



Review Article

# Methods to detect nitric oxide and its metabolites in biological samples

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## Abstract

Nitric oxide (NO) methodology is a complex and often confusing science and the focus of many debates and discussion concerning NO biochemistry. NO is involved in many physiological processes including regulation of blood pressure, immune response, and neural communication. Therefore its accurate detection and quantification are critical to understanding health and disease. Due to the extremely short physiological half-life of this gaseous free radical, alternative strategies for the detection of reaction products of NO biochemistry have been developed. The quantification of NO metabolites in biological samples provides valuable information with regard to in vivo NO production, bioavailability, and metabolism. Simply sampling a single compartment such as blood or plasma may not always provide an accurate assessment of whole body NO status, particularly in tissues. Therefore, extrapolation of plasma or blood NO status to specific tissues of interest is no longer a valid approach. As a result, methods continue to be developed and validated which allow the detection and quantification of NO and NO-related products/metabolites in multiple compartments of experimental animals in vivo. The methods described in this review is not an exhaustive or comprehensive discussion of all methods available for the detection of NO but rather a description of the most commonly used and practical methods which allow accurate and sensitive quantification of NO products/metabolites in multiple biological matrices under normal physiological conditions.

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**Keywords:** Nitrite; Nitrate; Nitrosothiols; Methods; Chemiluminescence; Nitrotyrosine

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**Abbreviations:** DAF-2, diaminofluorescein-2; DAN, 2,3-diaminonaphthalene; GSNO, *S*-nitrosoglutathione; HPLC, high performance liquid chromatography; MPO, myeloperoxidase; NEM, *N*-ethylmaleimide; NO<sub>2</sub><sup>-</sup>, nitrite; NO<sub>3</sub><sup>-</sup>, nitrate; NOHb, nitrosyl hemoglobin; NOS, nitric oxide synthase; 3NT, 3-nitrotyrosine; PBS, phosphate-buffered saline; sGC, soluble guanylyl cyclase; RNNOs, *N*-nitrosamines; RSNOs, *S*-nitrosothiols.

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## Introduction

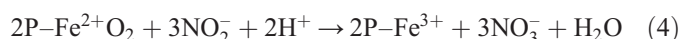
Nitric oxide (NO) is a diatomic free radical that is extremely short-lived in biological systems (less than 1 s in circulating blood) [1]. The established paradigm of NO biochemistry from production by NO synthases to activation of soluble guanylyl cyclase (sGC) to eventual oxidation to nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) may only represent part of NO's effects in vivo. The interaction of NO and NO-derived metabolites with protein thiols, secondary amines, and metals to form *S*-nitrosothiols (RSNOs), *N*-nitrosamines (RNNOs), and nitrosyl-heme respectively represent cGMP-independent effects of NO and are likely just as important physiologically as activation of sGC by NO. A true understanding of NO in physiology is derived from in vivo experiments sampling multiple compartments simultaneously. The ability to compare blood with select tissues in experimental animals will help bridge the gap between basic science and clinical medicine as far as diagnostic and prognostic utility of NO biomarkers in health and disease. In many cases NO status in blood does not accurately reflect the corresponding NO status in tissues of interest [2], highlighting the need for a systems based approach to NO analytical biochemistry. The elucidation of new mechanisms and signaling pathways involving NO hinges on our ability to specifically, selectively, and sensitively detect and quantify NO and all relevant NO products and metabolites in complex biological matrices. There are several methods for determining NO and its products/metabolites in biological fluids, many of which have been described in methodological review articles [3–6]. However, a comprehensive methodological description for the detection of NO and its products/metabolites in vivo in blood as well as multiple tissues and other compartments is still missing. We hope this review will bridge that gap.

## Physiological chemistry of nitric oxide

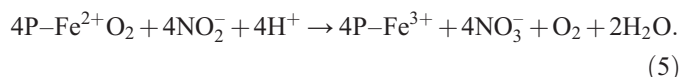
The endogenous production of NO by NOS has been established as playing an important role in vascular homeostasis, neurotransmission, and host defense mechanisms [7]. The major pathway for NO metabolism is the stepwise oxidation to nitrite and nitrate [8]. In plasma or other physiological fluids or buffers NO is oxidized almost completely to nitrite, where it remains stable for several hours [9,10]. The oxidation of NO by molecular oxygen is second order with respect to NO,



whereby  $\text{NO}_2$ ,  $\text{N}_2\text{O}_3$ , and  $\text{NO}_2^-$  represent nitrogen dioxide, dinitrogen trioxide, and nitrite, respectively. It should be noted that  $\text{N}_2\text{O}_3$  is a potent nitrosating agent by virtue of its ability to generate the nitrosonium ion ( $\text{NO}^+$ ). NO and nitrite are rapidly oxidized to nitrate in whole blood. The half-life of  $\text{NO}_2^-$  in human blood is about 110 s [11]. Nitrate on the other hand has a circulating half-life of 5–8 h [12,13]. Although the mechanisms by which NO and  $\text{NO}_2^-$  are converted to  $\text{NO}_3^-$  in vivo are not entirely clear, there are several possibilities. One mechanism, proposed by Ignarro et al., suggests that the  $\text{NO}_2^-$  derived from NO autoxidation is rapidly converted to  $\text{NO}_3^-$  via its oxidation by certain oxyhemoproteins ( $\text{P-Fe}^{2+}\text{O}_2$ ) such as oxyhemoglobin or oxymyoglobin [14]:



