest $S$-sulfuration. One possibility is that the carbamidomethyl disulfide was not stable under the extra sample handling steps. To the author’s knowledge this is the first time labeling with iodoacetamide and NEM has been used to try to simultaneously detect $S$-nitrosation and $S$-sulfuration. The method perhaps needs to be validated with a known $S$-sulfurated enzyme (eg. reduced GAPDH treated with polysulfides). The second possibility is that $S$-nitrosation does not promote $S$-sulfuration, and instead an unexpected chemical species was formed. Work by Filipovic et al. identified the product of the reaction of H$_2$S with GSNO as thionitrous acid (HSNO) and reduced glutathione (GSH) [135].

### 3.5.6 S-Sulfuration of Cys 152 in C156S GAPDH

During all our experiments with the wild-type enzyme no $S$-sulfuration of Cys 152 was observed. In order to observe the effects of Cys 152 $S$-sulfuration, a C156S mutant was purified. After treatment with NaSH the C156S mutant showed a gradual decrease in enzymatic activity (Figure 3.4.6-2). After polysulfide treatment the C156S mutant showed a rapid decrease in enzymatic activity at low concentrations and a gradual
increase at high concentrations. The response was more drastic to NaSH and polysulfides than the wild-type enzyme. When analyzed by mass spectrometry Cys 152 was S-sulfurated. These results correlate Cys 152 S-sulfuration with a decrease in enzymatic activity, contrary to previous literature results [141]. However, our results are in agreement with work by Valentine et al. from 1987 [258], where persulfides, generated through interaction of pyridoxal phosphate and cystine, inhibited GAPDH.

### 3.5.7 Role of GAPDH Cys 156

In our experiments with the wild-type GAPDH enzyme it was observed Cys 156 was S-sulfurated but Cys 152 was not. Results after GSNO treatment also indicate that Cys 156 is more readily S-nitrosated than Cys 152. After mutating Cys 156 to a serine, Cys 152 was S-sulfurated under our conditions. These experiments show Cys 156 is more reactive than Cys 152. We therefore propose a new role for Cys 156 where Cys 156 is a protective thiol for the adjacent catalytic Cys 152. This proposed function needs to be verified in vivo. Cys 156 is conserved and present in *E. coli*, *B. stearothermophilus*, lobster, rabbit, and human GAPDH [252], supporting our hypothesis. Cys 247 on the other hand is not conserved. If Cys 156 is more reactive than Cys 152, we can expect Cys 156 to have a lower $pK_a$ than Cys 152. This was not investigated in the present study, but is an interesting question that could be studied in the future.

### 3.5.8 NaSH and Polysulfide S-Sulfuration

In experiments with reduced and oxidized GAPDH, polysulfides produced S-sulfuration of Cys 156, while NaSH did not. In enzymatic assay experiments with reduced, GSNO-oxidized, and C156S mutant of GAPDH, polysulfides produced stronger decreases in enzymatic activity than NaSH. The results show that polysulfides are a stronger S-sulfurating agent than NaSH. Since NaSH was able to S-sulfurate reduced GAPDH at Cys 247, NaSH may be able to S-sulfurate thiols through NaSH-derived polysulfides. Based on these results, one can speculate that pure H$_2$S will be an even weaker S-sulfurating agent than NaSH, since NaSH is only ~60% pure once can expect it to form polysulfides more readily. Although it was not tested in the present study, Na$_2$S-induced S-sulfuration could be used to test this theory.
3.5.9 Effect of Enzyme Buffer on Treatments

In this study all treatments of GAPDH were performed in 20 mM Tris buffer at pH 7.8. Treatment of the GAPDH enzyme in GAPDH assay buffer (20 mM Tris pH 7.8, 100 mM NaCl, 1 mM NAD$^+$, 10 mM sodium pyrophosphate, 20 mM sodium arsenate, and 0.1 mg/mL BSA) may produce undesired reactions with buffer components such as arsenate. Since GAPDH was not supplemented with the co-enzyme NAD$^+$, we explored the possibility that NAD$^+$ binding may change the structure of GAPDH, and in turn change the response to NaSH or polysulfides. In an experiment where GAPDH was in 20 mM Tris buffer at pH 7.8 supplemented with NAD$^+$, treatment with NaSH produced the same results as the Tris buffer without NAD$^+$ (Appendix A).

3.5.10 Computational Chemistry

A molecular dynamics (MD) simulation was performed to examine the effect of Cys 156 S-sulfuration on GAPDH structure. The NAD$^+$ binding site was examined since the limiting step of GAPDH catalysis is NADH release [252]. It was found that the structure of the NAD$^+$ binding site does not change significantly after Cys 156 S-sulfuration. In the GAPDH active site, the hydrogen bond between the thiolate anion of Cys 152 and protonated imidazole of His 179 increases by 0.5 Å upon Cys 156 S-sulfuration. This increase in the hydrogen bonding distance would disrupt the ability of His 179 to stabilize the Cys 152 thiolate negative charge. Consequently, the p$K_a$ of the Cys 152 thiolate will increase, reducing its nucleophilicity. MD simulations show the p$K_a$ of Cys 152 increased by 0.5, from 5.81 in the unmodified enzyme to 6.31 in the S-sulfurated enzyme. This may help explain the reduced enzymatic rate upon Cys 156 S-sulfuration. In the phosphate binding site (Pi site) Thr 211 shifts 0.78 Å away from Thr 153. This may also affect the effectiveness of the Pi site to stabilize charges from inorganic phosphate. The MD simulation was performed on a single crystal structure. Since GAPDH is a homotetramer one subunit was S-sulfurated and compared to the wild-type subunit within the same enzyme homotetramer. After the simulation was completed the two subunits were compared. It would be interesting to perform two separate simulations, where the same subunit is modified, and compare the results to see if they agree with the single simulation.
3.6 Conclusions

In this chapter we investigated the reported S-sulfuration of GAPDH using a purified GAPDH enzyme. The effect of the initial redox state of the enzyme on S-sulfuration was examined. It was found that NaSH treatment did not significantly alter the enzymatic activity of reduced GAPDH. Treatment with polysulfides caused a decrease in GAPDH enzymatic activity. Treatment with NaSH caused S-sulfuration of Cys 247, while treatment with polysulfides caused S-sulfuration of Cys 156 and Cys 247. S-sulfuration of active site Cys 152 was not detected in the wild-type enzyme. Both NaSH and polysulfides were able to restore the activity of glutathionylated GAPDH with similar effectiveness as DTT. Similarly to the reduced enzyme, treatment of the glutathionylated enzyme with NaSH caused S-sulfuration of Cys 247, while treatment with polysulfides caused S-sulfuration of Cys 156 and Cys 247. Using a C156S mutant of GAPDH, NaSH and polysulfides were able to cause S-sulfuration of active site Cys 152, however a decrease in enzymatic activity was observed, and not an increase as previously reported. S-sulfuration of Cys 152 and Cys 156, both cause decreases in enzymatic activity. Based on our results, polysulfides are a stronger S-sulfurating agent than NaSH. S-sulfuration of reduced thiols by NaSH may be due to NaSH-derived polysulfides. We also propose a new role for GAPDH Cys 156, as a protective thiol for the catalytic residue Cys 152.
CHAPTER 4

ENRICHMENT OF SULFUR-CONTAINING PEPTIDES BY GOLD NANOPARTICLES FOR MASS SPECTROMETRY ANALYSIS
4.1 Introduction

Cysteines occur at a relative low frequency in proteins, however approximately 97% of human proteins contain at least one cysteine residue [259]. The relative amount of cysteine-containing proteins has been correlated with organism complexity. For example cysteines account for 0.5% of protein amino acids in archaebacterial, increasing to 2.26% in mammals. This suggests an important evolutionary role for cysteine residues, where the chemistry of cysteine residues allows for more complex protein functions. Cysteine residues serve a number of important roles in proteins. Cysteines are involved in maintaining protein structure by forming disulfide bonds and functional motifs, for example zinc fingers. Cysteine residues also function as redox switches, and are subject to a number of post-translational modifications [215, 257, 260-265]. Important cysteine modifications include disulfide formation, S-nitrosation, S-glutathionylation, sulfenic, sulfinic and sulfonic acid formation. Although once considered a mostly detrimental event in disease states and aging, macromolecule oxidation is now considered an important regulatory mechanism in healthy tissues [266]. Identification of modified cysteine residues and validation of their biological significance is important in elucidating the complex redox-dependent modifications that occur in the cell.

