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Analysis of S-nitrosothiols via Copper Cysteine (2C) and Copper Cysteine - Carbon Monoxide (3C) Methods

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Abstract

This chapter summarizes the principles of RSNO measurement in the gas phase, utilizing ozone-based chemiluminescence and the copper cysteine (2C) $\pm$ carbon monoxide (3C) reagent. Although an indirect method for quantifying RSNOs, this assay represents one of the most robust methodologies available. It exploits the NO $\cdot$ detection sensitivity of ozone based chemiluminescence, which is within the range required to detect physiological concentrations of RSNO metabolites. Additionally, the specificity of the copper cysteine (2C and 3C) reagent for RSNOs negates the need for sample pretreatment, thereby minimizing the likelihood of sample contamination (false positive results), NO species inter-conversion, or the loss of certain highly labile RSNO species. Herein, we outline the principles of this methodology, summarizing key issues, potential pitfalls and corresponding solutions.

1.0 INTRODUCTION

1.1 S-nitrosothiols (RSNOs)

S-nitrosothiols (RSNOs) are formed when an NO group binds to the sulfur atom of a thiol (i.e., a compound containing a carbon bonded sulfhydryl (-SH) group). This process occurs endogenously via both enzymatic [1] and non-enzymatic [2] reaction pathways [3]. The main target thiols involved in these reactions include protein (e.g., hemoglobin, albumin) or peptide (e.g., glutathione) cysteine residues, and cysteine itself [4].

1.1.1 Process of RSNO formation—Under physiological conditions, NO $\cdot$ (in its radical form) does not readily react with thiols, other than in exceptional circumstances when thiyl radicals are present (reaction [1]) [4–6]. For RSNO formation to occur, NO $\cdot$ must be oxidized to a nitrosonium (NO $^+$) equivalent (reaction [2]) [4, 5]. Several electron acceptors have been demonstrated to catalyze this reaction, including oxygen (O $_2$), Cu $^{2+}$, Fe $^{2+}$, and NAD $^+$ [4, 5, 7–9]. Once formed, the NO $^+$ equivalent can then react with a cysteine thiolate to form an RSNO bond (reaction [3]) [4].

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RSNOs can transfer NO^+ from one cysteine thiolate anion to another, in a process called transnitrosation (reaction [4]). Occasionally, transnitrosation involves the passing of a nitroxyl (NO^-) equivalent [5].

1.1.2 Detection strategy for RSNOs—The quantification of RSNOs in biological matrices is extremely challenging due to the diverse array and low concentrations of NO metabolite species. This complexity is increased by their labile and reactive nature. While a number of measurement techniques have been developed to quantify biological RSNOs, very few offer the specificity and sensitivity to measure physiological concentrations of these species.

Indirect methods are most commonly used, due to the scarcity of direct measurement techniques (i.e., only mass spectrometry and the anti-nitrosocysteine antibody directly detect intact RSNOs [3]). Indirect methods involve generation of a free $\text{NO}^\bullet$ group from the original RSNO species, in combination with a method of detecting the released $\text{NO}^\bullet$ [3]. To work successfully, such strategies require:

1. The means of releasing $\text{NO}^\bullet$ from the parent RSNO compound to be:
 - Specific to RSNO compounds, e.g. not releasing or producing $\text{NO}^\bullet$ from other potential contaminant species in the biological sample matrix.
 - Release $\text{NO}^\bullet$ in a 1:1 ratio to the parent RSNO group.
 - Demonstrate the above characteristics consistently for a diverse array of RSNO species and for individual RSNOs in a diverse array of biological matrices.
2. The means of $\text{NO}^\bullet$ detection to be:
 - Highly sensitive within the biological concentration range of the RSNO under study (i.e., capable of detecting very small amounts of free $\text{NO}^\bullet$).
 - Specific for $\text{NO}^\bullet$ alone (and not other potential interfering N-oxides, or gaseous components)
3. Biological samples to be handled:
 - Minimally, once removed from their physiological environment.
 - In a way that preserves protein chemistry, the redox environment, and other relevant physiological conditions encountered *in vivo* (i.e., pH and O_2 partial pressure) [10].