or



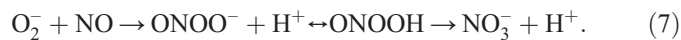
It should be noted, however, that these investigators used large concentrations of NO (300  $\mu\text{M}$ ) which will rapidly autoxidize to  $\text{NO}_2^-$ . Although the authors suggested that the  $\text{NO}_2^-$  would in turn react with the hemoproteins, this reaction is quite slow, requiring 2–3 h. A second, possibly more reasonable explanation for the presence of predominately  $\text{NO}_3^-$  in vivo may have to do with the fact that the levels of NO produced by nitric oxide synthase (NOS) in vivo would be much smaller and thus the half-life of NO would be much longer. In this case, NO would react directly and very rapidly with oxyhemoproteins ( $\text{P-Fe}^{2+}\text{O}_2$ ) to yield  $\text{NO}_3^-$  before it has an opportunity to autoxidize to  $\text{NO}_2^-$ :



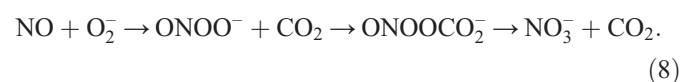
These mechanisms of autoxidation of NO would also be important in tissues and cell culture samples where NO may interact with a multitude of hemoproteins.

During fasting conditions with low intake of nitrite/nitrate, enzymatic NO formation from NOS accounts for the majority of nitrite [15]. On the basis of these studies, it was believed that NO is acutely terminated by oxidation to nitrite and nitrate. However, it is now appreciated that nitrite or nitrate can be recycled to produce NO in various ways. NO production has been described in infarcted heart tissue from nitrite [16]. The nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system [17–21], protonation [16,22], deoxyhemoglobin [22,23], and xanthine oxidase [24–26]. Both nitrite and nitrate have been shown to be reduced ultimately back to NO by commensal bacteria [27] and bacteria in the urogenital tract [28]. These pathways have been extensively reviewed elsewhere [29] in the vascular compartment but extend to all organ systems.

In addition to its reaction with oxygen, NO may rapidly interact with the free radical superoxide ( $O_2^-$ ) to yield the potent oxidants peroxynitrite ( $ONOO^-$ ) and its conjugate acid peroxynitrous acid ( $ONOOH$ ) [30]:

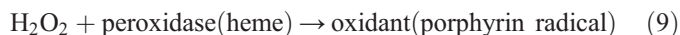


Although a large number of investigations have been performed which suggest that  $ONOO^-/ONOOH$  may play an important role in several pathophysiological situations, the question of whether  $ONOO^-/ONOOH$  is actually formed in vivo and exerts significant physiologic and/or pathophysiological activity remains the subject of vigorous debate. Peroxynitrite is known to promote both nitration and hydroxylation of different bioorganic molecules. It is thought that  $ONOOH$  exists as pair of the caged radicals  $NO_2$  and hydroxyl radical ( $OH^\cdot$ ). Furthermore, generation of both  $O_2^-$  and NO in the presence of physiological concentrations of  $CO_2/HCO_3^-$  will produce the potent nitrating species nitrosoperoxy carbonate anion ( $ONOOCO_2^-$ ).



Because both  $O_2^-$  and NO are known to be produced in large amounts during active inflammation, the ability to specifically detect  $ONOO^-$  and/or  $ONOOCO_2^-$  in vitro and in vivo would represent a major advancement in our understanding of the pathophysiological role of these oxidants and nitrating agents. Historically, it has been proposed that the ability of  $ONOO^-$  or  $ONOOCO_2^-$  to nitrate tyrosine residues in proteins and peptides to yield 3-nitrotyrosine (3NT) may be used as evidence for  $ONOO^-$  production in vivo. However, more recent evidence demonstrates that 3NT may be generated by multiple pathways, suggesting that the presence of 3NT is not necessarily a specific “footprint” for  $ONOO^-$  and/or  $ONOOCO_2^-$  formation in vivo (Fig. 1). For example, work by several laboratories have demonstrated that tyrosine can be nitrated by peroxidase (or heme/hemoprotein)-catalyzed,

hydrogen peroxide-dependent oxidation of nitrite to form  $NO_2$  (Fig. 1) [31–34]:



It has been shown that myeloperoxidase (MPO) as well as other peroxidases (and heme/hemoproteins) will mediate this reaction. Colocalization of MPO (or eosinophil peroxidase) with 3NT during inflammation strongly indicates that this reaction is the primary source of tyrosine nitration in vivo [31–33]. Although some studies have indicated the possibility of peroxidase-independent sources of 3NT formation [35], it should be remembered that virtually any hemoprotein (e.g., hemoglobin, myoglobin, cytochromes) possesses the potential to catalyze the  $H_2O_2$ -dependent oxidation of nitrite to yield 3NT [36]. In addition to heme or hemoprotein-mediated 3NT formation, the MPO-derived oxidant hypochlorous acid (HOCl) will interact with nitrite to yield the potent nitrating agent nitryl chloride ( $ClNO_2$ ) which will nitrate tyrosine to form 3NT [32]. Thus, the presence of 3NT in inflammatory foci most probably represents the sum total of all nitration reactions mediated by  $ONOO^-$ ,  $ONOOCO_2^-$ ,  $NO_2$  and  $ClNO_2$ .