The most accurate method for identifying post-translational modification sites is mass spectrometry. Mass spectrometry allows unambiguous identification of protein modification sites, without the need to generate and test mutants of a protein. Although mass spectrometry is an extremely sensitive technique, the relative abundance of proteins of interest may be very low in comparison to background protein. Protein of interest may be present in detectable quantities, however may not appear in the MS spectra due to high background. This is especially an issue in methods like MALDI-MS, where usually there is no chromatography step to separate peptides from each other. In addition to having low abundance, modified proteins may also be in low stoichiometry compared to unmodified. This makes sample preparation and enrichment strategies important, in order to reduce sample complexity by removing abundant structural and housekeeping proteins, which aids in detection of low-abundance proteins of interest [265].
In recent decades there has been tremendous growth in the area of nanoparticle research, due to advances in synthesis and characterization methods. One of the most widely studied types of metal nanoparticles are gold nanoparticles (AuNPs). Ease of synthesis, and useful properties such as surface plasmon resonance and inertness make them attractive for use in applications such as spectroscopy, sensing, imaging, and drug delivery [267-270]. Due to their high surface area, nanoparticles offer an attractive platform for mass spectrometric enrichment strategies of peptides. Gold can form strong bonds with soft ligands such as thiols, a property which is exploited in the stabilization and derivatization of AuNPs, as well as in the field of self-assembled monolayers [271]. Thiols also have the ability to displace weaker-binding ligands from Au, a property which is utilized in popular AuNP synthesis strategies [272]. Au-bound thiols can undergo thiol exchange with each other, the extent of thiol exchange depending on concentrations, and importantly, the chemical structure of the thiol ligands [273]. Much like ligand displacement and thiol exchange in preparation of nanoparticles and self-assembled monolayers, we tested the hypothesis that peptides containing thiols could displace weakly-bound citrate ligands, and be subsequently purified by thiol exchange using a small molecular weight thiol such as DTT. Unlike water-soluble PEG or carboxylate alkylthiol AuNPs, citrate capped AuNP provide low-affinity ligands with similar structure to peptides that could be displaced with thiol-containing peptides [274].

Our group developed a AuNP-based method for identifying protein S-nitrosation sites, which relies on the natural affinity of thiols for gold [275]. In the original publication the method was tested with a single purified protein. Here we use a mixture of 5 purified proteins. Three recombinant proteins were used in the study: cystathionine gamma-lyase (CSE), protein disulfide isomerase (PDI), and dual specificity phosphatase 12 (DUSP 12 or YVH1). Two proteins were commercially purchased: bovine serum albumin (BSA) and lysozyme. This generates a considerably more complex sample, however makes identification of individual peptides possible using post source decay (PSD) MALDI sequencing. PSD sequencing does not use a collision chamber to fragment peptides. As a result many peptides do not fragment well, preventing de-novo sequencing.
4.2 Materials

4.2.1 Reagents

Gold (III) chloride trihydrate (520918), bovine serum albumin (A-9647), lysozyme (L-7651), α-cyano-4-hydroxycinnamic acid (476870) were purchased from Sigma-Aldrich. DTT (100597) was purchased from MP Biomedicals. C18 Zip Tips® (ZTC18S096) were purchased from Millipore. Sequencing grade trypsin (V511A) was purchased from Promega. Zeba™ desalting columns (89882) were purchased from ThermoFisher. Acetonitrile and 0.1% trifluoroacetic acid solution were mass spectrometry grade reagents.
4.3 Methods

4.3.1 AuNP Synthesis

AuNPs were synthesized according to the protocol by Grabar et al. [276]. The synthesis was scaled down to produce 250 mL of AuNPs. A 500 mL round-bottom flask was cleaned with aqua regia (1:3 concentrated HNO$_3$:HCl) and rinsed with Milli-Q water before use. Gold (III) chloride trihydrate (HAuCl$_4$•3H$_2$O) was dissolved in Milli-Q water to a concentration of 1 mM. 250 mL of this solution was refluxed with vigorous stirring to ~100 °C. When the temperature stabilized, 25 mL of a 38.8 mM solution of trisodium citrate dihydrate (Na$_3$C$_5$H$_5$O$_7$•2H$_2$O) in Milli-Q water was very rapidly injected using a syringe. A rapid colour change from yellow, to clear, and to dark red was observed. Reflux and stirring was continued for 10 min after injection, the heat source was removed, and the solution was stirred for another 15 min. The resulting colloidal solution was cooled to room temperature and stored in a brown glass flask at 4 °C until usage. The UV-vis spectrum of the resulting AuNP solution showed a $\lambda_{\text{max}}$ of 520 nm. Long-term storage of AuNPs at 4 °C may be detrimental for AuNP stability, since precipitation was observed at a later date.

4.3.2 Protein Purification and Digestion

Recombinant human YVH1 was purified by the Vacratsis group (University of Windsor) according to the published protocol [277], and kindly donated for our experiments. Recombinant PDI and CSE were purified from *E. coli* containing the appropriate expression vector according to the detailed protocol by Faccenda et al. [185]. Bovine serum albumin and lysozyme were each dissolved in 50 mM Tris-HCl, 1 mM CaCl$_2$ pH 7.6. CSE, PDI, and YVH1 were each buffer-exchanged into 50 mM Tris-HCl, 1 mM CaCl$_2$ using Zeba™ Desalt Spin Columns. Each protein was trypsin-digested for 18 h at 37 °C while shaking, using a trypsin:protein ratio of 1:40 by mass (protein concentration 0.2 µg/µL). BSA, lysozyme, CSE, PDI, and hYVH1 were mixed in a 1:1:1:1:1 ratio after digestion.
4.3.3 Peptide Purification by AuNPs

500 µL of AuNPs were centrifuged at 16,000 g for 10 min and 450 µL of the supernatant was removed to concentrate the AuNPs. 40 µg (0.2 µg/µL) of the unquenched tryptic protein digest was added to the AuNPs. The digest was incubated with AuNPs for 1 h at room temperature. The AuNPs were centrifuged at 16,000 g for 10 min. The supernatant was removed and the AuNPs were resuspended in 500 µL of 10 mM sodium phosphate buffer (pH 7.0) with sonication to redisperse aggregates. The AuNPs were centrifuged at 16,000 g for 10 min and the supernatant was removed. Washing with 500 µL of 10 mM sodium phosphate buffer (pH 7.0) was repeated two more times. After the last wash 40 µL of 50 mM DTT was added to the AuNP pellet. The AuNPs were redispersed with sonication, and incubated in DTT for 1 h. The AuNPs were centrifuged at 16,000 g for 15 min and the supernatant was removed for analysis.

4.3.4 Mass Spectrometry

The DTT elution was acidified with 20 µL 0.1% trifluoroacetic acid and purified with Millipore C18 ZipTips®. 1 µL of sample was spotted with 1 µL of 10 mg/mL matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.1% trifluoroacetic acid) on a MALDI plate, and analyzed by an Applied Biosystems Voyager DE-Pro Mass Spectrometer. Linear mode was used to acquire fingerprint spectra. The fingerprint and PSD (post-source decay) spectra were manually compared to the masses predicted by UCSF Protein Prospector (http://prospector.uchicago.edu).
4.4 Results

4.4.1 Sulfur-Containing Peptide Enrichment by AuNPs

In the control digest, before AuNP purification six sulfur-containing peptides are observed (Figure 4.4-1 and Table 4.4-1). Three of the peptides are thiols (cysteine containing). A high baseline signal is observed in the control sample indicating a complex mixture. After AuNP purification thirteen sulfur-containing peptides are observed, including seven cysteine-containing peptides (Figure 4.4-2 and Table 4.4-2). The baseline signal has considerably decreased (Figure 4.4-2) and sulfur-containing peptides constitute a much larger proportion of the detected peptides versus the control. Some non-sulfur peptides are still present after AuNP purification.

