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# $H_2S$ regulation of nitric oxide metabolism

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## Abstract

Nitric oxide (NO) and hydrogen sulfide ($H_2S$) are two major gaseous signaling molecules that regulate diverse physiological functions. Recent publications indicate the regulatory role of $H_2S$ on NO metabolism. In this chapter, we discuss the latest findings on $H_2S$-NO interactions through formation of novel chemical derivatives, and experimental approaches to study these adducts. This chapter also addresses potential $H_2S$ interference on various NO detection techniques, along with precautions for analyzing biological samples from various sources. This information will facilitate critical evaluation and clearer insight into $H_2S$ regulation of NO signaling and its influence on various physiological functions.

## 1. Introduction

### 1.1 Nitric oxide (NO) and Hydrogen sulfide ($H_2S$) –Key gaseous signaling molecules and their interactions

Nitric oxide (NO), the endothelium-derived relaxing factor (EDRF) and the recently identified Hydrogen sulfide ($H_2S$), which has been dubbed as endothelium-hyperpolarizing factor (EDHF), are the gaseous signaling molecules with similar signaling functions. NO and $H_2S$ regulate complex biological processes including cardiovascular, neuronal, cytoprotection, inflammation and immune functions (Kolluru et al., 2013a). These gasotransmitters have gained recent attention for their 1) unique regulation of physiological functions through common signaling cascades 2) chemical interactions between the molecules leading to production of novel compounds, that regulate physiological functions and 3) interaction with thiols and other biological compounds. However, biochemical mechanisms that regulate these two gasotransmitter interactions have only recently been examined and require further investigation. This chapter describes the detection and quantification methods involved in identifying the effect of $H_2S$ on NO and its metabolites mediated through nitric oxide synthases and non-enzymatic pathways (Kolluru et al., 2013b; Liu and Huang, 2008) (Figure 1).

### 1.2 How does $H_2S$ influence NOS and production of NO and its metabolites?

Pathophysiological effects of NO have been well established over many years (Hirst and Robson, 2011; Liu and Huang, 2008). Similar to NO, the biological effects of $H_2S$ have been demonstrated in numerous publications (Kolluru et al., 2013a; Wang, 2012; Zamora et al., 2000). However, only recent studies suggest that $H_2S$ influences the bioavailability of NO and activity of its synthesis enzymes. The production of NO can be accomplished either through enzymatic or non-enzymatic pathways (Lundberg et al., 2008). It has been reported

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that $H_2S$ induces phosphorylation of eNOS and also prevents its degradation (King et al., 2014; Kondo et al., 2013; Lei et al., 2010). Conversely, exogenous $H_2S$ donors diallyl trisulfide (DATS) and NaHS, reduces iNOS expression and corresponding inflammation process (Benetti et al., 2013; Liu et al., 2006). Furthermore, non-NOS induction of NO production via sulfide dependent nitrite reduction to NO under ischemic conditions has also been demonstrated (Bir et al., 2012). Together, these reports suggest that $H_2S$ clearly influences NO production and it's metabolites.

Studies with specific knockouts or heterozygous mutants of $H_2S$ producing enzymes cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) further substantiate $H_2S$ influence on vascular functions including vasodilation that involves NO (Yang et al., 2008). However, no studies have been reported showing the influence of 3-mercaptopyruvate sulfurtranferase (3MST) on NO metabolism. In a recent I/R study, the Lefer laboratory has demonstrated that the bioavailability of NO and eNOS expression decreases in CSE knockout mice. However, acute $H_2S$ therapy restored both eNOS function and NO bioavailability (King et al., 2014). Similarly, studies in animal models of hyperhomocysteinemia with heterozygous disruption of the cystathionine β-synthase (CBS +/−) gene resulted in impairment of vascular functions (Eberhardt et al., 2000). Further investigations in this model have demonstrated that this dysfunction is the result of decreased eNOS activity and bioavailability of NO and its metabolite S-nitrosothiol (Eberhardt et al., 2000; Upchurch et al., 1997; Zhang et al., 2000). Upregulation of other reactive oxygen species such as superoxide, peroxynitrite and hydrogen peroxide in these models may also impair the biological activity of NO (Eberhardt et al., 2000; Upchurch et al., 1997).

Both $H_2S$ and NO regulate several vascular functions through common signaling mechanisms (VEGF/Akt/PKB/p38 MAPK) (Altaany et al., 2013; Bucci et al., 2010; Kondo et al., 2013; Lei et al., 2010; Yong et al., 2008; Yusof et al., 2009). This is a relatively new area of research and is not without controversies. There are studies on $H_2S$-NO interactions that otherwise indicate $H_2S$ mediated downregulation of NOS expression and subsequently NOS/NO mediated physiological functions (Geng et al., 2007; Kubo et al., 2007; Oh et al., 2006). However, it is increasingly clear that $H_2S$ influences NOS/NO signaling in regulating various biological functions that requires further to understand specific mechanistic interactions.

### 1.3 Novel adducts formation from $H_2S$-NO interactions

Apart from influencing NO metabolism, H2S has been recently identified to interact with NO metabolites (Kolluru et al., 2013b). $H_2S/HS^-$ (hydrosulfide ion), a strong nucleophile can indirectly react with electrophilic NO through its oxidized or nitrosating forms to produce distinct biological compounds (Figure 2). Recently there have been several studies on novel $H_2S$-NO reactants. Whiteman et al., previously revealed that $H_2S$ may reduce cytotoxicity by reacting with peroxynitrite ($ONOO^-$) forming a novel unknown compound (Whiteman et al., 2006a). Later, Sebastian et al., reported that $HS^-$reacts with peroxynitrite to form sulfenic acid (HSOH) and $NO_2^-$ (Sebastián et al., 2011). Sulfenic acid may further form dihydrogen disulfide via reaction with $HS^-$ (Sebastián et al., 2011). $HS^-$ may react

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with S-nitrosothiol (SNO) or S-nitrosoglutathione (GSNO) to form novel metabolites such as thionitrous acid (HSNO), sulfinyl nitrite (HS(O)NO or $HSNO_2$) or nitroxyl (HNO) (Filipovic et al., 2013; Filipovic et al., 2012a; Filipovic et al., 2012b). However, a recent study reported that sulfide reacts with nitrosothiols rather to form nitrosopersulfide ($SSNO^-$) that may contribute to sulfide induced extended effects of NO mediated through nitrosothiols (Cortese-Krott et al., 2014b). Nonetheless, a key conundrum remains in that the formation of these compounds has been reported using supra physiological concentrations of both $H_2S$ and NO donors and their variants. Further studies are needed to investigate the formation of NO-$H_2S$ adducts considering physiological conditions of pH and concentration.