1.2 Chapter Summary

This chapter will summarize the principles of RSNO measurement in the gas phase, utilizing ozone-based chemiluminescence as the means to detect NO• and the copper cysteine (2C) ± carbon monoxide (CO) (3C) reagent as the means by which NO• is released from parent RSNO compounds. Additionally, sample-handling procedures will be outlined to demonstrate how the artificial loss or formation of RSNOs during processing is avoided.

2.0 MATERIAL AND METHODS

2.1 Experimental Apparatus

The measurement of NO• in the gas phase requires an enclosed gas tight system, comprising (1) a carrier gas (an inert gas such as nitrogen, argon, or helium for 2C, or CO for 3C), which continually purges the system. Carrier gas flow must be regulated via (2) a flow meter, controlling and stabilizing flow between the gas tank and the rest of the system. From the flow meter, gas travels through (3) a water-jacketed purge vessel, in which the copper cysteine cleavage reagent is held and heated (50° C). The gas stream carries released NO• groups to the ozone-based chemiluminescent nitric oxide analyzer (NOA). Between the purge vessel and the NOA, at least (4) one condenser and (5) one solvent filter should be placed in-line (Figure 1).

Samples are injected into the purge vessel, via a rubber septum covered injection port, utilizing a gas-tight Hamilton syringe. In the purge vessel, the SNO bond is cleaved from the original RSNO species by the copper cysteine reagent (reaction details below). The free NO• group is then carried at a constant flow rate to the NOA reaction chamber, where it reacts with ozone (O₃). The reaction chamber is coupled to a photomultiplier tube, which generates a signal (mV) directly proportional to NO• concentration in the inert gas flow. Resultant peaks are integrated and the RSNO concentration is determined by comparison to a previously generated standard curve.

All components of this system are joined via gas-impermeable ¼ inch tubing and gas tight fittings. This design minimizes “dead space”, ensuring the efficient transfer of NO• from the purge vessel to the NOA [10]. Any dead space will disrupt gas flow, increasing the delay in signal detection, broadening the resulting peaks and limiting measurement sensitivity.

2.1.1 Carrier Gas—The carrier gas, which transports NO• from the purge vessel to the NOA, also maintains an anaerobic environment within the experimental apparatus, thereby preventing loss of NO• signal resulting from reaction with O₂ (the autoxidation reaction – reaction [8]).

Carrier gas type should be selected according to the RSNO sample being quantified and the surrounding biologic matrix. Samples free of heme proteins permit use of an inert gas, such as argon, helium, or nitrogen. CO however, must be used when quantifying samples rich in heme proteins. By competing with NO• for binding at heme, CO prevents auto-capture of NO• released from heme-containing SNO proteins (such as SNO-hemoglobin) or prevents capture by non-SNO heme-proteins in a complex biological matrix, when other RSNOs are the analyte of interest [11–13]. Such inhibition by CO (3C method) of otherwise avid NO•-heme binding is required since physiological levels of NO•-heme go undetected in the copper cysteine (2C) reagent [14], NO•-heme auto-capture results in a significant reduction in measurement sensitivity (section 2.3.2.1).

2.1.2 Flow Meter—Carrier gas flow must be tightly regulated (ideally via a sensitive and stable, gas-specific mass flow meter) to ensure flow is appropriately balanced against the NOA draw, thus optimizing both headspace pressure in the purge vessel (influencing dissolved gas concentrations in the 2C or 3C reagent) as well as chamber pressure for the $\text{NO}^\bullet + \text{O}_3$ reaction within the NOA reaction cell [15].

2.1.3 Purge Vessel—The purge vessel should be water jacketed, enabling the copper cysteine reagent to be held at 50°C (for 2C and 3C) [16]. The vessel itself should hold 10–20ml of reagent solution and be easily filled/flushed/emptied (via a drainage port). This enables the processing of a large number of biological samples, which requires reagent exchange between each sample injection.

2.1.4 Condenser and Filters—Gas from the purge vessel should flow through a condenser (maintained at 4°C, by chilled water bath) prior to entering the NOA. Additionally, at least one solvent filter should be placed in-line between the condenser and the NOA (Whatman, solvent IFD). The condenser(s) and filter(s) ensure that the gas stream from the purge vessel to the NOA is as dry as possible [10], eliminating any possible quenching of $\text{NO}^\bullet$ signal by water vapor [17], while also protecting the NOA reaction cell from moisture and possible damage [18]. The condenser also optimizes the $\text{NO}^\bullet + \text{O}_3$ reaction in the NOA, which is reduced at higher temperatures [19].