4.4.2 Additional MALDI-MS PDS Spectra

Individual MALDI-MS PSD spectra have been included in Appendix B.
Figure 4.4-1 MALDI-MS spectrum of a tryptic digest of a mixture of 5 proteins: BSA, CSE, lysozyme, PDI, and YVH1. Six sulfur-containing peptides were identified in the sample.

Figure 4.4-2 MALDI-MS spectrum of a tryptic digest of a mixture of 5 proteins: BSA, CSE, lysozyme, PDI, and YVH1, subject to sulfur enrichment by AuNPs. Thirteen sulfur-containing peptides were identified in the sample.
Table 4.4-1 Sulfur-containing peptides identified by MALDI-MS in a tryptic digest of a mixture of 5 proteins; BSA, CSE, lysozyme, PDI, and YVH1.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Observed Avg. m/z</th>
<th>Predicted Avg. m/z</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVAFIGQAR</td>
<td>965.94</td>
<td>965.17</td>
<td>YVH1</td>
</tr>
<tr>
<td>MNEGFEWQLK</td>
<td>1282.84</td>
<td>1282.47</td>
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</tr>
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<td>LGSFNWYGEQSCGR</td>
<td>1706.28</td>
<td>1707.89</td>
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</tr>
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<td>IKPHLMSQELPEDWKQPK</td>
<td>2419.36</td>
<td>2419.82</td>
<td>PDI</td>
</tr>
<tr>
<td>GSPEFMLEAPGPSDGCELSNPSASR</td>
<td>2536.16</td>
<td>2536.77</td>
<td>YVH1</td>
</tr>
</tbody>
</table>
Table 4.4-2 Sulfur-containing peptides identified by MALDI-MS after AuNP-based purification of a tryptic digest of a mixture of 5 proteins: BSA, CSE, lysozyme, PDI, and YVH1.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Observed Avg. m/z</th>
<th>Predicted Avg. m/z</th>
<th>Protein</th>
</tr>
</thead>
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</table>
4.5 Discussion

4.5.1 Spectra Interpretation

The proteins used in this study have various abundances of sulfur-containing residues, ranging from 12 sulfur-containing residues in lysozyme to 40 sulfur-containing residues in BSA, 35 of which are thiols (cysteines). MALDI-TOF-MS remains a qualitative technique where many factors such as abundance, ionization efficiency, and ion suppression due to sample composition affect which peptides are observed in the MS spectra. As a result only six sulfur-containing peptides are observed in the five protein mixture, which increases to thirteen sulfur-containing peptides after AuNP purification. The proportion of sulfur-containing peptides is greatly improved after AuNP purification as the majority of non-sulfur peptides are removed from the spectra.

4.5.2 Interaction of Peptides and AuNPs

Free thiols can bind to Au through gold-thiolate bonding. Disulfides are generally broken upon binding to the Au surface, resulting in a gold-thiolate bond [278, 279]. Thioethers cannot form gold-thiolate bonds but are adsorbed intact on Au surfaces [279-281]. Although Au binds preferentially to thiols, AuNPs can be stabilized using citrate, and Au has some affinity for the carboxyl groups in citrate. Since peptides are large, complex, molecules, with multiple charges, other functional groups likely contribute to binding to AuNPs. The binding of peptides to Au is likely not as strong as for alkyl-thiols since alkyl thiols are further stabilized with hydrophobic interactions from alkyl chains, which are not present in peptides. Low binding affinity is beneficial for allowing disulfide exchange with small molecular weight thiols like DTT. However, the binding may not provide high enough affinity to purify only thiol-containing peptides. If strong Au-peptide binding was occurring one might expect only cysteine-containing peptides (thiols) to be purified, and not methionines (thioethers). However, purification of thioethers may be useful for studies where alkylation agents like iodoacetamide and N-ethylmaleimide are used, which generate a thioether linkage.
4.6 Conclusions

AuNPs were used to purify sulfur-containing peptides from a mixed tryptic digest of 5 purified proteins: BSA, CSE, lysozyme, PDI, and YVH1. Using MALDI-MS analysis six sulfur-containing peptides were identified before AuNP enrichment, and thirteen after AuNP enrichment. Importantly, the proportion of sulfur-containing peptides in the spectra increases after AuNP purification. Both cysteines and methionines are purified by AuNPs, however non-sulfur peptides are not fully removed. The results demonstrate AuNPs may be used for sample purification in mass spectrometric studies of sulfur-containing proteins.
CHAPTER 5

DEVELOPMENT OF NO-RELEASING MATERIALS FOR WOUND HEALING APPLICATIONS
5.1 Introduction

NO is an important mediator of wound healing, and NO-releasing materials have the potential to be used as therapeutic agents to improve wound healing [206, 282], both in disease states and healthy tissues. In this chapter we aimed to develop NO-releasing wound dressings to improve cutaneous wound healing. Two different designs of wound dressings have been proposed. In the first design the chemistry of NO release is due to the denitrosation of small molecular weight $S$-nitrosothiols by gold nanoparticles (AuNPs). In the second design the NO release is due to the reduction of $NO_2^-$ ions to NO by copper. The proposed $S$-nitrosothiol based design is activated by a pressure-sensitive membrane. Upon breaking a pressure-sensitive membrane, mixing of the two solutions would occur, and initiate the reaction of $S$-nitrosothiols and metals, which would release NO at the wound site (Figure 5.1-1). It has been proposed AuNPs denitrosate $S$-nitrosothiols by forming a gold-sulfur bond and releasing NO in the process (Eq. 5.1-1) [283, 284].

$$\text{(5.1-1) } \text{AuNP} + \text{RSNO} \rightarrow \text{RS-AuNP} + \text{NO}$$

The second design features a chitosan polymer doped with either CuNPs or copper ions. Cu(I) can reduce $NO_2^-$ to NO (Eq. 5.1-2) [285], and can also denitrosate $S$-nitrosothiols to generate NO (Eq. 5.1-3) [286, 287]. A large number of studies indicate that chitosan has inherent antimicrobial, and wound healing abilities [288]. In this design chitosan is not an inert compound for suspending copper, but is also expected to improve the wound healing process.

$$\text{(5.1-2) } 2\text{Cu}^+ + 2\text{NO}_2^- + 2\text{H}_2\text{O} \rightarrow 2\text{Cu}^{2+} + 2\text{NO} + 4\text{OH}^-$$

$$\text{(5.1-3) } \text{Cu}^+ + \text{RSNO} \rightarrow \text{Cu}^{2+} + \text{NO} + \text{RS}^-$$

Chitin is the second most abundant natural polymer after cellulose [289]. Chitin is a derivative of glucose ($N$-acetyl-$\beta$-D-glucosamine) synthesized by many organisms, and forms the exoskeleton of arthropods (e.g. insects, crustaceans), as well as the cell walls of fungi and yeast. Treatment of purified chitin with NaOH causes deacetylation of chitin forming chitosan (D-glucosamine) (Figure 5.1-2). Different types of chitosan are
characterized by their degree of deacetylation (DD), distribution of acetyl groups on the main chain, and the molecular weight. The amine groups on chitosan allow chitosan to be soluble in acidic solutions, although the exact solubility properties depend strongly on how the chitosan was purified from chitin [289]. Due to the abundance of amine groups in chitosan, chitosan has an ability to chelate metal ions. For divalent ions chitosan shows the highest selectivity towards Cu$^{2+}$, while for trivalent ions chitosan shows highest selectivity towards Eu$^{3+}$ and Nd$^{3+}$ [290]. Since chitosan is a cationic polymer it could be also used to absorb and remove anionic dyes from solution [291]. Numerous studies have used chitosan to remove metal ions, and dyes from waste water [291, 292].