## 2. Techniques determining enzymatic activity and expression of NOS

NO is synthesized by three isoforms of nitric oxide synthases (NOS) – endothelial, neuronal and inducible (Moncada and Higgs, 1993). Measurement of NOS activity and expression is essential to demonstrate the role of enzymatic synthesis of NO from various biological sources. This section describes currently available methods to quantify NOS activity and expression.

### 2.1 High-sensitive radiolabeled detection of NOS

Bredt and Snyder first reported the citrulline assay for quantification of NOS isoforms (Bredt and Snyder, 1990). The principle of this high sensitive assay is based on the conversion of L-arginine to L-citrulline and NO formation as a byproduct. This assay is performed either on fresh samples such as cells and tissues or from biopsies (Bredt and Schmidt, 1996; Rachmilewitz et al., 1994; Steil et al., 1995). The assay can also be performed on homogenates of frozen tissue or cells, provided the material is non-fixed, snap-frozen in liquid nitrogen, and stored at −80 °C. Activity of NOS in comparison with that of control preparations indicates the level of induction of NOS activity.

Prior to starting the assay samples are treated with addition of several cofactors and substrates, including calmodulin, tetrahydrobiopterin ($BH_4$) and NADPH. NOS activity is determined based on the amount of 1mol radiolabeled L-arginine converted to radiolabeled L-citrulline. The samples are prepared by giving KCl-wash to reduce nonspecific synthesis of L-citrulline. Cationic L-arginine is retained by AG 50 ion-exchange resin column in its $Na^+$ form, while L-citrulline is eluted. Either L-[$^3$H]arginine or L-[$^{14}$C]arginine is used as a precursor of L-citrulline. While $^{14}$C-has a better radioisotope stability, L-[$^3$H]arginine has a better specific activity and economical. With few minor modifications this assay can be performed to measure activity of all the three isoforms of NOS based on their dependence of $Ca^{2+}$ mediated activation (Salter et al., 1991). Selective measurement of iNOS or nNOS activity is achieved by performing reactions with $Ca^{2+}$ chelating agent (1 mM EGTA) or in combination with NOS inhibitor (1 mM EGTA with 1 mM L-$N^G$-methylarginine).

#### Key points and limitations

1. Highly specific assay for determination of NOS enzymatic activity.
2. Works with biological materials containing high enzyme levels

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3. Additional metabolic pathways such as arginase and ornithine transcarbamylase can also indirectly synthesize L-citrulline from L-arginine in the absence of NOS.
4. Presence of endogenous L-arginine in the samples that may vary the results by affecting the specific activity of the enzymes.
5. Radioactive materials are hazardous and have to be handled with extreme caution

### 2.2 Western blotting for detection of NOS expression

As mentioned earlier, sulfide influences NO bioavailability by upregulating the activity of its enzymes (King et al., 2014; Kondo et al., 2013; Lei et al., 2010). Levels of NOS proteins have been determined using western blot technique. For analysis, tissue/organs should be homogenized on ice using a tissue homogenizer in RIPA buffer (25 mM Tris–HCl pH 7.4 and protease inhibitors) to yield a 10% (weight/volume) homogenate. The required amount of homogenate can be added to an equal volume of Laemmli buffer for SDS-PAGE analysis. Remaining sample can be snap frozen in liquid nitrogen for future analysis. Tissue/cellular extracts should be separated by centrifugation at 10,000 x g for 10 min at 4°C. Supernatants are collected and measured for total protein concentrations will be determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Same amounts of proteins should be taken to separate on a 10% SDS – polyacrylamide gel and transferred to PVDF membrane. Membranes should be probed with corresponding antibodies against eNOS, phospho-eNOS, nNOS or iNOS (Abcam) (1:1000) respectively with loading control β-tubulin or β-actin (cell signaling). The ECL western blot analysis system (Bio-Rad Laboratories or Amersham Life Systems) can be used for detection of the primary antibody signal and included a peroxidase-labeled secondary antibody. Our lab has achieved better specificity with primary antibodies purchased from Cell signaling or Abcam. The immunoreactivity of NOS is detected by changes in chemiluminescence. After transfer to an autoradiogram, signals of NOS isotypes can be quantified by densitometry using Image J software ver 1.48 (NIH).

### 2.3 Determination of mRNA expression of NOSs by qRT-PCR

To check the aspects of NOS gene expression mediated by sulfide, levels of NOS mRNA can be quantified using a RT-PCR reaction. The samples isolated from various tissue sources placed in RNA later 10% (weight/volume) should be homogenized using a tissue homogenizer. Total RNA will be obtained using the RNeasy Fibrous Tissue Kit (Qiagen). A total of 1 μg of DNase I treated RNA reverse should be transcribed into cDNA using the Bio-rad iscript cDNA supermix kit (Bio-rad) with random hexamers in a 20 μL reaction. PCR should be performed in duplicate for each sample using 1 μL of cDNA as a template, Bio-rad SYBR green assay kit (Bio-rad) in a 20μL reaction. Specific primers should be used to probe for eNOS, nNOS and iNOS with GAPDH controls should be designed (Beacon Designer 2.0 software). The sequences of the primers used for mouse as follows: eNOS accession number NM_008713 (sense-5′-CAGCATCACCTACGACAC-3′; A-5′-GGCTCTGTAACTTCCTTGG-3′); nNOS accession number NM_008712 (sense-5′-GTCTTCCACCAGGAGATG-3′; A-5′-AAAGGCACAGAAGTGGGGGTA-3′); iNOS, accession number NM_010927 (sense-5′-ACGGACGAGACGGATAG-3′; A-5′ GGGCTTCAAGATAGGGA-3′). The primers should be verified by a gel electrophoresis after obtaining a single PCR product with a conventional PCR. A reaction mixture lacking

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cDNA should beused as the negative control. Quantitative RT-PCR will be carried out in a Bio-Rad CFX96 real-time PCR using the following conditions: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The expression level of the three NOS isotypes should be calculated using the comparative threshold cycle method normalized to housekeeping gene GAPDH.

## 3. Detection of NO and its metabolites

NO can react with oxygen, hemoglobin and various other molecules to form various metabolites such as nitrite, nitrate, *S*-nitrosothiols (RSNOs), *S*-nitrosohemoglobin etc. In view of difficulties inherent to direct NO detection, measurement of accumulated nitrite and nitrate, stable metabolites of NO, is often used to monitor cumulative NO synthesis, cells in culture, tissue homogenates, perfused organs, and organisms (Kevil et al., 2011). $H_2S$ can regulate the bioavailability and generation of NO and its metabolites. $H_2S$ also affect the release of NO through other species of NO such as nitrosothiol and metal nitrosyl complexes (Ondrias et al., 2008).