### Biological activity of nitrite and nitrate

Nitrite is a central homeostatic molecule in NO biology and serves as an important signaling molecule in its own right [2,37]. Nitrite and nitrate in blood have been widely used as an index of endothelial NO synthase activity [38,39] as routine indirect measures of NO levels. Metal-containing proteins can catalyze the oxidation of NO to nitrite [40]. The recent discoveries that nitrite can be reduced back to NO under appropriate physiological conditions and nitrite itself can directly nitrosate thiols to form RSNOs [37] has caused intense interest in this molecule [41]. Furthermore the association between NO and nitrite is not as straightforward as once thought [37]. Nitrite is found in high abundance throughout the mammalian organ system [42]. It is normally a short-lived, highly regulated ion in the circulation (200–600 nM) with a half-life in whole blood of 110 s [11]. There is a significant contribution of both nitrite and nitrate from the diet which varies substantially from diet to diet, not only in humans but in experimental animal chow (as much as 12-fold differences in NOx between standard rodent chows [Bryan unpublished observations]). Nitrite has emerged as an important molecule in physiology and recent discoveries have opened a new avenue for the diagnostic and therapeutic application of nitrite, especially in cardiovascular diseases, using nitrite as marker as well as an active agent. Furthermore, dietary nitrate has been recently shown to reduce diastolic blood pressure in healthy volunteers [43]. Therefore it is prudent at this juncture to carefully account for all nitrite and nitrate in biological samples.

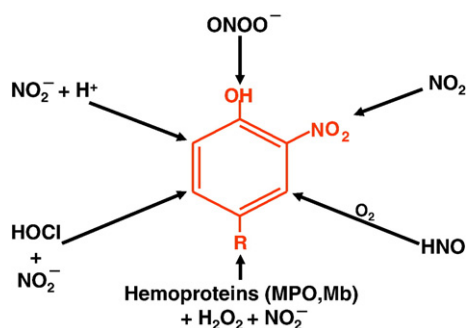


Fig. 1. Multiple pathways for the formation of 3-nitrotyrosine. 3-Nitrotyrosine may be generated by peroxynitrite ( $ONOO^-$ ),  $NO_2$  (from autoxidation of NO), nitroxyl (HNO) in the presence of oxygen,  $NO_2$  produced by heme or hemoprotein catalyzed,  $H_2O_2$ -dependent oxidation of nitrite ( $NO_2^-$ ), nitryl chloride ( $ClNO_2$ ) produced by the interaction between  $NO_2^-$  and HOCl, and  $NO_2$  generated from acidified  $NO_2^-$ .

## Sample preparation

Sample preparation may be one of the most important steps in quantifying any and all NO metabolites regardless of the method employed. Extreme care must be taken to preserve what you are looking for and more importantly, not to artifactually create NO products or metabolites during sample preparation. Nitrite is rapidly metabolized to different extents in different biological compartments by thiols and redox-active metals [37]. Nitroso/nitrosyl products are unstable and rapidly decompose over time [42]. Therefore it is crucial to collect blood and tissue while preserving their integrity. This is best accomplished by perfusing tissues with an air equilibrated isotonic solution of PBS containing 10 mM *N*-ethylmaleimide (NEM) and 2.5 mM EDTA and subsequent homogenization in the same solution. The addition of NEM/EDTA serves the purpose of blocking SH groups and inhibiting transition metal-catalyzed transnitrosation reactions, preventing artificial nitrosation, as well as thiolate- and ascorbate-mediated degradation of endogenous RSNOs and nitrite [37,44]. This also holds true for in vitro cell culture systems. For compartmental comparison and multicompartment analysis it is absolutely critical that the investigator become very efficient at the procedure before any data are interpreted. For example, if blood and heart are to be analyzed for NO metabolites, of course it is ideal to obtain both blood and heart from the same animal without sacrificing the integrity of both samples. In this case, blood must be withdrawn as rapidly as possible from anaesthetized animals without causing hemolysis and immediately centrifuged to separate plasma from RBCs. Once blood is obtained, one must immediately begin perfusion of the tissues with NEM/EDTA buffer to stop metabolic activity. If the blood withdrawal takes too long (>20 s) then the animal is rendered hypoxic and if too much blood is taken then the animal is hypovolemic, both of which alter steady-state NO products [42]. Rapid perfusion is best accomplished by inserting a needle into the left ventricle and using roller pump or gravity do a full blood exchange once blood is obtained. The inferior vena cava or right atrium should be cut to provide an exit for the blood. A full blood exchange of all tissues can be achieved in ~1 min. The tissues should be extracted and homogenized within 2 min of perfusion in order to minimize any hypoxia or degradation of NO species. Hypoxia signals blood and tissue to reduce nitrite to NO, at which time nitrite will be underestimated and there will be an overestimation of nitroso/nitrosyl products as a result [42]. All steps of blood and tissue preparation should occur under reduced ambient lighting conditions (<15 lx) to minimize photolytic decomposition of tissue NO products [45]. Tissue homogenates should be kept on ice in the dark and immediately analyzed within 2 min. It is best to analyze samples immediately on harvesting.