Chitosan has numerous possible biomedical applications due to the fact it’s a biodegradable and non-toxic polymer with intrinsic wound-healing properties. Chitosan also has intrinsic antimicrobial and antifungal properties [293]. The antimicrobial properties of chitosan are related to its DD, and higher DD chitosan produces stronger antimicrobial effects [294]. Although the exact mechanism is not known, it has been theorized that the positive charge on chitosan destabilizes and permeates the cell membrane of gram-negative bacteria [288, 293]. In gram positive bacteria chitosan may chelate multivalent cations which stabilize the cell wall [293]. Chitosan has been shown to promote blood clot formation, and commercial chitosan-based wound dressings have been shown to be more effective at stopping hemorrhage than standard cotton gauze in animal studies [295]. At least one chitosan-containing bandage has been commercialized (HemCon®) and has been tested as a hemostatic by EMS services [296] and the US Army [297]. A study by Gopal et al. in 2014 tested a solution of copper nanoparticles and chitosan dissolved in 0.1% acetic acid in an animal model of wound healing [298]. The solution showed improved wound healing compared to chitosan without copper nanoparticles, and the control. However, the control was 0.1% acetic acid solution and no untreated control was shown. A study by Khan et al. showed acetic acid chitosan films caused skin irritation, while lactic acid chitosan films did not [299].

Studies show that copper has antimicrobial [300, 301], and wound healing properties [302-304]. Copper is biocidal, and far less likely to produce adverse skin reactions than silver. In recent years, the rise of antibiotic-resistant bacteria has led to
renewed interest in the antimicrobial properties of copper [300, 301]. The use of copper touch surfaces in hospitals may help the spread of bacteria. The wound healing effects of copper are less widely-studied. In 2008 Borkow et al. hypothesized that persistence of chronic wounds may be caused by low local levels of copper [303]. Copper is an essential trace element, and many cellular processes important in wound healing are copper-dependent. Borkow et al. tested copper oxide impregnated wound dressing in diabetic mouse, and showed copper oxide dressings improved wound healing [302]. The mechanism of action was hypothesized to be generation of $\text{H}_2\text{O}_2$ and hydroxyl radicals through redox cycling between CuO and Cu$_2$O. Analysis by real-time PCR and histochemistry showed numerous proangiogenic growth factors were upregulated with the use of copper oxide wound dressings.
Figure 5.1-1 Proposed design of an $S$-nitrosothiol based NO-releasing wound dressing. Upon breaking of the disruptable membrane $S$-nitrosothiol solution and Ag- or Au-composite material would come in contact. Reaction of Ag or Au with $S$-nitrosothiols will cause denitrosation of $S$-nitrosothiols and NO release through the gas permeable membrane.

![Diagram of proposed design](image)

A. \[ \text{Chemical structure of chitin.} \]

B. \[ \text{Chemical structure of chitosan.} \]

Figure 5.1-2 (A) Chemical structure of chitin. (B) Chemical structure of chitosan. Treatment of chitin with NaOH causes partial deacetylation resulting in chitosan. 50% deacetylation is shown in this example.
5.2 Materials

5.2.1 Reagents

Gold (III) chloride trihydrate (520918), Sephadex® G-75 (G-75-120), cellulose (C8002),
tetraethyl orthosilicate (TEOS) (86578), triethyl phosphate (TEP) (538728), Ca(NO$_3$)$_2$
(20-296-7), AgNO$_3$ (209139), diethylenetriaminepentaacetic acid (DTPA) (D6518),
hydrazine (225819), ethylene glycol (102466), NaNO$_2$ (237213), lactic acid (252476),
CuCl (212946), Minimum Essential Medium (MEM), reduced glutathione were
purchased from Sigma-Aldrich. Polydimethylsiloxane (PDMS) Sylgard® 184 Silicone
Elastomer Kit was purchased from Dow Corning. Chitosan (AZ-78-6101) was purchased
from Dungeness Environmental Solutions. Ethylene glycol diglycidyl ether (EGDE)
(01479-100) was purchased from Polysciences. Gibco® Fetal Bovine Serum was
purchased ThermoFisher.

5.2.2 Materials

Septa-sealed vials (Supelco 27533) were purchased from Sigma-Aldrich. 1 mL syringes
(309659) were purchased from BD. Polystyrene columns (29920) were purchased from
Fisher Scientific.
5.3 Methods

5.3.1 AuNP Synthesis

AuNPs were synthesized according to the protocol by Grabar et al. [276]. The synthesis was scaled down to produce 250 mL of AuNPs. A 500 mL round-bottom flask was cleaned with aqua regia (1:3 concentrated HNO$_3$:HCl) and rinsed with Milli-Q water before use. Gold (III) chloride trihydrate (HAuCl$_4$•3H$_2$O) (Sigma Aldrich) was dissolved in Milli-Q water to a concentration of 1 mM. 250 mL of this solution was refluxed with vigorous stirring to ~100 °C. When the temperature stabilized, 25 mL of a 38.8 mM solution of trisodium citrate dihydrate (Na$_3$C$_5$H$_7$O$_7$•2H$_2$O) (Sigma Aldrich) in Milli-Q water was very rapidly injected using a syringe. A rapid colour change from yellow, to clear, and to dark red was observed. Reflux and stirring was continued for 10 min after injection, the heat source was removed, and the solution was stirred for another 15 min. The resulting colloidal solution was cooled to room temperature and stored in a brown glass flask at 4 °C until usage. The UV-VIS spectrum of the resulting AuNP solution showed a $\lambda_{\text{max}}$ of 520 nm. Long-term storage of AuNPs at 4 °C may be detrimental for AuNP stability, since precipitation was observed at a later date.

5.3.2 AuNP-PDMS

0.2 mL of PDMS mixture was cast directly in vials. A ratio of 0.04 curing agent to monomer base was used. This was previously determined by Adam Faccenda to be the optimum for AuNP synthesis on PDMS surfaces. PDMS was cured for 40 minutes at 70 °C. The vials were cooled to room temperature. 0.5 mL of a solution containing 10 mM HAuCl$_4$ and 5 mM citrate was added to each vial. The vial was placed on a shaker at room temperature for 3 h. The vial was removed and any gold flakes were pipetted off the surface before the solution was removed. The resulting AuNP-PDMS was washed with H$_2$O three times. Control vials were not treated with the HAuCl$_4$ and citrate solution.

5.3.3 AuNP-G75 Sephadex

PBS was added to G-75 Sephadex to make a slurry. An equal volume of AuNPs was added to the slurry, and mixed. The slurry was poured into a chromatography column,
and washed with PBS to remove unbound AuNPs. 100 µL of the slurry was taken out of the column and added to each septa-sealed vial. Control G-75 did not contain AuNPs.

5.3.4 AuNP-Cellulose

1 g of α-cellulose was added to 40 mL of AuNPs. The mixture was placed on a nutating mixer. Excess AuNPs were removed as in the AuNP-G75 Sephadex protocol. 150 µL of the cellulose was added to each septa-sealed vial.

5.3.5 AuNP-Gauze

A ~1 x 1 inch piece of gauze was placed in a petri dish. AuNPs were added, and the gauze was soaked overnight. The excess AuNPs were removed, and the AuNP-gauze was washed with H₂O until no more AuNPs eluted from the gauze into the H₂O (no red colour). The gauze was dried at ~50 °C. The gauze was compared by weight to a piece of control gauze. Pieces with approximately equal weight (~0.5 x 1 cm for AuNP and ~0.5 x 1.5 cm for control) were cut and placed inside septa-sealed vials.

5.3.6 AuNP-Glass Synthesis

AuNP doped glass was synthesized according to the sol-gel protocol published by Lusvardi et al. [305]. 3.542 g of Ca(NO₃)₂•4H₂O and 0.788 g of HAuCl₄•3H₂O was dissolved in 12.96 mL of HCl (0.0091 M). 17.84 mL TEOs and 1.71 mL of TEP were added to 40 mL of ethanol. The Ca/Au solution was added to the TEOs/TEP solution and stirred well. The stirred solution was transferred to an airtight Teflon container and kept at room temperature for 24 h. The gel was transferred to an open crucible and heated for 3 h at 600 °C. The control glass was prepared the same way except without the addition of HAuCl₄•3H₂O. 5 mg of AuNP-glass or control glass was placed in each septa-sealed vial for NO measurements.