Detection of NO from biological samples is a challenge as this gaseous molecule is very unstable with a half-life in seconds. There are many discrepancies in the literature that reported the levels of NO often underestimated the actual NO produced from the biological samples. So, it is warranted to estimate accurate levels of NO by minimizing its loss through proper preservation/stabilization of the sample. Now there are several techniques available that determine the most accurate measurements of this gasotransmitter, and its metabolites with minimum error (Bryan and Grisham, 2007; Cai et al., 2007; Weissman and Gross, 2001). NO detection has evolved from the earlier measurements of Griess assay to the high sensitive chemiluminescence and direct electrode detection methods. This has reduced the erroneous detection levels of NO and its metabolites from millimolar to nanomolar over many years (Bryan and Grisham, 2007; Gomes et al., 2006; Hetrick and Schoenfisch, 2009).

### 3.1 Griess Assay – Classic biochemical assay for nitrite/nitrate/nitrosothiol detection

This is a spectrophotometric assay based on the formation of an aromatic amine, diazo derivative from nitrite ions in acidic conditions (Giustarini et al., 2008; Weissman and Gross, 2001). Oxidized NO or nitrite under acidified environment reacts with sulfanilamide to produce a diazonium ion, which further reacts with *NNED* to form a chromophoric azo product. For measuring NO synthesis, one potential drawback is that under normal conditions only a fraction of NO is oxidized to $NO_2^-$(in the absence of hemoglobin, usually greater than half). Moreover, there are studies suggesting the involvement of sulfide on interfering with the assay (van der Zee et al., 2001; Wedmann et al., 2014). Possible interference of sulfide through decolorization of diazo derivatives, the prime end product (van der Zee et al., 2001), thereby affecting the detection assay.

#### Protocol

1. This assay can be performed on most of the samples including tissue extracts, cell culture medium, plasma, urine and solutions etc.

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2. Prepare working Griess reagent by mixing equal parts of A and B (sulfanilamide and *N*-naphthyl ethylenediamine (NNED)). Care has to be taken to avoid any dry material while mixing reagents A and B, which may react with Griess reagents to give increased background absorbance thereby affecting the sensitivity of the assay.
3. Prepare nitrite/nitrate standards from stock and add 25 μl to triplicate wells in a 96-well plate in different wells. On the same plate add 25 μl volumes of unknowns and appropriate blanks to triplicate wells. A useful standard of nitrite/nitrate is prepared as: 0, 12.5, 25, 50, 75, and 100 μM.
4. Dilute nitrate reductase to a final concentration of 0.1 U/ml and add 25 μl of nitrate reductase buffer to all wells.
5. Add 100 μl of working Griess reagent to all wells. Quantify A540 or A550 in each well using a microtiter plate reader. A pink to purple color will develop within seconds, which is proportional to the combined concentrations of nitrite and nitrate in all samples.
6. Plot standard curves for nitrite and nitrate from all unknown samples nitrite/nitrate concentrations using a combined standard curve.

Some modified Griess reactions further reduce nitrate to nitrite using chemical reductants including cadmium ($Cd^{2+}$), zinc ($Zn^{2+}$), mercuric chloride (HgCl2) and Vanadium chloride or enzymes such as bacterial nitrate reductase (NR). However, some of these methods are not reliable as they further reduce nitrite (zinc) or limited shelf life of the compounds and additionally, slows the reaction kinetics. Cadmium in presence of copper is a most favorable choice (Cortas and Wakid, 1990; Green et al., 1982), where the conversion of nitrate to nitrite can be achieved rapidly as represented below:

$$NO^{3-} + H_2O + Cd \rightarrow NO^{2-} + Cd^{2+} + 2NH_2OH$$

It has been suggested that prior treatment of biological samples with $Cd^{2+}$, $Zn^{2+}$ can reduce the interference of sulfide with the Griess reaction (Wedmann et al., 2014).

## Key points and limitations

1. Less sensitive than any other assay such as chemiluminescence and does not perform a direct detection of NO.
2. Only a fraction of NO is oxidized to $NO_2^-$ the remainder goes to $NO_3^-$
3. It is a simple chemical/enzymatic assay for quantifying the sum of nitrate and nitrite in biological samples with colorimetric read out
4. It can be performed in 96 well plates.
5. Does not require specialized equipment, following diazotization with the Griess reagent
6. Reduced azo dye signal by a possible interference of sulfide with the Griess reagents.

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7. Reactivity of different compounds varies with NO among various biological samples. So the measurement of NO must be interpreted with caution considering the variations in the settings. Wang et al reported no changes in plasma NO levels upon $H_2S$ therapy during tissue ischemia (Wang et al., 2010). However, this study only examined plasma nitrite/nitrate levels with the Griess reaction methodology, which does not account for NO, nitrosothiols, nitrosoheme).

### 3.2 Chemiluminescent detection of NO metabolites

Advancement in techniques for sensitive and specific detection of NO species has further revealed the physiological and biochemical role of NO and its metabolites including RSNOs and Hb-NO. Detection of NO metabolites using chemiluminescence technique is a widely used approach. The principle of this detection method is based on specific reaction of NO with ozone (O3) to form excited-state NO2*. Spontaneous return to ground-state NO2 results in photon emission in the range of red to infrared energy.

As the reaction between NO and O3 proceeds at a near-diffusion-limited rate and in the absence of a significant activation barrier, this reaction goes to rapid completion at room temperature. Conventional photomultiplier tubes (PMTs) are sensitive to the lower end of this range of emitted light and afford an enormous amplification (>106 fold) of the current arising from a single-photon-induced displacement of an electron from the photosensitive PMT surface. Thus, chemiluminescent detection provides a very sensitive method for direct quantification of NO in picomolar quantities.

#### Protocol

**Stabilization Solution preparation:** Biological samples should be collected and placed in the stabilization buffer as homogenized in a solution designed to stabilize NO and all its derivations (Dejam et al., 2005; Kumar et al., 2008; Lang et al., 2007). Composition of stabilization buffer varies based on the sample to be processed.

**a.** Stabilization solution 1 (SS1) comprising of N-Ethylmaleimide (NEM) (5mM final concentration) and DTPA (100μM final concentration) along with an anti-coagulant sodium citrate (1.5% w/v) or Heparin (1 unit/ml 10% v/v).

**b.** Stabilization solution 2 (SS2) comprising of NEM (1mM final concentration) and DTPA (100μM final concentration) along with an anti-coagulant sodium citrate (1.5% w/v) or Heparin (1 unit/ml 10% v/v), NEM = 1mM and DTPA = 100μM.

**c.** Stabilization solution 3 (SS3) comprising of Potassium Ferricyanide ($K_3(FeCN)_6$) (133 mM final concentration), NEM (1.67mM final concentration) and detergent Nonidet NP-40 (1% v/v), prepared in PBS.