It may be necessary to harvest samples and send off for analysis for laboratories that do not contain the instrumentation for NO analysis. In this case, blood should be collected, spun down to separate plasma from RBCs, and then both snap-frozen in liquid nitrogen. Tissues should be perfused free of blood with NEM/EDTA PBS and whole tissues immediately snap-frozen in an aliquot of the NEM/EDTA perfusion buffer. The tissues can

then be thawed and homogenized immediately before analysis. An article by Daiber et al. [46] reports on freezing artifacts. The study revealed that when freezing nitrite containing biological samples in the presence of sodium and phosphate, a process of tyrosine nitration and *S*-nitrosocysteine formation is observed. The underlying mechanism is based on the already described pH decrease in sodium phosphate-buffered solutions during a slow freezing process and probably involves nitrous acid as an intermediate [47]. Irrespective of the mechanism, the data warrant caution for the analysis of nitration or nitros(yl)ation products following freezing of nitrite containing biological material. It is worth noting that their results were obtained under conditions that were optimized for freezing artifacts but care should be taken and proper controls for all frozen samples be performed. This artifact can be avoided in biological samples by rapidly freezing in liquid nitrogen. It is also critical that samples of the perfusion buffer are analyzed for nitrite content which can interfere with analysis of the biological samples and that samples are immediately frozen in liquid nitrogen. All buffers should be made fresh daily with ultrapure MilliPore water to avoid any nitrite contamination and should serve as the blank for all analyses. Leaving buffer solutions uncapped throughout the day can lead to nitrite accumulation from the ambient NO in the air.

Another problem with sample preparation relates to the preparation of plasma. We have found that heparinized plasma may form a precipitate on addition of the highly acidic Griess reagent, rendering these samples unusable for analysis [48]. The use of citrate or EDTA/DTPA as an anticoagulant or allowing plasma to clot to yield serum prevents this problem. If heparinization of blood is required, protamine sulfate-mediated precipitation of heparin prior to the addition of the Griess reagent will, in many cases, allow for determinations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in blood. In addition to blood, urinary levels of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  provide a useful method to quantify systemic NO production in vivo. Analysis of urine for the presence of nitrogen oxides remains one of the easiest methods to assess noninvasively systemic NO metabolism. As noted previously, it should be remembered that urinary (or plasma)  $\text{NO}_2^-$  and  $\text{NO}_3^-$  levels reflect not only endogenous NO production but also total  $\text{NO}_2^-/\text{NO}_3^-$  ingestion from the diet as well as the minor contribution made by bacteria found in the gut. Thus, animals should either be fasted or allowed to ingest  $\text{NO}_3^-/\text{NO}_2^-$  free diets prior to determinations. It has been our experience that a 24-h fast will reduce plasma  $\text{NO}_3^-$  levels by 60–80%, demonstrating that the majority of the circulating nitrate in rats is contributed to their diet. Furthermore, it is important to include “antibiotics” such as propanol or penicillin/streptomycin in collection tubes, to prevent bacterial growth during collection [49].

## Determination of nitrite and nitrate using the Griess reaction

### Colorimetric determination

One method for the indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . This method requires that  $\text{NO}_3^-$



first be reduced to  $\text{NO}_2^-$  and then  $\text{NO}_2^-$  determined by the Griess reaction as shown in Fig. 2. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent, dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO), reacts with sulfanilamide to produce a diazonium ion which is then coupled to *N*-(1-naphthyl)ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm [48]. For quantification of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in extracellular fluids, we have found that enzymatic reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  using a commercially available preparation of nitrate reductase is a satisfactory method. *Aspergillus* nitrate reductase is highly efficient at reducing small amounts of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . Following the incubation, any unreacted NADPH is oxidized by addition of lactate dehydrogenase and pyruvic acid because reduced pyridine nucleotides (NADPH, NADH) strongly inhibit the Griess reaction. An alternative method for oxidizing any unreacted NADPH is to replace the LDH/pyruvate system with 1 mM potassium ferricyanide [48]. A known volume of premixed Griess reagent is added to each incubation mixture and incubated for 10 min, and the absorbance of each sample determined at 543 nm.

#### High performance liquid chromatography (HPLC) determination

A dedicated HPLC system developed by EiCom Corporation allows sensitive and selective measurement of nitrite and nitrate in all biological matrices with ease and high throughput. The ENO-20's high sensitivity is attained by the combination of a diazo coupling method and HPLC. The system uses reverse-phase chromatography to separate nitrite from nitrate and then nitrate is reduced to nitrite through a reaction with cadmium and reduced copper inside a reduction column (Fig. 3). The two resolved peaks then mix with the Griess reagent inline to form the classical diazo compound which can be detected spectro-