5.3.7 Ag₂S-Paper

Filter paper was soaked twice in a methanol solution saturated with AgNO₃. Filter paper was then rinsed in methanol solution three times, and air dried at room temperature. 10 mM Na₂S solution was added to the filter paper to form Ag₂S. The filter paper was rinsed
in H₂O solution three times, and air dried at room temperature. 9 mg of Ag₂S-paper or control paper was used per vial for NO measurements.

5.3.8 NO Measurements from AuNP and Ag Composites

Vials containing the AuNP-composite and controls were sealed and degassed with argon for 7 minutes each. Sievers Nitric Oxide Analyzer (NOA 280i) was set up using the standard NO₂⁻ measurement mode and standardized with NO₂⁻ according to manufacturer protocol. GSNO was dissolved in 100 µM DTPA. 200 µL of GSNO was added to a degassed vial, the vial was incubated 10 minutes, and 1 mL of the headspace was injected into the reaction chamber. Separate syringes were used for GSNO and headspace injections. When gas from the vial headspace was injected into the NOA reaction chamber, it was injected into the reaction chamber headspace, and the syringe needle did not touch the solution inside.

Each vial was timed exactly 10 minutes between injection of GSNO into vials and measurement of vial headspace. The addition of GSNO to individual vials was done in 2 min intervals, and the subsequent injection of NO into the NOA was done in 2 min intervals (exactly 10 minutes later). By the 5th injection of GSNO, the injections of GSNO and NO will start overlapping. For simplicity and more accurate timing, the incubations and measurements were done in sets of 4 or 5 samples, so the injections of NO and GSNO did not overlap.

5.3.9 CuNP Synthesis

CuNPs were synthesized according to protocol by Zhu et al. [306]. 20 mL of N₂H₄ and NaOH in ethylene glycol was added to 20 mL of 0.1 M CuSO₄ in ethylene glycol while stirring. The molar ratio of N₂H₄ to CuSO₄ was 1.5, while the molar ratio of NaOH to CuSO₄ was 0.05. The solution was irradiated in a household microwave oven and cycled 18 s on, 12 s off, for 3 min. Nanoparticles were isolated by centrifugation and washed with anhydrous ethanol.
5.3.10 Chitosan and CuNP-Chitosan Film Preparation

To prepare chitosan films, 0.2 g of chitosan was added to 9.6 mL H₂O while stirring. Stirring was continued for 5 minutes. For CuNP-chitosan 10 mg of solid CuNPs were added. Stirring was continued for 5 minutes. 200 µL of acetic acid or lactic acid was added to the chitosan dropwise while stirring. Stirring was continued for 1 h. The CuNPs may slowly disperse and dissolve, however the process will not occur to completion. Chitosan was poured into weighing boats and dried for at least 48 h. Drying time will vary depending on temperature and humidity. Control chitosan and CuNP-chitosan, used for NO release experiments examining the effect of the addition of thiol to the films, was prepared using this method. Later an improved method was used (vide infra). The amounts may be scaled up depending on how much chitosan one wants to make. 0.2 g of chitosan in ~10 mL results in a 2% chitosan mixture. The volume added to weighing boats may be changed to control film thickness.

5.3.11 Cu-Chitosan Film Preparation, Glucose-Chitosan Film Preparation, and Improved CuNP-Chitosan Film Preparation

An improved protocol was used to prepare CuNP-chitosan films in order to reduce time and improve CuNP dispersion. To prepare chitosan films, 0.2 g of chitosan was added to 9.6 mL of H₂O while stirring. Stirring was continued for 5 minutes. The solution was now transferred from a beaker to a falcon tube. 10 mg of solid CuNPs was added to the falcon tube, and the falcon tube was sonicated in a water bath sonicator to disperse the CuNPs. Afterwards, the solution was poured back into the same beaker, and stirring was continued. 200 µL of lactic acid was added to the chitosan dropwise while stirring. Stirring was continued for 5 minutes. 10 mL of 0.33 M glucose solution was now added, and stirred for 5 minutes. 5 mL of the resulting mixture was poured into each weighing boat, and dried for at least 48 h.

The amount of copper salt to be added to chitosan was calculated to be equivalent in mol Cu to 10 mg of CuNPs. For CuCl it was 0.0156 g, for Cu₂O it was 0.0112 g, etc. To prepare chitosan, 0.2 g of chitosan was added to 9.6 mL of H₂O while stirring. Stirring was continued for 5 minutes. Cu was added to the solution and stirring was continued for
5 min. 200 µL of lactic acid was added to the chitosan dropwise while stirring. Stirring was continued for 5 minutes. 10 mL of 0.33 M glucose solution was now added, and stirred for 5 minutes. 5 mL of the resulting mixture was poured into each weighing boat. When comparing chitosan with or without glucose, 10 mL of H₂O was added instead of 0.33 M glucose to keep the % of chitosan the same. Using this protocol the resulting chitosan concentration in the slurry was 1%, although the concentration can be easily adjusted if so desired. 6 mL of the resulting mixture was poured into each weighing boat, and dried for at least 48 h.

5.3.12 Cross-linked Chitosan Film Preparation

Chitosan films were cross-linked by placing them in 1% EGDE solution in methanol on a shaker for 18 h. Films were washed with methanol to remove excess EGDE, and air dried.

5.3.13 Solution Concentrations for NO Measurements of Chitosan and CuNP-Chitosan, With, and Without GSH

In the first experiment, 7 mg of chitosan, or CuNP-chitosan was placed in septa-sealed vials and degassed with argon for 5 minutes. 100 µL of 10 mM sodium phosphate buffer pH 7.0 and 100 µL of 100 µM NO₂⁻ was injected into vials during measurements. 100 µL of GSH in 10 mM sodium phosphate buffer pH 7.0 was used for testing CuNP-chitosan in the presence of thiol, instead of buffer alone Time between injection of NO₂⁻ and NO measurement was 10 minutes.

In the second experiment the amount of CuNP-chitosan was reduced to 2.5 mg. 100 µL of 10 mM sodium phosphate buffer pH 7.0 and 100 µL of 50 µM NO₂⁻ was injected into vials during measurements. 100 µL of 200 µM GSH in 10 mM sodium phosphate buffer pH 7.0 was used for the CuNP-chitosan in testing presence of thiol, instead of buffer alone.
5.3.14 Solution Concentrations for NO Measurements in the Comparison of Different Sources of Cu in Cu-Glucose-Chitosan

0.5 x 0.5 cm chitosan squares were cut, placed in septa-sealed vials, and degassed with argon for 5 minutes. 100 µL of minimum essential media (MEM) supplemented with fetal bovine serum (FBS) and 100 µL 50 µM NO\textsubscript{2} was injected into the vials. Time between injection of NO\textsuperscript{2} and NO measurement was 15 minutes.

5.3.15 Solution Concentrations for NO Measurements Determining the Effect of Glucose on Cu-Chitosan

0.6 x 0.6 cm chitosan squares were cut, placed in septa-sealed vials, and degassed with argon for 5 minutes. 200 µL of 10 µM NO\textsubscript{2} in PBS was injected into vials. Time between injection of NO\textsuperscript{2} and NO measurement was 15 minutes.

5.3.16 Solution Concentrations for NO Measurements of Cross-linked Chitosan and Cu-Chitosan

0.5 x 0.5 cm chitosan squares were cut, placed in septa-sealed vials, and degassed with argon for 5 minutes. 200 µL of NO\textsuperscript{2} in PBS and 100 µL of 2 g/L glucose in PBS was injected into vials. Time between injection of NO\textsuperscript{2} and NO measurement was 15 minutes.

5.3.17 NO Measurements from Chitosan and Cu-Chitosan Films

Vials containing the Cu-chitosan and control chitosan were sealed and degassed with argon for 5 minutes each. Sievers Nitric Oxide Analyzer (NOA 280i) was set up using the standard NO\textsuperscript{2} measurement mode and standardized with NO\textsuperscript{2} according to manufacturer protocol. NO\textsuperscript{2} in PBS was injected into the degassed vials, and vials were incubated 10 minutes or 15 minutes depending on the experiment. 1 mL of the headspace was injected into the reaction chamber.