2.2 Detecting NO: The Nitric Oxide Analyzer (NOA)

The chemiluminescent NOA is one of the most sensitive $\text{NO}^\bullet$ detection methods available [20]. It exploits the luminescent nature of the reaction between $\text{NO}^\bullet$ and O_3 [18]. An O_2 source tank is used to generate O_3 in the NOA reaction cell [18]. O_3 subsequently reacts with $\text{NO}^\bullet$, generating nitrogen dioxide (NO_2), a portion of which arises in an electronically excited state (NO_2^*) (reaction [5]) [21].

As the NO_2^* returns to its more stable ‘ground’ state, it releases energy. This energy is either observed as a photon ($h\nu$) (reaction [6]), or quenched following its interaction with other gas molecules (M) (reaction [7]) [21, 22].

Generated light is filtered, prior to amplification and detection via a photomultiplier. The resulting current is then converted to a recordable mV signal [22].

2.2.1 Sensitivity of the NOA—Several features of the NOA ensure optimized reaction with O_3 [6], thereby maximizing the sensitivity of $\text{NO}^\bullet$ measurement. Firstly, the system is held under vacuum, minimizing the concentration of gas molecules in the NOA reaction cell that could potentially quench energy from NO_2^* (M in reaction [7]) [22]. Secondly, the NOA utilizes a photomultiplier detector, which is highly sensitive to the wavelengths of light emitted by NO_2^* [22]. Finally, the photomultiplier and reaction cell are deeply cooled (–20° C), reducing dark current, which minimizes background noise, whilst also maximizing the output of light from the $\text{NO}^\bullet + \text{O}_3$ reaction [15, 17, 19].

2.2.2 Specificity of the NOA—Specificity for NO• measurement is maintained by filtering wavelengths not specific to the NO• + O₃ reaction (only those in the 640 – 900 nm band are detected). A red cut-off filter, positioned between the NOA reaction cell and the photomultiplier tube [22], ensures that light generated by reaction with potential contaminants (including NO₂, CO₂, CO, C₂H₄, NH₃, or H₂O), go undetected [20]. Provided O₃ is present in excess and reaction conditions are held constant, the intensity of light emitted from the NO• + O₃ reaction is directly proportional to the NO• concentration [20].

2.2.3 Calibrating the NOA: Standard Curve—A calibration curve should be generated daily to account for variability in system sensitivity. The standard used should be representative of the species under study, and within its biological concentration range. A set volume of each standard concentration in triplicate (and ddH₂O or standard-free buffer as a ‘blank’ control) should be injected. The area under curve (AUC) of each resulting peak should be analyzed and plotted against SNO concentration (or absolute molar amount, as preferred), with the blank signal being subtracted from all standards. With the resulting AUC data, a linear trend line can then be plotted and the resulting line gradient used to calculate SNO concentration from the AUC of unknown samples.

2.3 Cleavage Reagent

2.3.1 2C—The CuCl/cysteine (2Cs) reagent, developed to specifically measure RSNOs under anaerobic conditions, forms NO• stoichiometrically from low mass RSNOs [16]. Excess cysteine (1mM) is used to drive the formation of CSNO (by transnitrosation) from parent RSNO compounds (analytes of interest) [10, 16], while Cu⁺ (100μM) homolytically cleaves the CSNO bonds thus formed, generating NO• and Cu²⁺ (reaction [9]) [23–25]. The Cu²⁺ is then recycled back to Cu⁺ by excess cysteine (reaction [10]) [16, 24, 26].

The 2C reagent must be produced fresh, each day of experimentation. Additionally, care must be taken to ensure the viability of the chemicals used. In particular, while stored (particularly if not stored in a desiccator), cysteine can readily undergo auto-oxidation to cystine, rendering it ineffective for this assay.

2.3.1.1 Sensitivity of 2C: The 2C reagent has been shown to be highly sensitive in quantifying low molecular weight RSNO species, including GSNO and CSNO (down to 5–10 pmole) [1, 16]. However, the efficiency of albumin-SNO detection is only approximately 35%. This lack of sensitivity is thought to result from incomplete transnitrosation between the sheltered SNO bond of albumin-SNO and CSH [16].