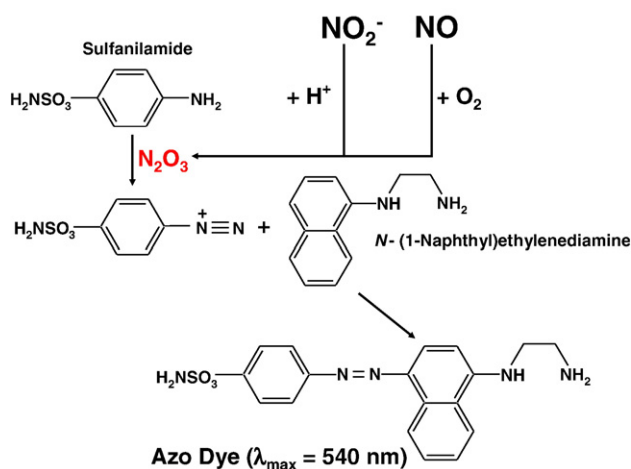


Fig. 2. The Griess reaction. The nitrosating agent dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ) generated from acidified nitrite (or from the autoxidation of NO) reacts with sulfanilamide to yield a diazonium derivative. This reactive intermediate will interact with *N*-1-naphthylethylenediamine to yield a colored diazo product that absorbs strongly at 540 nm.

photometrically. This system allows for easy sample preparation and little if any cross-reactivity and along with an auto-sampler allows for high-throughput analysis. There are limitations which includes chemicals or reagents that may not be compatible with the separation or reduction column. One such agent is ferricyanide used as a nitrite stabilization solution in whole blood samples [50]. Samples containing millimolar concentrations of ferricyanide and metals with lower ionization energy than the cadmium, such as  $\text{Hg}^{2+}$  and  $\text{Fe}^{3+}$ , and millimolar concentrations of dithiothreitol are not compatible with the reduction column and therefore will not effectively reduce nitrate. Lower (micromolar) concentrations of metals do not pose a problem to this system. This system is viable for a wide range of nitrite and nitrate concentrations regardless of their respective ratios. This system allows sensitivity to  $1 \text{ nM} \times 100\text{-}\mu\text{l}$  injections for each anion with no interference from protein or colored species.

#### Fluorometric determination

##### Diaminonaphthalene assay

In attempts to enhance the sensitivity of measuring NO-derived  $\text{NO}_2^-$  or NO generated under physiological conditions, different fluorometric methods have been developed. One method has employed the use of the aromatic diamino compound 2,3-diaminonaphthalene (DAN) as an indicator of NO formation [51]. The relatively nonfluorescent DAN reacts rapidly with  $\text{N}_2\text{O}_3$  generated from acidified nitrite (nitrous acid) or from the interaction of NO with oxygen (Eqs. (1)–(3)) to yield the highly fluorescent product 2,3-naphthotriazole (Fig. 4). This assay offers the additional advantages of specificity, sensitivity, and versatility. This assay is capable of detecting as little as 10–30 nM (i.e., 10–30 pmol/ml) naphthotriazole and may be used to quantify NO generated under biologically relevant conditions (e.g., neutral pH) with minimal interference by nitrite decomposition [51]. As with the Griess reaction, the DAN assay can be used to quantify NO production in physiological fluids as well as tissue culture media and organ culture supernatants.

##### Diaminofluorescein-2 assay

In addition to the DAN assay, more recent studies demonstrated that diaminofluorescein-2 (DAF-2) may be used to determine the presence of NO in vitro and in situ [52,53]. Like DAN, nitrosation of the diamino group results in a nitrosamine which through an internal rearrangement forms the fluorescent triazole (Fig. 5). The advantage of this compound is that wavelength associated with fluorescein can be used making equipment currently used for other bioassays as well as cell and tissue imaging easily adapted to detect NO in vitro and in vivo. Although NO-derived nitrosation is thought to be the primary mechanism by which cells and tissue generate the triazole derivative, recent investigations suggest that oxidative nitrosylation may represent an alternative pathway for triazole generation [54–56]. Since DAF-2 can be oxidized by one electron by species such as  $\text{NO}_2$  it can first oxidize the diamino complex to an aromatic radical which then undergoes radical–radical coup-