Separate syringes were used for NO\textsuperscript{2} and headspace injections. When aspirating the gas from the vial headspace one must be extremely careful not to touch the NO\textsuperscript{2} solution inside. If the syringe comes in contact with NO\textsuperscript{2}, the NO\textsuperscript{2} will be converted to
NO in the NOA reaction chamber and give a false signal. When gas from the vial headspace was injected into the NOA reaction chamber, it was injected into the reaction chamber headspace, and the syringe needle did not touch the solution inside. Even when following this protocol previous contact with NO$_2^-$ may produce a false signal.

Each vial was timed exactly 10 minutes between injection of NO$_2^-$ into vials and measurement of vial headspace. The addition of NO$_2^-$ to individual vials was done in 2 min intervals, and the subsequent injection of NO into the NOA was done in 2 min intervals (exactly 10 minutes later). By the 5$^{th}$ injection of NO$_2^-$, the injections of NO$_2^-$ and NO will start overlapping. For simplicity and more accurate timing, the incubations and measurements were done in sets of 4 or 5 samples, so the injections of NO$_2^-$ and NO did not overlap. When following the protocol with 15 minutes between injections sets of 6 samples were used.

Different types of samples were measured first, and subsequently replicates were measured. For example samples were injected in the order: control, copper, control, copper, control, copper, and not control, control, control, copper, copper, copper. This was done in case contamination of the syringe needle occurred, it would be easier to spot, and less likely to be mistaken for genuine NO release.
5.4 Results

5.4.1 NO Release from GSNO by Au and Ag Composites

All the AuNP and Ag composites tested (AuNP-PDMS, AuNP-G75, AuNP-Cellulose, AuNP-Gauze, AuNP-Glass, Ag$_2$S-Paper) were able to denitrosate S-nitrosothiols, producing gaseous NO (Figures 5.4.1-1 to 5.4.1-6). These results support the previously-described denitrosation mechanism of S-nitrosothiols by AuNPs [283]. It is likely Ag can denitrosate S-nitrosothiols using a similar mechanism as Cu or Au. All the materials tested also displayed at least some de-nitrosation without any Au or Ag present. This is most likely due to metal ion contamination. However, the presence of AuNPs or Ag does provide significant increase in released NO.
Figure 5.4.1-1 NO release from GSNO by AuNP-PDMS compared to control PDMS. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.

Figure 5.4.1-2 NO release from GSNO by AuNP-G75 Sephadex compared to control G75 Sephadex. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.
Figure 5.4.1-3 NO release from GSNO by AuNP-cellulose compared to control cellulose. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.

Figure 5.4.1-4 NO release from GSNO by AuNP-gauze compared to control gauze. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.
Figure 5.4.1-5 NO release from GSNO by AuNP-glass compared to control glass. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.

Figure 5.4.1-6 NO release from GSNO by Ag₂S-paper compared to control paper. 1 mL of headspace gas was measured 10 min after the addition of GSNO to a septa-sealed vial.
5.4.2 NO Release from NO$_2^-$ by Cu-Chitosan

Chitosan embedded with CuNPs was able to generate NO from NO$_2^-$ (Figure 5.4.2-1). Copper is expected to be oxidized in this reaction from Cu(I) to Cu(II). GSH was added to the chitosan to test whether it could reduce Cu(II) back to Cu(I). In an experiment with 10 mg chitosan there was no significant difference between copper and copper with GSH (Figure 5.4.2-1). When the amount of chitosan was reduced to 2.5 mg the addition of GSH did increase NO release (Figure 5.4.2-2), although the total NO released was much lower.

Different sources of copper were tested to determine whether they can replace microwave synthesized CuNPs. In this experiment, glucose was added to the chitosan mixture before casting in order to attempt to improve the physical properties of the films. Minimum Essential Medium (MEM) supplemented with fetal bovine serum (FBS) was added to chitosan during NO release experiments to test whether cell culture media would interfere with potential cell culture experiments. It was found different copper sources (CuNP, CuSO$_4$, CuCl, Cu$_2$O) can facilitate release of NO from NO$_2^-$ in chitosan (Figure 5.4.2-3). This is likely due to the chitosan being supplemented with glucose. When using a CuCl as a source of copper, it was determined the addition of glucose to the chitosan before casting a film was required for NO release (Figure 5.4.2-4).

Chemical cross-linking was used to try to improve the physical properties of the chitosan films and prevent cracking of the film after drying. It was found cross-linking prevented NO release by the Cu-chitosan films (Figure 5.4.2-5). In this experiment glucose was added to chitosan films alongside NO$_2^-$ during NO measurements.
Figure 5.4.2-1 NO release from NO$_2^-$ by 10 mg CuNP-chitosan with and without reduced glutathione (GSH). 1 mL of headspace gas was measured 10 min after 100 µL of pH 7.0 10 mM PO$_4^{3-}$ buffer (with or without GSH) and 100 µL of 100 µM NO$_2^-$ was injected into a septa-sealed vial. Error bars represent standard error (n=3).
**Figure 5.4.2-2** NO release from NO$_2^-$ by 2.5 mg CuNP-chitosan with and without reduced glutathione (GSH). 1 mL of headspace gas was measured 10 min after 100 µL of pH 7.0 10 mM PO$_4^{3-}$ buffer (with or without GSH) and 100 µL of 50 µM NO$_2^-$ was injected into a septa-sealed vial. Error bars represent standard error (n=3).
Figure 5.4.2-3 NO release from NO$_2^-$ by Cu-glucose-chitosan prepared using different sources of copper in the presence of minimum essential media (MEM) supplemented with fetal bovine serum (FBS). 1 mL of headspace gas was measured 15 min after 100 µL of MEM and 100 µL 50 µM NO$_2^-$ was injected into a septa-sealed vial. Error bars represent standard error (n=3).
Figure 5.4.2-4 Effect of glucose and CuCl on NO release from NO$_2^-$ by chitosan. CuCl and glucose were incorporated into chitosan films. 1 mL of headspace gas was measured 15 min after 200 µL of 10 µM NO$_2^-$ in PBS was injected into a septa-sealed vial. Error bars represent standard error (n=3).
**Figure 5.4.2-5** Effect of EGDE cross-linking of chitosan on NO release from NO$_2^-$. 1 mL of headspace gas was measured 15 min after 200 µL of NO$_2^-$ in PBS and 100 µL of 2 g/L glucose in PBS was injected into a septa-sealed vial. Error bars represent standard error (n=3).
5.5 Discussion

5.5.1 NO Release from GSNO by AuNP and Ag Composites

All of our AuNP and Ag composites tested were able to denitrosate S-nitrosothiols, and produce NO since they contain either Au or Ag. The presence of AuNPs or Ag does increase the NO release versus the control materials. The control materials did facilitate some NO release, which is likely due to trace metal contamination. PDMS uses a proprietary metal catalyst to cure the PMDS polymer, which can likely denitrosate S-nitrosothiols. Other materials used are likely contaminated with trace metals. It is also possible the vials used were contaminated with trace metals. The relative NO release cannot be reliably compared, since the amount of Au or Ag in each material has not been quantified.

5.5.2 Proposed SNO-Based NO-Releasing Wound Dressing

The proposed design of the wound dressing uses S-nitrosothiols as a source of NO. While the chemistry of S-nitrosothiols is convenient, this is problematic for storage of the wound dressing, as S-nitrosothiols are not stable at high temperatures. During use of the proposed wound dressing, the ambient temperatures could also affect NO release. S-nitrosothiols are also unstable when exposed to light, and the external packaging would require the S-nitrosothiols to be protected from light. The proposed design uses gold to denitrosate S-nitrosothiols. Copper or silver could replace gold, which is an expensive material. The kinetics of NO release may be changed by changing the amount of metal in the composites. Although the kinetics of NO release may be prolonged, it is unknown if they could be prolonged for the extended periods of time that are required for wound healing.