2.3.1.2 Selectivity of 2C: The major benefit of using the copper cysteine reagent (2C and 3C) is that it offers specific RSNO detection without the need for sample pretreatment. Many other reductive assays convert more than one NO metabolite species back to NO•, necessitating a means of isolating the individual metabolite species of interest prior to injection. This normally entails the addition of chemicals (to remove or make inactive certain metabolites) or sample separation by various means (e.g., HPLC). Ultimately, these pre-treatments increase the likelihood of either sample contamination (false positive results), NO species inter-conversion, or the loss of certain highly labile SNO species.

Of note, selectivity of the 2C reagent is highly pH dependent. At pH > 6, nitrite, nitrate and 3-nitrotyrosine are undetected [16]. However, if the pH falls < 6, nitrite is converted to NO[•] via acidic reduction (reactions [11] and [12]) [27].

The overall reaction being:

Maintenance of pH during assay is perhaps the most important practical consideration regarding use of the copper cysteine reagent. Extra vigilance is necessary when measuring samples to which HgCl₂ has been added (to generate negative controls, see 2.3.1.3), due to Cl⁻ release in water and H⁺ generation (following formation of Hg⁺ - reduced thiol complexes), or when antifoam is added to the reagent (which normally decreases the pH) [3, 10]. In order to minimize issues with pH, we suggest (1) making up the reagent in buffer (i.e., MOPS) and (2) then correcting the 2C reagent to pH 6.5 (at 50°C). In the absence of any buffering capacity, the pH of the 2C reagent will fall steadily over time [3, 10].

2.3.1.3 Assay Controls: In order to ensure the selectivity of RSNO measurement, several assay controls should be performed during each experiment. We recommend that:

1. All samples are treated with/without HgCl (buffered in PBS), prior to injection (Figure 2). This pre-treatment (under oxygenated conditions) hydrolyses the RSNO bond, leading to the formation of NO⁺, which is rapidly converted to nitrite. As nitrite is undetected in the copper cysteine (2C or 3C) reagent, this pretreatment should fully quench RSNO-attributable signal. Mercury reacts in such a way that it captures the thiol (forming a covalent bond with the sulfur – a mercaptan), thereby preventing further thiol reaction [28]. It is essential to realize that mercury concentration must be in excess of both RSNO and other free thiol groups in the sample; however, significant excess will lead to protein precipitation in samples – therefore, preparatory evaluation of sample:HgCl ratios should be undertaken. Both operator and environmental care should also be taken when handling this toxic chemical [29].
2. For further controls, samples should be spiked (separately) with nitrite and nitrate (10μM) to ensure that the (2C or 3C) reagent maintains specificity for RSNO measurement. If signals are observed following injection of these spiked samples, the most likely cause is a drop in the reagent pH (see section 2.3.1.2).

2.3.1.4 Measurement of biological samples: The injection of biological samples high in protein and lipid content into the copper cysteine reagent (under constant gas perfusion) results in significant foaming. This ultimately leads to peak broadening and loss of sensitivity. However, foaming can be minimized by adding antifoam to the (2C or 3C) reagent. Care must be taken when choosing the antifoam, to ensure it does not interfere with either selective NO[•] generation from RSNOs in the copper cysteine reagent, or with the detection of NO[•] by the NOA [30].

2.3.2 3C—The modified 2C assay, with substitution of CO as the carrier gas (hence 3C), was originally developed to assay total RSNO content of red blood cells (RBC) [11]. Avid

CO – heme interaction (with CO in significant excess to NO[•]) is exploited to block the interaction of NO[•] with deoxygenated hemoglobin in the purge vessel, thereby enabling NO[•] cleaved from RSNOs to be carried by the gas stream to the NOA for detection.