**Collection and storage of the samples:** Whole blood (500μl) has to be immediately (~15–30sec) mixed with SS3 (100μl); Cell/tissue samples should be collected and homogenized in SS3. Blood is centrifuged at 5000rpm for 5min at 4°C to separate the plasma and RBC. Plasma is stored in SS1 (1:1 vol), while RBC is stored in SS2 (1:5 vol). Samples should be mixed vigorously and incubated on ice for 5 min to allow reaction with K3(FeCN)6 to reach

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completion and then snap frozen in liquid $N_2$ and stored at −80°C until analysis on a Siever's 280i Nitric Oxide Analyzer (General Electric, California).

**Preparation of samples for analysis—**Samples are allowed to completely thaw on ice in the dark. Samples are vortex mixed briefly and centrifuged at 15,000xg for 2min. Supernatants are to be taken for further measurements as described. Aliquots of samples will be tested for sulfanilamide resistance or mercuric chloride ($HgCl_2$), by addition of an acidic sulfanilamide solution or $HgCl_2$ to a final concentration of 0.5% v/v, and allowed to sit in the dark for 15 min prior to injection into the analyzer.

### Process for analyses of Nitrate, Nitrite, S-nitrosothiols and XNO

1. Assemble the purge vessel with 1 ml of Sodium Iodide (NaI) catalyst and 7 ml of glacial acetic acid, for nitrite/nitrate detection. For nitrite/nitrate use 8 ml of acidic vanadium chloride and 100 μl Antifoam B in the purge vessel.
2. Connect the purge vessel to a helium tank and begin gassing through the porous glass frit at a rate that causes the liquid level to approach the top of the purge vessel (~10 ml/min). A continuous supply of water is provided into the purge vessel. The outflow of the purge vessel should be connected through a trap containing 1M NaOH. Connect the trap to the inflow valve of the chemiluminescence detector. If trap is not used the purge vessel directly connects to the chemiluminescence detector.
3. The vacuum, ozone generator and the photomultiplier tube (PMT) are turned ON. The vacuum introduces the sample into the reaction chamber and also purges the gases that may quench the light generated by NO2*. The vacuum should be preferably between 6 and <200 torr.
4. The PMT output signal can be seen on a monitor integrated to the chemiluminescence detector via the data-analysis system. Once the PMT output signal stabilizes, the system is ready for measurements.
5. Before measuring the samples, it is important to generate a calibration curve with known volume for known standard concentrations. Prepare a calibration curve with appropriate sodium nitrite standards and take the readings in duplicate or triplicate to establish linearity and reproducibility of peak areas.
6. Samples are introduced into the purge vessel with acidified NaI buffer by injection using a gas-tight Hamilton syringe through a septum on top of the apparatus. Helium rapidly sweeps the aqueous sample in the gaseous form into the chemiluminescence detector. The relatively small space of the purge vessel provides sharper peaks and high sensitivity. All the levels of NO metabolites have to be normalized to protein concentrations.

**Experimental protocol to identify interference of $H_2S$ on NO detection—**To address possible effects of $H_2S$ on chemiluminescent detection of NO, we have experimentally demonstrated using a cell free sample with donors of NO and $H_2S$ using reductive acidic triodiode (KI/I3) as described below:

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1. Prepare various working concentrations (0.1, 1, 10, 100, 200, 500μM and 1mM) of NO donors – Sodium nitrite (NaNO2; Sigma Chemicals) and Diethylenetriamine NONOate (DETA NONOate; Sigma chemicals); Sulfide donors – Sodium sulfide ($Na_2S$) and GYY4137 (Cayman chemicals). Prepare another set of samples with a pretreatment of CuSO4 to reduce any reactive thiols present in the samples.
2. Assemble the purge vessel of the chemiluminescence detector and prepare sodium nitrite calibration curve as mentioned in the protocol above.
3. Inject the 50μl samples, as indicated above in step 1 into the purge vessel containing a solution of potassium iodide and glacial acetic acid. Samples are run by replacing the purge vessel with fresh reaction solution between each individual experiments.
4. Signals generated from the samples are integrated and compared to sodium nitrite standard curves to calculate concentrations. Signals from the samples reflect total NOx concentrations (nitrite + S-nitrosothiol + XNO).

Figure 3 indicates distinct peaks of NO levels obtained from various concentrations (0.1, 1, 10 and 100 μM) of NO donors sodium nitrite ($NaNO_2$) and DETA-NONOate (Fig 3A and B). In contrast, no signal peaks were observed using sulfide donors $Na_2S$ at 0.1, 1.0, and 10 μM) (Fig 3C) or GYY4137 at 0.1, 1.0, 10, 100, 500 μM and 1mM (Fig 3D). However, very small signal peaks were observed using 200, 500 μM and 1mM concentrations of $Na_2S$ but no signals from GYY4137 at the same concentrations (inset Fig 3C and D) using chemiluminescent detection. Together, these data demonstrate that chemiluminescent detection is 50 times more sensitive in detection of 10 μM $NaNO_2$ than an equal molar concentration of $Na_2S$. Nonetheless, high concentrations of $H_2S/HS^-$ (>500 μM) might possibly interfere with measurements of low NO concentrations using chemiluminescence. Teng et al. have also reported similar observations, but under alkaline pH using higher concentrations of $H_2S$ (1mM to 10mM) (Teng et al., 2008). Thus, care should be taken to employ proper controls and appreciate the potential concentration parameters of possible sulfide levels when measuring NO using chemiluminescence.

### Key points and limitations

1. Chemiluminescence assay is a highly sensitive detection method for measurement of NO metabolites.
2. Chemiluminescence assay can measure multiple forms of NO metabolites like free nitrite, nitrate, nitosothiols and nitosoheme upon simple pretreatment of the samples with certain chemicals/inhibitors.
3. One of its disadvantages is that it cannot measure real time NO levels directly emanating from intact tissues.
4. Minimal interference of signals with higher concentrations $H_2S$ > 1mM (Figure 3).

## 3.3 Real-time detection of NO by electrode probe

Malinski et al., has first devised a carbon fiber ‘microsensor’ in amperometric or voltammetric mode for detection of NO gas in biological systems, with a detection limit of

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10nM (Malinski and Taha, 1992). Presently there are World precision instruments (WPI) and other commercial companies like Innovative Instruments Inc., offer this electrode probe for NO detection with sensitivity as low as 0.03 pA/nM. Amperometric detection of NO is based on oxidation of NO on the surface of an electrode and subsequent generation of current. Gaseous NO diffuses across the NO selective membrane and into sensor containing Ag/AgCl electrode pair. Upon applying of potential equivalent to 900 mV on the working electrode, small redox current is generated due to the oxidation of NO at the electrode, which is measured by an amplifier and recorded. The Principle of this detection is represented in the equation below:

$$NO+e^{-}-->NO^{+}+OH^{-}-->HNO2-->H^{+}+NO_{2}--->NO_{3^{-}}+2H_{2}O+3e^{-}$$

NO in the presence of $OH^{-}$, it is converted into nitrite ($NO_2^{-}$) further converted to nitrate.