The design of the wound dressing itself introduces packaging challenges. The dressing design requires NO to diffuse through a gas-permeable membrane, and then through a traditional wound dressing, such as cotton gauze, to reach the site. The amount of NO actually released at the wound site would also have to be tested if a prototype was constructed. Furthermore, effective bandages allow gas-exchange at the wound site [307]. This could be a challenge due to the proposed design. The multi-layered design might not
effectively facilitate gas-exchange at the wound site. To facilitate gas exchange, a permeable membrane could be incorporated on both sides of the wound dressing, the side facing the wound and the side facing away. This would facilitate more gas exchange but also cause NO loss to the external environment.

5.5.3 NO Release from NO$_2^-$ by Cu-Chitosan Films

The Cu-chitosan films were able to release NO from NO$_2^-$, which is a salt, and more stable than S-nitrosothiols. During our initial experiments, CuNPs were used as the source of copper. CuNPs were synthesized by microwave synthesis [306] and added to the chitosan films. The CuNPs used are uncapped, meaning they have no capping agent like citrate in classic AuNPs. The copper in the CuNPs is in the Cu(0) oxidation state after synthesis. After the addition of acetic acid or lactic acid to CuNP-chitosan solution, the solution turns green indicating oxidation of the copper. The CuNPs do not fully dissolve, and when cast into a film do not disperse evenly, resulting in an uneven distribution of CuNPs in the film. Sonication improves this only to a certain extent.

Since the copper partially oxidizes during casting of the films, it was tested whether the addition of thiols can increase the NO release by reducing Cu(II) to Cu(I). At high amounts of chitosan (10 mg) the addition of GSH did not significantly increase the amount of NO release (Figure 5.4.2-1). However, at lower amounts of chitosan (2.5 mg), the amount of reduced copper in the film becomes limiting and the addition of GSH does increase NO release (Figure 5.4.2-2). When glucose is cast with chitosan, other sources of copper can be used to generate NO from NO$_2^-$ (Figure 5.4.2-3). Glucose is a reducing sugar and can reduce Cu(II) to Cu(I), however it was incorporated into the film rather than added with NO$_2^-$. The reduction of Cu(II) by glucose is the basis of Benedict's reagent, a reagent used in a colourimetric test for reducing sugars. When using copper salts like CuCl, copper was oxidized from Cu(I) to Cu(II) when incorporated into chitosan films, and glucose was required for NO release (Figure 5.4.2-4).

5.5.4 Physical Properties of Cu-Chitosan Films

It was tested whether lactic acid can replace acetic acid for making chitosan films. Lactic acid is expected to be more biocompatible than acetic acid [299]. During mixing of a
chitosan solution, the acid is used to protonate the amino groups on chitosan and increase the solubility of chitosan in solution before casting of the film. The specific acid used for mixing the chitosan slurry is therefore interchangeable. When performing NO release experiments it is important that $\text{NO}_2^-$ is added to chitosan in a buffered solution, since residual acid can reduce $\text{NO}_2^-$ to NO. After casting of the chitosan slurry, the water evaporates to form a chitosan film. Prolonged evaporation results in a film that is extremely brittle. The addition of glucose allows the film to be soft for a longer period of time; however the film will eventually become brittle with prolonged drying. The browning reaction of glucose is also observed when drying the chitosan films. When placed in water the chitosan films swell and break apart. Chemical cross-linking was therefore used to try to improve the physical properties of the films, and prevent the films from breaking apart. Ethylene glycol diglycidyl ether (EGDE) was used as the cross-linker, however cross-linking prevented NO release from chitosan films (Figure 5.4.2-5). Chemical cross-linking may also alter the chemical and biological properties of chitosan in an undesired way.

### 5.5.5 Proposed Design of Chitosan-Based NO-Releasing Wound Dressing

The proposed NO releasing chitosan-based bandage combines therapeutic effects from chitosan, copper, and NO release. In this design the chitosan can be in contact with the wound, and does not require complex packaging unlike our previous AuNP and Ag based design. The use of chitosan also allows for gas exchange at the wound site. The use of copper allows two pathways for NO release, the reduction of $\text{NO}_2^-$ to NO, and the denitrosation of $S$-nitrosothiols. The copper may be able to react with native $\text{NO}_2^-$ and $S$-nitrosothiols to produce NO. Additional $\text{NO}_2^-$ may also be applied to the wound dressing, perhaps between the chitosan film, and an adhesive backing. Since the use of copper salts required the incorporation of glucose into the chitosan films, CuNPs may be the best copper source. Incorporating large amount of glucose, to reduce Cu(II), would likely be detrimental to wound healing. However, thiols and glucose present in biological fluids may provide a pathway for the regeneration of Cu(I) from Cu(II).
5.5.6 Future Directions

In this work we studied the release of NO from copper-chitosan films in an endpoint manner. The next step is to study the kinetics of NO release, and determine how much NO the copper-chitosan films release over time. This would also help optimize the chemical composition of the films. Ultimately, to test the efficacy of the proposed wound healing dressings animal studies are required.
5.6 Conclusions

In this chapter we sought to develop NO-releasing wound dressings that may be used to improve wound healing. The first wound dressing design is based on the denitrosation of S-nitrosothiols by gold or silver composites. The chemistry of S-nitrosothiols is convenient; however this design produces packaging challenges. The second design uses chitosan embedded with copper, which can reduce NO$_2^-$ to NO, as well as denitrosate S-nitrosothiols. The use of chitosan and NO$_2^-$ eliminates the packaging challenges present in the first design. Our experiments show chitosan films embedded with copper are effective at reducing NO$_2^-$ to NO. Although our therapeutic agent is NO, numerous studies show that the use of chitosan or copper alone can improve wound healing in animal models. NO-releasing copper-chitosan may be used as a wound dressing to improve wound healing.
6.1 Discussion and Perspectives

The discovery of NO as a gasotransmitter in the late 1980’s has resulted not only in discovery of an important biological pathway, but the establishment of a whole new field of biological signaling. One of the major recent discoveries in the field of gaseous signaling is \( \text{H}_2\text{S} \) signaling. Many similarities between NO and \( \text{H}_2\text{S} \) exist: biological effects in the cardiovascular and nervous systems, interaction with biomolecules through cysteine residues and heme iron, complex chemistry due to oxidation by \( \text{O}_2 \), and potential challenges when performing *in vivo* measurements. The study of gasotransmitters, like NO and \( \text{H}_2\text{S} \), requires a strong understanding of their underlying chemistry. The overestimation of \( \text{H}_2\text{S} \) concentrations in biological samples highlights this issue. It also highlights the need for detailed understanding of analytical techniques used to detect \( \text{H}_2\text{S} \), and the need for improved \( \text{H}_2\text{S} \) detection methods. Driven by the need for accurate and specific methods for detecting \( \text{H}_2\text{S} \), in Chapter 2 we developed a simple and specific assay for \( \text{H}_2\text{S} \) gas. This assay requires no specialized equipment, and provides researchers an additional tool for studying \( \text{H}_2\text{S} \).

Similarly to \( S \)-nitrosation by NO, \( \text{H}_2\text{S} \) can modify cysteine residues by \( S \)-sulfuration, affecting protein functions. In Chapter 3 we investigated the reported \( S \)-sulfuration of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Our work on GAPDH \( S \)-sulfuration provides more insights into how \( \text{H}_2\text{S} \) may modify GAPDH cysteine residues, and in-turn affect GAPDH enzymatic activity. Although our work was performed *in vitro*, it provides researchers clues about what could be potentially occurring *in vivo*. We also demonstrate the importance of the initial cysteine redox state of an enzyme, which will affect the response to gasotransmitters like NO and \( \text{H}_2\text{S} \), highlighting the importance of basic biochemistry in studying gasotransmitters.