This assay involves the use of dangerous components and thus requires vigilance. We recommend the following safety precautions when utilizing this assay:

1. Ensure the experimental apparatus is set up in a well-vented area, ideally with a broad cone (linked to a fume hood) proximal to the purge vessel.
2. Check all fixtures and fittings in the experimental apparatus setup to ensure they are gas tight and there are no leaks [3].
3. Use an environmental CO alarm to warn operators of a CO leak [3].
4. Unhook the O₃ trap from the chemiluminescent NOA and instead vent the machine to a well-functioning fume hood. The O₃ trap contains hopcalite, which reacts exothermically with CO. The heat produced from this reaction can melt or ignite the O₃ scavenging cassette [3].
5. At the cessation of CO gas flow, purge the system with an inert gas before opening it to the atmosphere [3].

Despite the complexity associated with setting up the 3C assay, we recommend that 3C be used whenever measuring RSNO species in biological matrices containing significant amounts of heme proteins.

2.3.2.1 Sensitivity of 3C: The sensitivity of the 3C reagent for low molecular weight thiols has been demonstrated to be the same as that for the 2C assay [11]. The success of this assay in preventing heme autocapture of NO[•] in the purge vessel necessitates full saturation of the cooper cysteine reagent with CO. This requires the purge vessel pressure to be maintained at approximately 120 Torr above atmospheric pressure [3, 10, 18]. This headspace pressure ensures that the heme interacts with CO rather than NO[•], despite its greater affinity for NO[•] over CO [31, 32]. Once the 3C solution is fully saturated with CO, any further increase in headspace pressure does not improve detectability. Rather, NOA sensitivity is adversely affected; even when maintaining headspace pressure ~ 120 Torr it is necessary to place a flow restrictor between the NOA and condenser to protect the reaction chamber from the high-pressure up-system. Moreover, it is important to ensure that the flow meter employed to control CO delivery is accurate at this system pressure (sections 2.1.2 and 2.2.1) (Figure 3).

2.3.2.2 Selectivity of 3C: Like the 2C reagent, if the pH of the 3C solution remains above 6, nitrite, nitrate, 3-nitrotyrosine, or peroxynitrite are undetected [11]. However, 3C selectivity is affected by metal carbonyls, which accumulate when CO is stored in steel source tanks (even research grade CO contains metal carbonyls at around 0.7ppm). These generate light in the same band as NO[•] after reaction with O₃, thereby interfering with selective chemiluminescent detection of NO[•] [33]. To remove metal carbonyls, the CO source gas must be passed through iodine crystals and (in series) activated charcoal [11], prior to entry into the purge vessel. Of note, other components known to produce artifactual signals in the 3C system are the detergents Nonidet P-40 and Triton X-100 [11]. A detergent screen (for artifact) is highly recommended prior to assay of experimental samples.

2.3.2.3 Assay controls: If non-heme containing RSNOs are used as reference standards, we recommend comparing GSNO standard curves in the presence/absence of heme protein to ensure assay fidelity.

2.4 Sample Handling

Proper sample handling minimizes artifactual generation or degradation of RSNO metabolites. We recommend that samples are:

1. Collected and treated in solutions containing either ethylene diamine tetraacetic acid (EDTA), or diethylene triamine pentaacetic acid (DTPA). These metal chelators bind transition metals, which are capable of catalyzing RSNO decomposition [29].
2. Protected from UV light, which is also capable of catalyzing RSNO decomposition [34]. To achieve this, we cover vacutainers with silver foil, and use amber colored tubes for all sample processing.
3. Kept on wet ice, when possible.
4. Processed at pH and gas tensions similar to the physiological conditions observed at the time of collection; sample handling in an appropriate glove box under nitrogen or controlled gas blends is frequently necessary for this purpose.

2.4.1 Manipulation of O₂ tension—Red blood cell (RBC) hemoglobin conformation can be rapidly manipulated utilizing a rotating glass tonometer submerged in a water bath (37°C) (Figure 4). The spinning of the tonometer generates a thin film of buffer-suspended RBCs (or whole blood) on its internal surface, thereby optimizing conditions for rapid gas exchange [35]. The tonometer chamber is joined via a vacuum-tight, motorized couple (Rotavapor R110; Buchi, Flawil, Switzerland) to a gas source, permitting simultaneous chamber rotation and flushing by gas blended from O₂, N₂, and CO₂ tanks (OxyStreamer; BioSpherix, Lacona, NY, USA). Careful adjustment of the CO₂ content ensures the maintenance of a physiological pCO₂ and pH in Krebs buffered samples. This system not only allows controlled RBC oxygenation or deoxygenation, but also sample extraction without atmospheric exposure [11].