However, a recent study indicates the response exhibited by this electrode upon addition of $H_2S$ (Wedmann et al., 2014). This study revealed that the NO specific electrode displays signals as response to addition of $H_2S$ (1mM). It has to be further investigated with various concentrations of $H_2S$ at physiological range. However, the usage of this direct NO detection has to be carried out with caution considering the electrode response to other radicals/gaseous molecules such as $H_2S$.

**Calibration—**Like most other electrodes NO electrode should be calibrated before quantitative measurements can be made. Calibration based on a chemical reaction of nitrite with iodide in acid that generates known concentrations of NO as the following equation:

$$2\,KNO_2+2\,KI+2\,H_2SO_4-->2\,NO+I_2+2\,H_2O+2\,K_2SO_4$$

A calibration curve demonstrating changes in current or peak height as a function of NO concentration can be produced. The generation of NO is based on the following equation where a known amount of $KNO_2$.

The electrode probe has to be gently placed on to the surface of the cells with Hank's balanced salt solution (supplemented with 2 mM CaCl 2 and 20mM Hepes, pH 7.4); or can be used on the surface of tissues to measure the at variable concentrations the peaks in NO.

## Key points and limitations

1. Usage of this electrode probes for sensitive NO detection real time; it is very simple and easy to perform.
2. Studies looking for $H_2S$ effects on real time NO variations can be performed using this probe.
3. However, this method can measure NO levels on the periphery and not suitable for detection of NO metabolites in deeper tissues.

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### 3.4 ESR detection of NO

ESR (or electron paramagnetic resonance (EPR) – spin trapping) spectroscopy is a useful tool to detect NO from various sources – in vitro, ex vivo and in vivo. Detection of NO using ESR has been detailed in the literature (Cai et al., 2007; Hogg, 2010). NO is a relatively stable radical and not directly detectable by EPR spectroscopy and should be conjugated with compounds such as 'spin traps'. Upon reacting with spin traps, NO becomes detectable by EPR. Different spin traps can used for NO detection by EPR, such as Iron (Fe) based hydrophobic diethyldithiocarbamate ($Fe^{2+}(DETC)_2$), which is more compatible with biological fluids such as blood and hydrophilic *N*-methyl-d-glucamine dithiocarbamate ($Fe^{2+}(MGD)_2$) spin traps for extracellular detection of NO such as cells and tissues (Cai et al., 2007; Vanin and Poltorakov, 2009). Nitronyl nitroxides (NNO), or imidazolinoxyl-N-oxides have been identified as both NO detectors and scavengers (Akaike et al., 1993; Joseph et al., 1993). One of the disadvantages using with dithiocarbamates is copper interference with the Fe/NO signal.

One of the commonly used probe in this category is 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5, tetramethyl-1H- imidazoyl-1-oxyl-3-oxide (CPTIO). Recent study demonstrated that CPTIO directly react with $H_2S$ to form hydroxylamine (Wedmann et al., 2014). Usage of NNOs for NO detection for biological samples with $H_2S$ has to be performed with caution. However, the concentrations used (CPTIO 1mM and $Na_2S$ 10mM) to understand this interaction requires further examination using physiological ranges of sulfide and relevant levels of CPTIO used for experimental purposes.

### 3.5 Fluorescent detection of NO

Several fluorescence indicators for direct NO-imaging in vitro or in vivo have been commercially developed based on aromatic diamino derivatives of fluorescent chromophores. These fluorescent probes have distinct excitation/emission (ex/em) wavelengths. Some of the popular fluorescent probes and their ex/em include 2,3-diaminonaphthalene (DAN; 365/415nm), 4,5-diaminofluorescein (DAF-2) and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) both with same 495/515nm, whereas diaminorhodamine-4M (DAR-4M; 560/575) and 1,2-diaminoanthraquinone (DAA) (Lacza et al., 2005; von Bohlen und Halbach, 2003). These substances interact with NO to form a fluorescent triazole complex. However, these fluorescent probes have issues with the specificity (detection of other thiols, nitrosating species) and autofluorescence based on the biological substances such as tissues. Therefore, use of these fluorescent probes have to be carried out with caution as there is a possibility of $H_2S$ influence that may produce erroneous NO levels.

## 4. Novel adducts from $H_2S$-NO interactions

The sulfur of $H_2S/HS^-$ has the lowest valence (−2) and therefore serves as a reductant. NO, on the other hand, is a poor one-electron oxidant is as monomer in biological conditions (Fukuto et al., 2012). Additionally, the paramagnetism of NO makes it even less possible for direct interaction with the diamagnetic $H_2S/HS^-$ (Bruce, 2013). Although recent studies suggested direct reaction between $H_2S$ and NO is possible (Eberhardt et al., 2014), mounting

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evidence supports NO adducts as important intermediates of $H_2S$ and NO interactions. We discuss here various -NO adducts that are discussed in recent research publications.

### 4.1 Peroxynitrite (ONOOH/$ONOO^-$)

NO and superoxide react with a near diffusion rate constant (Huie and Padmaja, 1993) to give rise to peroxynitrite (ONOOH/$ONOO^-$), a potent oxidant with important roles in neurodegenerative diseases, inflammation and ischemia-reperfusion. Moore et al. first reported $H_2S$ scavenges $ONOO^-$ and prevents $ONOO^-$ induced cytotoxicity, similarly to GSH (Whiteman et al., 2004). Carballal et al. proposed a nucleophilic attack by $H_2S$ on $ONOO^-$ to form HSOH and $NO_2^-$ (Sebastián et al., 2011). Filipovic et al., on the other hand, observed $H_2S$ scavenges $ONOO^-$ with a second order rate constant of $8 \pm 2 \times 10^3$. The major product is sulfinyl nitrite (mainly HS(O)NO) as the major product, which further decomposes to NO (Milos et al., 2012). The antioxidant effects of $H_2S$ have been partially attributed to the direct scavenging of oxidants. However, this is questionable considering the relatively low physiological concentration of $H_2S$ (Sebastián et al., 2011).