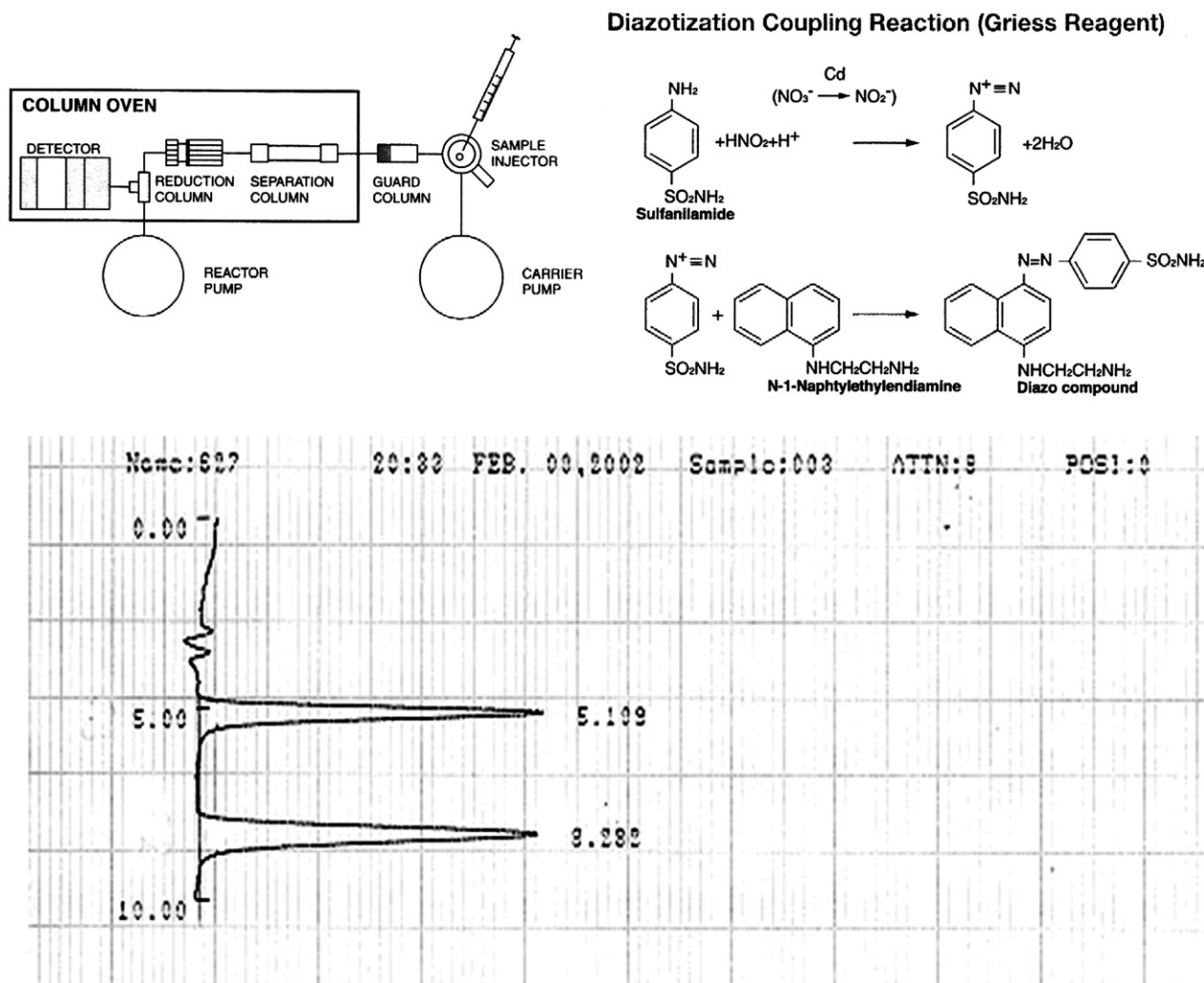


Fig. 3. (Top) Schematic of the ENO-20 method of detection for nitrite and nitrate. (Bottom) Standard chromatogram of 10 pmol nitrite and nitrate injected into ENO-20 (100  $\mu\text{l}$  of 100 nM solution of nitrite and nitrate). Sensitivity of 1 nM for each anion with 100  $\mu\text{l}$  injection volume. No interference with protein or colored species.

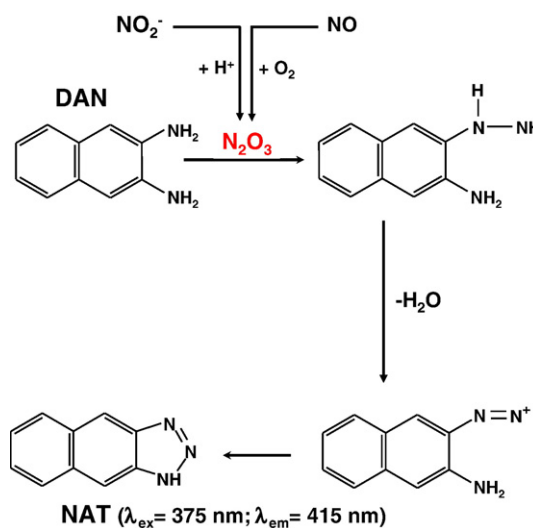


Fig. 4. Fluorometric detection of nitrite or NO using diaminonaphthalene. The nitrosating agent  $\text{N}_2\text{O}_3$  generated from acidified nitrite or from the autoxidation of NO reacts with diaminonaphthalene (DAN) to yield the highly fluorescent product naphthotriazole (NAT).

ling to form the nitrosamine which subsequently rearranges to the fluorescent triazole. This implies under biological conditions that the triazole can be formed from either nitrosative or oxidative chemistry.

In solution these two pathways can be differentiated. The use of nitrosative scavengers such as azide will prevent nitrosative but not oxidative nitrosation [55]. Conversely, urate will quench oxidative nitrosylation but not nitrosative [57]. Using different scavengers, the chemistry of DAF-2 formation can tease out in different experiments [54–56]. Though DAF-2 has been thought to be an indicator of NO, it recently has been shown that nitroxyl (HNO) reacts with DAF-2 giving even higher yields of triazole than NO [56]. In addition, an in-depth investigation into the performance of DAF-2 by Rodriguez et al. [58] reveals that there are important limitations to this method when used to determine basal levels of NO products/metabolites in tissues. Autofluorescence of DAF-2 and tissue limits the sensitivity of the probe to low micromolar levels of accumulated DAF-2 triazole which is higher than endogenous levels of NO metabolites found in animal tissues [42]. Compartmentalization of DAF-2 limits its potential to characterize the subcellular