2.4.2 Maintenance of O₂ tension—Depending on the oxygen tension of the samples under study, we either process samples on the bench or (in the case of low oxygen tensions) in a hypoxic chamber (glove box) (Coy Laboratories, Grass Lake, MI). All processing of samples at low oxygen tension must be performed with deoxygenated buffers and reagents.

2.4.3 SNO transfer experiment—While it is readily agreed that RBCs export SNO bioactivity in an oxygen sensitive manner, the mechanism via which this occurs has yet to be fully elucidated. To further probe this signaling pathway, we have used the pharmaceutical antioxidant N-acetylcysteine (NAC) as a bait reactant to measure NO transfer reactions between RBCs and plasma [36].

In this experimental process, heparinized blood is oxygenated and then treated with N-acetylcysteine (NAC; 100 μ M). Samples are then deoxygenated in the tonometer (Figure 4) (0% O₂; 95% argon; 5% CO₂) and drawn at different time intervals - to obtain a range of HbO₂ saturations spanning the physiological gradient. Sample co-oximetry is performed on each sample (Bayer RapidLab 845 blood gas analyzer and co-oximeter, Bayer Corp, East Walpole, MA), and the sample is centrifuged, with the plasma and RBC fractions separated for analysis. In Figure 5, plasma accumulation of SNO NAC was measured via Mass Spectrometry, while RBC SNO hemoglobin was assayed utilizing 3C. Similar studies are currently being performed with various bait thiols, utilizing 3C to measure both plasma accumulation and RBC depletion of SNO.

3.0 CONCLUSIONS

As highlighted in this chapter, the 2C/3C reagent used in conjunction with ozone based chemiluminescence is one of the most sensitive, specific and robust assays available for the quantification of RSNOs. However, we still recommend that this assay be used as one of several complementary assays to verify RSNOs quantification. Only with consensus of measurement methodologies will the true significance of RSNO signaling be uncovered.

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Figure 1. Experimental apparatus used to quantify NO in the gas phase
Apparatus used to quantify NO released from RSNOs in the gas phase.

Figure 2. Specificity of RSNO quantification using mercury chloride

Raw chemiluminescent traces from red blood cell samples (1mM Hb), treated with/without HgCl_2 . HgCl_2 hydrolyzes the RSNO bond, leading to the formation of NO^+ , which is rapidly converted to nitrite. As nitrite goes undetected in the copper cysteine reagent, the difference in signals with/without HgCl_2 represent RSNO. Baseline signals have been normalized for comparison. Taken from [11].

Figure 3. Inhibitory effect of carbon monoxide on nitric oxide autocapture by cell free heme in the copper cysteine reagent

Increasing headspace pressure in the purge vessel improves the effectiveness of CO in blocking heme interaction with NO (A). However, increasing headspace pressure in the purge vessel also increases NOA reaction cell pressure (B). This has an adverse effect on the detectability of NO, as demonstrated from the raw chemiluminescence traces of the top standard from each standard curve at each purge vessel pressure (C). Chemiluminescent trace baseline signals have been normalized for comparison. Taken in part from [18].

Figure 4. Experimental apparatus used to manipulate O₂ tension

Red blood cell (RBC) hemoglobin conformation can be rapidly manipulated utilizing a rotating glass tonometer submerged in a water bath. The tonometer is joined via a vacuum-tight, motorized couple to a gas source permitting simultaneous chamber rotation and flushing by gas blended from O₂, N₂, and CO₂ tanks.

Figure 5. Transfer of NO from RBC to bait plasma thiol

Accumulation of SNOAC (yellow circles) and decrease in the concentration of *S*-nitrosothiol-modified Hb (SNOHb; black circles) as a function of oxyhemoglobin saturation (Hb SO₂; dotted line). Experiment performed using heparinized whole blood, with NAC (100μM) added as a bait thiol. Samples (deoxygenated in the tonometer; 95% argon/5% CO₂) were removed at set time intervals and underwent co-oximetry. Samples were centrifuged and the plasma and RBC separated. SNOAC measurements were performed by Mass Spectrometry and SNORBC measurement using 3C. Taken in part from [36].