### 4.2 $H_2S$ interactions with NO donors

Bian's group and Moore's group studied the interaction of $H_2S$ and NO using NaHS and a variety of NO donors. In the study of Yong et al., while NaHS (50μM) does not affect myocyte contractility, it reversed the negative inotropic effect introduced by NO donors (L-arginine, SNP and DEA/NO) (Yong et al., 2010), suggesting formation of a product other than NO. They suggested that this product to be HNO/$NO^-$ by using Angeli's salt ($Na_2N_2O_3$) to mimic observed effects and using HNO/$NO^-$ scavengers (NAC, L-cysteine and GSH) to block response mediated by NaHS and SNP. Similarly, Filipovic et al. have found that SNP blocks $H_2S$ induced contractility of rat uteri and increased cellular level of HNO/$NO^-$ in HUVECs treated with 100μM $Na_2S$ and 100μM SNP. However, they indicated that this is a multi-step direct reaction between $H_2S$ and SNP, involving formation of $[(CN)_5FeN(O)SH]^{3-}$, reduction of HSNO/$SNO^-$, and reaction between polysulfides and $[(CN)_5Fe(HNO)]^{3-}$ (Filipovic et al., 2013).

Multiple endogenous sources of HNO/$NO^-$ have been proposed. An early work suggested that S-nitrosothiols may react with thiols to yield HNO/$NO^-$ (Wong et al., 1998). HNO was evaluated according to the product of the reaction between HNO cyclohexyl mercaptan, by HPLC, LC-MS and GC-MS. However, the study used GSNO and GSH were used at mM concentrations, which may lack physiological relevance. It is also shown that NOS may generate HNO/$NO^-$ instead of NO when electron transfer to $H_4B$ is hindered (Wei et al., 2003). Oxidative heme proteins have shown to oxidize hydroxylamine derivatives to release HNO/NO measured by methemoglobin$^-$ (Reisz et al., 2010). Recently, improved analytical methods using phosphine ligation have also been reported (Reisz et al., 2009; Reisz et al., 2011). A novel fluorescent probe has also been developed to measure the cellular HNO/$NO^-$ level. Improved analytical methods using phosphine ligation have also been reported (Filipovic et al., 2013; Rosenthal and Lippard, 2010; Wrobel et al., 2014; Zhou et al., 2011).

In another study Moore et al. found $HgCl_2$ and $CuCl_2$ restore the increase of cGMP by 100μM SNP, which is inhibited by 100μM NaHS (Whiteman et al., 2006b). Since $HgCl_2$

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enhances the decomposition of S-nitrosothiol to nitrite, they proposed S-nitrosothiol as a product of $H_2S$ and NO reaction. Despite the fact that SNP can react with $H_2S$ directly, another NO donor, SIN-1, releases $O_2^-$ along with NO (Feelisch et al., 1989), leaving the reaction to be between $H_2S$ and oxidative nitrogen species. Moreover, as mentioned previously (Qian and Jack, 2013), Moore's study used various NO donors, including SNP, SIN-1, spermine NONOate, DD1 (4-Bromo-3,3,4-trimethyl-1-oxo-1,2-diazetidin-1-ium-2-olate), DEA NONOate and EDTA NONOate; yet only nitrite introduced by spermine NONOate and DEA NONOate have been inhibited by NaHS. Also, in the case of SNP, SIN-1 and DD1, nitrite level "restored" by $HgCl_2$ has been much higher than NO donor alone.

### 4.3 S-nitrosothiols

Unlike the unfavorable reaction between $H_2S$ and NO, $H_2S$ reacts with S-nitrosothiol to yield a 'yellowish product', which has been observed by different groups (Cortese-Krott et al., 2014a; Filipovic et al., 2012b; Munro and Williams, 2000; Seel F., 1988). The mechanism of this reaction remains unclear. Munro and others have assigned this yellow product to $HSSNO/SSNO^-$ (Munro and Williams, 2000). On the contrary, Filipovic and his colleagues have demonstrated using ultrahigh-resolution ESI-TOP mass spectrometry, that $HSSNO/SSNO^-$ has been absent from the product of the reaction between $Na_2S$ and GSNO (Filipovic et al., 2012), and identified HSNO as the product. Moreover, using FTIR spectrometry, they also observed the disappearance of characteristic N=O vibration with retaining of the yellow color. Therefore, it has been concluded that HSNO mediates $H_2S$ and RSNO interaction instead of $HSSNO/SSNO^-$.

On the contrary, Feelisch and his group suggested that the mobile hydrogen in HSNO makes the molecule to be present as HNSO, HOSN and HONS as well (Cortese-Krott et al., 2014). Instead of releasing NO, these isomers tend to form gas or polymers (Müller R.P., 1984). Although, the complexity of UV/Vis spectra implies the existence of multiple reactions, the formation of HSNO and its long-term presence is debated. It has been proposed that HSNO should react with $HS^-$ to yield $HSSNO/SSNO^-$ eventually (Cortese-Krott et al., 2014a). However, more direct evidence is needed to prove these potential mechanisms.

### 4.4 Experimental Procedures

**4.4.1 UV-Visible and Stopped-flow Spectroscopy—**Stopped-flow is a spectroscopic technique used for kinetics study in solutions. Test reagents are mixed rapidly and contained in the sample cell for absorption and fluorescence measurements at a specific wavelength. Recordings over time can help analyze reaction rate and mechanism. The following protocol, adopted from Filipovic's study (Milos et al., 2012), uses $Na_2S$ and $ONOOH/ONOO^-$ as examples to illustrate how to use stopped-flow spectroscopy to study $H_2S$ interaction with NO adducts.

**Protocol:** $Na_2S$ powder is kept in a desiccator at 4°C and dissolved in degased ultrapure water for experiment freshly. Working stock solution of $Na_2S$ concentration is verified with monobromobimane (MBB) and RP-HPLC as we have published (Shen et al., 2012). $ONOOH/ONOO^-$ is prepared as reviewed elsewhere (Robinson and Beckman, 2004). The

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measurements are achieved by μSFM-20 Bio-Logic stopped-flow module, Huber CC90 cryostat, J&M TIDAS high-speed diode array spectrometer with deuterium and tungsten lamps. 10ml Hamilton syringes sealed with Isolast perfluoroelastomer O-rings controlled by different drives. Data are analyzed by Bio-Kine software and Specfit/32™. $Na_2S$ and $ONOO^-$ solutions are diluted in 300mM phosphate buffer (pH 7.4). While ONOO- concentration is fixed at 140μM, $Na_2S$ is kept excessive (0.5–3mM).

### Key points

1. UV-visible light spectroscopy is one of the oldest forms of spectroscopy. It is simple to use and able to study reaction kinetics with stopped-flow setup.
2. Stopped-flow spectroscopy is especially useful to capture transient intermediate in reactions and reveal a whole spectrum of products. However, it does not confirm the identity of an intermediate or product, but only electronic and vibrational properties.
3. Absorption of the structure has to be known to make an intelligent speculation. Interpretations of the data may vary due to the complexity of the reaction.

**4.4.2 Mass Spectrometry—**Mass spectrometry is one of the most powerful techniques for probing biological samples. Assays based on mass spectrometry have been used to analyze a variety of NO and $H_2S$ derivatives. The following is an example using LC-MS for direct GSNO measurement.