Mass spectrometry is an extremely powerful and versatile tool for studying protein post-translational modifications, including modifications induced by gasotransmitters. In Chapter 4 we used the natural affinity of thiols and thioethers towards gold nanoparticles in order to purify sulfur-containing tryptic peptides for mass spectrometric analysis. Since cysteine residues are common targets of gasotransmitters
NO and H₂S, this technique could be used for studying post-translational modifications of proteins induced by gasotransmitters.

Gasotransmitters like NO and H₂S may also be used as therapeutic agents to treat disease. For example, nitroglycerin is used to treat angina pectoris, and its activity is due to the release of NO. Application of exogenous NO can improve cutaneous wound healing, and there is a possible therapeutic application of NO for treatment of chronic and persistent wounds. In Chapter 5 we developed NO-releasing materials that could be used as wound dressings to improve wound healing. Chitosan is a natural, biocompatible, and biodegradable polymer, which has been previously used as a wound dressing. By incorporating copper into chitosan films, chitosan films can generate NO from inorganic nitrite, and S-nitrosothiols. Although further studies are required, copper-chitosan films could be potentially used as NO-releasing wound dressing.

Our understanding of H₂S signaling is a lot less complete than NO signaling. While NO is well-established to be a major determinant of vascular tone, H₂S signaling could even be described as controversial. Although H₂S and NO are similar, some unique challenges exist when studying H₂S. The lack of specific enzyme inhibitors, as well as detection methods has been a major challenge in H₂S research. H₂S-producing enzymes, CBS, CSE, CAT, and 3-MST catalyze many different reactions in addition to H₂S generation. It is difficult, but very important, to separate these functions from H₂S-related effects. H₂S can oxidize to numerous different sulfur species, and react with other redox signaling molecules, including NO. Determining which reactions occur physiologically will aid our understanding of H₂S, and even NO biochemistry. Going forward, the development of improved methods to study H₂S, and furthering our knowledge of H₂S chemistry would greatly improve our understanding of H₂S signaling. When drawing biological conclusions it is paramount to appreciate the chemistry of gasotransmitters, the chemical tools used to study gasotransmitters, and consider the big picture.
REFERENCES


[84] Taoka, S.; Lepore, B. W.; Kabil, O.; Ojha, S.; Ringe, D.; Banerjee, R. Human cystathionine beta-synthase is a heme sensor protein. evidence that the redox sensor is heme and not the vicinal cysteines in the CXXC motif seen in the crystal structure of the truncated enzyme. *Biochemistry* **41**:10454-10461; 2002.


[199] Turko, I. V.; Ballard, S. A.; Francis, S. H.; Corbin, J. D. Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (type 5) by sildenafil and related compounds. Mol Pharmacol 56:124-130; 1999.


APPENDICES
Figure A-1 MALDI-MS spectra of vasopressin treated with 1,000 fold (1 mM) NaSH and labeled with iodoacetamide. m/z 1084 represents unmodified vasopressin, m/z 1117 represents unlabeled singly S-sulfurated vasopressin, m/z 1175 represents singly S-sulfurated and iodoacetamide labeled vasopressin, m/z 1232 represents singly S-sulfurated and doubly iodoacetamide labeled vasopressin, m/z 1264 represents doubly S-sulfurated and iodoacetamide labeled vasopressin.
Figure A-2 GAPDH protein purification gel. Lane 1: protein ladder, lane 2: purified GAPDH, lane 3: *E. coli* crude lysate. Image was taken using a desktop scanner.
Figure A-3 C156S GAPDH protein purification gel. Lane 1: protein ladder, lane 2: crude lysate, lane 3: column flow-through, lane 4: wash #1, lane 5: wash #2, lane 6: purified GAPDH. Image was taken using a commercial gel imager.
Figure A-4 Example plot of GAPDH enzyme activity. Conversion of NAD$^+$ to NADH was measured at 340 nm. The initial rate up to 42 s was used for calculations.
Figure A-5 GAPDH enzyme inactivation by oxidized glutathione (GSSG).
Figure A-6 Enzymatic activity of purified GAPDH (1 µg/mL) as a function of increasing NaSH concentration. GAPDH was supplemented with NAD\(^+\) before treatment with NaSH. The addition of NAD\(^+\) did not change the effect of NaSH on GAPDH activity.
Figure A-7 MS/MS spectrum of GAPDH tryptic peptide (m/z 765.90) containing carbamidomethylated Cys 247, sequenced from reduced GAPDH sample (no NaSH or PS treatments).

Figure A-8 MS/MS spectrum of GAPDH tryptic peptide (m/z 781.89) containing S-sulfurated and carbamidomethylated Cys 247, sequenced from reduced and NaSH-treated GAPDH sample.
Figure A-9 MS/MS spectrum of GAPDH tryptic peptide (m/z 737.39) containing unlabeled Cys 247, sequenced from GSSG-treated GAPDH sample (no NaSH or PS treatments).

Figure A-10 MS/MS spectrum of GAPDH tryptic peptide (m/z 889.92) containing glutathionylated Cys 247, sequenced from GSSG-treated GAPDH sample (no NaSH or PS treatments).
Figure A-11 MS/MS spectrum of GAPDH tryptic peptide (m/z 593.61) containing glutathionylated Cys 247, sequenced from GSSG-treated GAPDH sample (no NaSH or PS treatments).

Figure A-12 MS/MS spectrum of GAPDH tryptic peptide (m/z 888.95) containing carbamidomethylated Cys 152 and free Cys 156, sequenced from GSSG and NaSH-treated GAPDH sample.
Figure A-13 MS/MS spectrum of GAPDH tryptic peptide (m/z 799.91) containing NEM-labeled Cys 247, indicating S-nitrosation of Cys 247, sequenced from GSNO-treated GAPDH sample subject to differential labeling.

Figure A-14 MS/MS spectrum of GAPDH tryptic peptide (m/z 951.48) containing carbamidomethylated Cys 152 and NEM-labeled Cys 156, indicating S-nitrosation of Cys 156, sequenced from GSNO-treated GAPDH sample subject to differential labeling.
Figure A-15 MS/MS spectrum of GAPDH tryptic peptide (m/z 985.49) containing NEM-labeled Cys 152 and NEM-labeled Cys 156, indicating S-nitrosation of Cys 152 and Cys 156, sequenced from GSNO-treated GAPDH sample subject to differential labeling.

Figure A-16 MS/MS spectrum of C156S GAPDH tryptic peptide (m/z 852.45) containing free Cys 152, sequenced from C156S GAPDH sample (no NaSH or PS treatments).
**Figure A-17** MS/MS spectrum of C156S GAPDH tryptic peptide (m/z 880.96) containing carbamidomethylated Cys 152, sequenced from C156S GAPDH sample (no NaSH or PS treatments).

**Figure A-18** MS/MS spectrum of C156S GAPDH tryptic peptide (m/z 896.95) containing S-sulfurated and carbamidomethylated Cys 152, sequenced from PS-treated C156S GAPDH sample.
Appendix B

**Figure B-1** PSD-MS spectrum of YVH1 peptide (m/z 937.96) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

**Figure B-2** PSD-MS spectrum of YVH1 peptide (m/z 966.36) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
Figure B-3 PSD-MS spectrum of YVH1 peptide (m/z 1183.16) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

Figure B-4 PSD-MS spectrum of YVH1 peptide (m/z 1283.31) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
Figure B-5 PSD-MS spectrum of lysozyme peptide (m/z 1436.16) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

Figure B-6 PSD-MS spectrum of PDI peptide (m/z 1537.41) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
Figure B-7 PSD-MS spectrum of lysozyme peptide (m/z 1677.65) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

Figure B-8 PSD-MS spectrum of YVH1 peptide (m/z 1708.41) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
Figure B-9 PSD-MS spectrum of lysozyme peptide (m/z 1805.44) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

Figure B-10 PSD-MS spectrum of BSA peptide (m/z 1825.68) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
**Figure B-11** PSD-MS spectrum of PDI peptide (m/z 2419.85) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.

**Figure B-12** PSD-MS spectrum of YVH1 peptide (m/z 2536.71) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
Figure B-13 PSD-MS spectrum of CSE peptide (m/z 2563.19) from a 5 protein mixture enriched for sulfur-containing peptides by AuNPs.
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