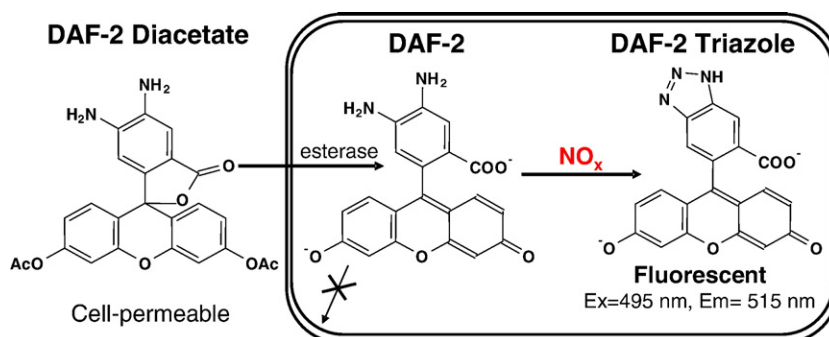
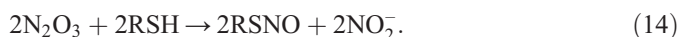


Fig. 5. Fluorometric detection of NO using diaminofluorescein-2 diacetate. DAF-2 diacetate diffuses into cells where esterases hydrolyze the diacetate residues, thereby trapping DAF-2 within the intracellular space. NO-derived nitrosating agents (NO<sub>x</sub>) nitrosate DAF-2 to yield its highly fluorescent product DAF-2 triazole.

locations of NO production. Furthermore mercuric chloride, a reagent commonly used to characterize RSNOs, reacts with DAF-2 and causes spectral changes that may be misinterpreted as NO signals.

#### Detection of *S*-nitrosothiols by colorimetric and fluorometric methods

The formation and biological properties of NO-derived *S*-nitrosothiols play an important part of the biology of NO [59–61]. It is well appreciated that autoxidation of NO in the presence of thiols (RSH) generates RSNOs via the following mechanism:



However, the concentrations of NO required to drive this reaction would be very large and most probably would not be present *in vivo* at least under normal physiological conditions. A more likely physiological scenario (i.e., low micromolar levels of NO) would be the following [62,63]:



More recently, endogenous RSNOs have been reported to be formed in a first-order reaction directly from nitrite *in vivo* [37]. These data alone suggest that tissue nitrosation from nitrite may not be occurring through the intermediacy of higher N-oxides created via the standard NO autoxidation reaction, since the latter is known to be second order in NO [64]. These data illustrate that nitrite may be in steady-state equilibrium with endogenous RSNOs and one should consider background nitrite when attempting to quantify RSNOs [65].

The use of mercury for specifically probing thiols has been used analytically for years [66]. The original method of Saville was developed to assay thiols, and later adapted to RSNOs. The quantification of the NO group, displaced from the thiol by mercuric ion, forms the basis of this highly sensitive method. The mercuric ion always reacts with accessible thiols and thus always captures the general behavior for any given protein. The detection of RSNO may utilize the Saville reaction in which mercuric chloride displaces the NO<sup>+</sup> (Fig. 6). The resulting NO<sub>2</sub><sup>-</sup> generated from the spontaneous decomposition of NO<sup>+</sup> in the presence of O<sub>2</sub> may be detected using a colorimetric method such as the Griess reaction to measure the nitrite formed from the treatment of RSNO with mercuric chloride. However, samples which contain large amounts of NO<sub>2</sub><sup>-</sup> can interfere with and limit the detection range of these methods under acidic conditions. To overcome these problems, two methods were developed to detect RSNO-derived nitrosating species *at neutral pH* [67]. The colorimetric method uses the components of the Griess reaction while the fluorimetric method utilizes the conversion of DAN to its fluorescent triazole derivative [67]. These methods are conducted at neutral rather than acidic pH, which eliminates the interference of contaminating nitrite and allows for the detection of RSNO.

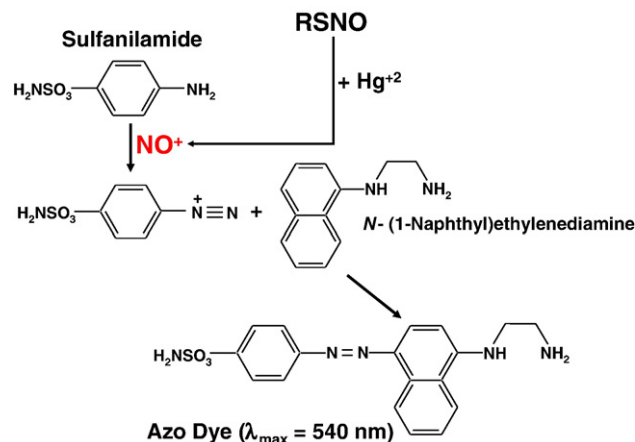


Fig. 6. Detection of *S*-nitrosothiols (RSNO) by the Saville reaction. Liberation of the nitrosating agent nitrosonium (NO<sup>+</sup>) by interaction of RSNO with Hg<sup>2+</sup> in the presence of the Griess reagents at pH 7.4 results in the formation of a diazo product that absorbs strongly at 540 nm.