**Protocol:** Desalt the biological samples by eluting through a C18 column (90% formic acid [0.1% v/v], 10% methanol, 1.5 ml/min). Lyophilize the eluted samples and reconstitute. Add $^{15}$N-Glutathione as a control to avoid artificial on-column formation of GSNO. Photolyze the samples or pre-treat with $HgCl_2$ samples to use as a negative control. Analyze the eluted samples by an electrospray ionization MS (Finnigan LCQ Deca XP, Xcalibur software). GSNO cations are detected by selective ion monitoring at 336.9 (m/z).

### Key comments

1. SNO species are liable to photolysis and oxidation. Biological samples have to be processed rapidly in cool and inert solution.
2. If samples are processed in acidic condition, a corresponding reduced thiol (in this example $^{15}$N-glutathione) has to be used as a control for artificially increase SNO signal. Other SNO species can be measured with this protocol at appropriate m/z values.
3. The detection limit of this method for SNO is reported to be ~200nM (Gow et al., 2007). Protein SNO can be biotin-labeled before MS measurement. However, the biotin switch assay can be unreliable if samples are not processed properly, as discussed above.
4. Other NO and $H_2S$ derivatives can be measured using isotope labeled NO and $H_2S$ donors, such as GS$^{15}$NO and Na2$^{34}$S (Filipovic et al., 2012b; Shen et al., 2014). It is also reported that HNO can be measured by phosphine based labeling *in vitro*

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and *in vivo*. However, only AS derived and enzymatically synthesized HNO is tested. Its application in biological samples remains to be investigated.

**4.4.3 HNO/$NO^-$ Imaging with CuBOT1—**CuBOT1 is the first fluorescent probe detecting HNO/NO- over other ROS/RNS in live cells. It has absorption and excitation wavelength at 518nm and emission wavelength at 526nm (Rosenthal and Lippard, 2010).

**Protocol:** Sub-culture Human umbilical vein endothelial cells (HUVECs, passage 3–7) in 35 μ dishes to confluence in low serum medium overnight. Incubate the cells with 10μM CuBOT1 in cell culture medium for 20 min (Eberhardt et al., 2014; Filipovic et al., 2013; Filipovic et al., 2012b). Wash the cells with fresh medium three times before the treatments. Real-time image can be captured with an inverted epifluorescent microscope with green fluorescent filters.

### Key points

1. CuBOT1 is shown to be highly specific to HNO, with no interference by $NO_3^-$, $NO_2^-$, $ONO_2^-$, $OCl^-$, $O_2^-$ or NO. Angeli's salt activates CuBOT1, so does the combination of DEA NONOate and $Na_2S$ in cell culture (Eberhardt et al., 2014). Interestingly, neither DEA NONOate nor $H_2S$ itself increases fluorescence.
2. Exogenous cysteine at 200 μM increased fluorescence of the probe rapidly, while the normal intracellular cysteine level did not activate the probe (Rosenthal and Lippard, 2010). Consistently, recent study showed that depletion of either arginine or cysteine decreases fluorescence significantly (Eberhardt et al., 2014). These evidence suggest that NO and $H_2S$ may interact to give rise to HNO.
3. Moreover, as a fluorescence probe, CuBOT1 is able to do real-time imaging and reveal subcellular location. However, it does not measure absolute concentration of HNO.

**Conclusion:** It is beyond contention from available literature that there is an interaction between $H_2S$ and NO. Proposed interactions between these two molecules involve formation of various adducts. The information provided in this chapter details the precautions to be taken while performing NO measurements, in lieu with the possible $H_2S$ interference.

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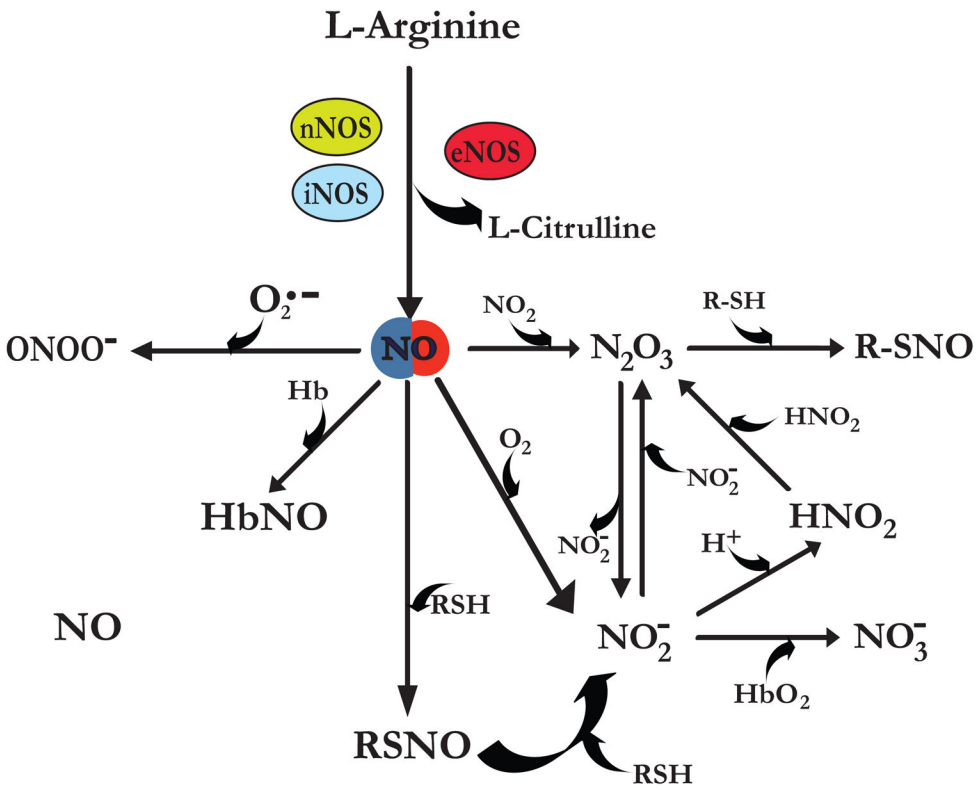


**Figure 1. Biosynthesis of NO and its metabolism**

NO is enzymatically synthesized by NOS isoforms – neuronal, inducible and endothelial NO synthase (nNOS, iNOS and eNOS) that catalyze the conversion of L-arginine to L-citrulline. NO undergoes oxidation to form nitrite ($NO_2^-$) and nitrate ($NO_3^-$). NO reacts with free thiol (R-SH) to form nitrosothiol (R-SNO). NO may react with either oxyhemoglobin ($HbO_2$) to form nitrate, or with hemoglobin (Hb) to form nitrosylhemoglobin (HbNO). NO can also be formed non-enzymatically from reduction of nitrite. Nitrite upon reacting with NO or another nitrite forms dinitrogen trioxide ($N_2O_3$) that further reacts with free thiol to form R-SNO or reduces to form nitrite. $N_2O_3$ can also be formed from nitrous acid ($HNO_2$) that in turn is formed from nitrite. Additionally, NO may also react with superoxide to produce peroxynitrite.

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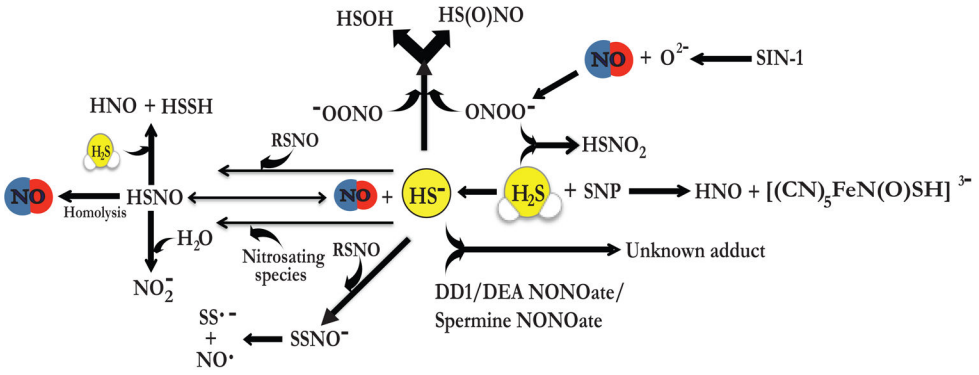


**Figure 2. Novel adducts from NO–$H_2S$ interactions**

Sulfide radical $HS^-$ can react with NO to form thionitrous acid (HSNO). Reaction of $HS^-$ either with a thiol (RSNO) or nitrosating species can also from HSNO. Alternatively, $HS^-$ can react with RSNO to form nitrosopersulfide ($SSNO^-$), which further disintegrates into NO and disulfide radical ($SS^{\bullet-}$). HSNO can undergo hemolysis to form NO or react with $H_2S$ to form a disulfide (HSSH) and HNO. It can also form nitrite upon hydrolysis ($H_2O$). NO donors can directly or indirectly involve to form new adducts. SIN-1 can generate both NO and $O_2^{\bullet-}$ that leads to formation of peroxynitrite ($ONOO^-$). Peroxynitrite can react with either $H_2S$ or its radical $HS^-$ to form sulfinyl nitrite ($HSNO_2$ or HS(O)NO). $HS^-$ can also form HSOH upon reacting with $ONOO^-$. Other NO donors such as DD1, DEA NONOate or Spermine NONOate are known to react with $HS^-$ to form an unknown adduct. Sodium nitroprusside (SNP)($Na_2[Fe(CN)_5\ NO]$) reacts with $H_2S$ to form nitroxyl (HNO) and an intermediate $[(CN)_5FeN(O)SH]^{3-}$.

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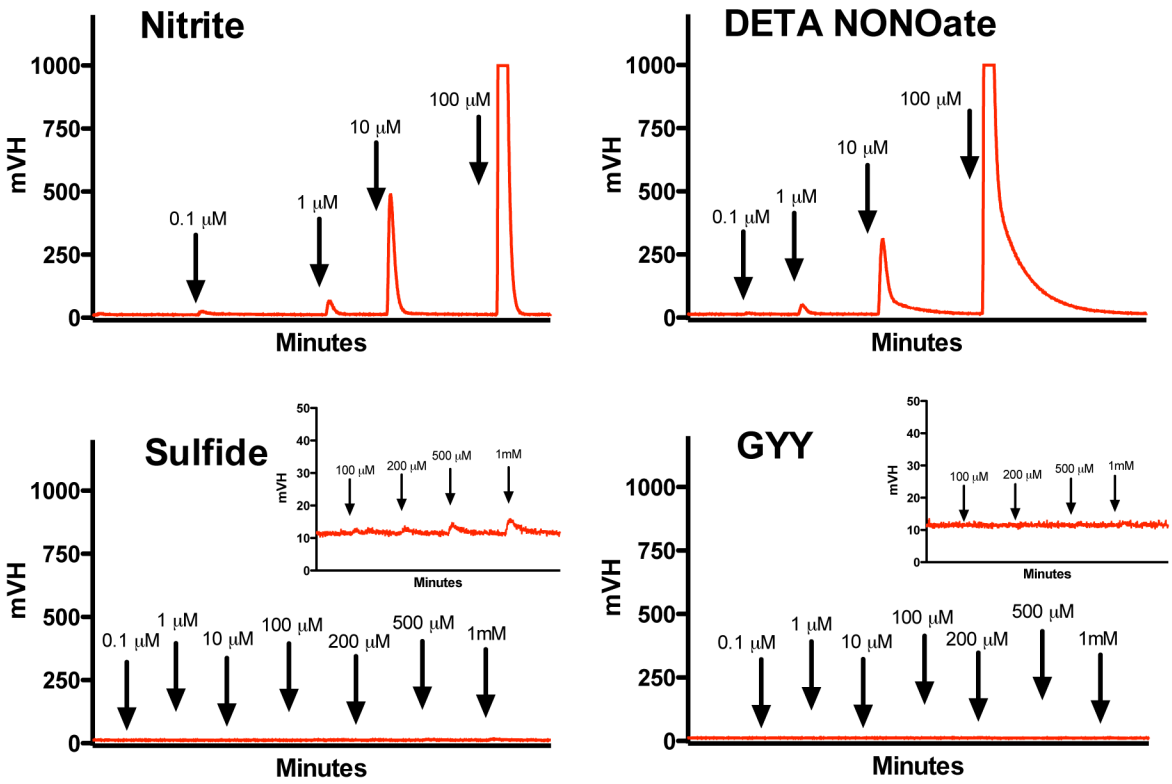


**Figure 3. $H_2S$ interference in chemiluminescence detection of NO**

Using the tri-iodide method in the Sievers 280i chemiluminesence detector, 50μl of increasing concentrations (0.1μM to 1mM) of either A) Sodium Nitrite, B) DETA NONOate, C) Sodium sulfide or D) the sulfide donor GYY were injected. As shown in A) and B) NO donors resulted in increasing NOx generation in a dose dependent manor. Panels C and D illustrate little to no NOx production with increasing concentrations up to 1mM when sulfide donors were injected. The insets in panels C and D show a magnified section of the higher concentrations. The small peaks shown in the magnified inset with Sulfide (C) could indicate the limitation of filter system used in the Sievers 280i to eliminate sulfide chemiluminesence. These small peaks are not visible in the GYY samples (D inset).