The colorimetric reaction utilizes the same chemistry as described previously in which the nitrosating specie nitrosonium ( $\text{NO}^+$ ) generated from the interaction between RSNO and mercuric chloride interacts with sulfanilamide to form a diazonium ion (Fig. 6). The resulting diazonium salt then reacts with naphthylethylenediamine to form the colored azo complex (Fig. 6). The fluorometric assay is based on the reaction of DAN with  $\text{NO}^+$  liberated from RSNO following mercuric chloride addition to yield a primary nitrosamine which is converted rapidly to a fluorescent triazole (Fig. 7). The colorimetric assay has a detection range of 0.5–100  $\mu\text{M}$ , while the fluorometric assay is effective in the range of 50–1000 nM RSNO [67]. The combination of the two assays provides a detection range from 50 nM to 100  $\mu\text{M}$  RSNO, required for most biological experiments. Variations of these methods have been used successfully to quantify high and low-molecular-weight RSNOs in human and rat plasma as well as the *S*-nitrosated derivatives of human and rat hemoglobin [63,68,69].

#### Determination of nitrite, nitroso, and nitrosyl products using chemiluminescence

A method for the determination of nitrite, RSNOs, nitrosamines (RNNOs), and nitrosyl heme production in biological samples has been developed that utilizes chemiluminescence. The ozone-based chemiluminescent detector (CLD) detects only NO by the following reaction:



NO reacts with ozone to give an excited state of nitrogen dioxide. On return to ground state, nitrogen dioxide emits light, which is then detected by a photomultiplier tube. A glass purge vessel is connected to the CLD and purged with an inert gas, i.e., nitrogen or helium. A chemical trap containing ~ 15 ml of 1

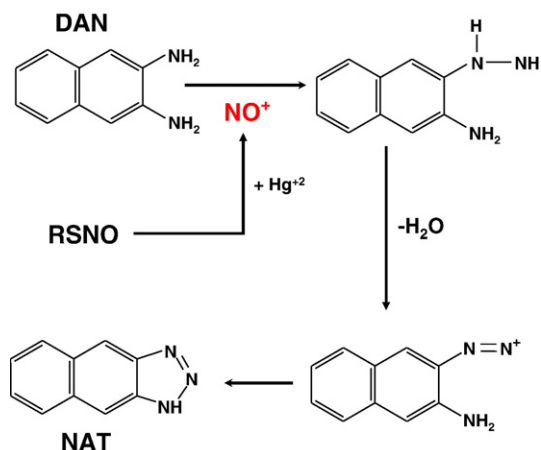


Fig. 7. Fluorometric detection of RSNO. Liberation of  $\text{NO}^+$  by interaction of RSNO with  $\text{Hg}^{2+}$  in the presence of the diamionaphthene (DAN) at pH 7.4 results in the formation of the fluorometric derivative naphthotriazole (NAT).

N sodium hydroxide in an ice bath is connected between the reaction vessel and the CLD. Specific detection of RSNOs by CLD was first described by Samouilov and Zweier [70] by a reductive denitrosation solution of alkaline quinone-hydroquinone. There have been many other reductive denitrosation solutions since including iodine/iodide mixture, vanadium chloride, copper chloride/cysteine, and modifications to each [71–75]. Whereas the quinine, vanadium chloride, and copper cysteine assay are specific for RSNO detection, the tri-iodide assay will reduce nitrite, RSNO, RNNO, and to a lesser extent NO-hemes. Since nitrite is usually present in much higher concentrations than nitroso/nitrosyl products [42], and is also a ubiquitous trace contaminant of chemical and laboratory glassware [76], it is absolutely critical to account for all nitrite and remove it prior to analysis of nitroso and/or nitrosyl content of a biological sample [65] when using an assay that also reduces nitrite. Acidified sulfanilamide eliminates nitrite without affecting the stability of other NO products/metabolites [77]. Acidified sulfanilamide is used as the first step in the Griess reaction whereby nitrite reacts in a stoichiometric diazotization reaction to form a purple azo product. This nitrite/sulfanilamide product produces no signal in the tri-iodide assay or any of the other aforementioned chemical CLD assays. In any assay that reduces nitrite and several species of nitroso/nitrosyl compounds, it is necessary to further discriminate between the chemical species for accurate labeling of specific nitroso/nitrosyl species. One way to discriminate among nitrite, RSNO, and RNNOs is by the use of group-specific reagents in the pre-treatment of the samples prior to injection. Three aliquots of the same sample are taken separately. The first aliquot is directly injected in the reaction vessel. The peak represents the sum of nitrite, RSNO, and RNNO compounds (Fig. 8A). The second aliquot is preincubated with acidified sulfanilamide (0.5% final) for 10 min and then injected. Peak 2 below from this sample represents RSNO and RNNO compounds since sulfanilamide specifically removes nitrite (Fig. 8A). The difference between peaks 1 and 2 can be quantified as the amount of nitrite in the sample. This value can and should be validated by Griess or HPLC as described above as any acid labile nitroso product would contribute to this peak and would be misidentified as nitrite. The third aliquot is preincubated with mercuric chloride (0.2% final) for 15 min followed by 10 min of sulfanilamide to quench nitrite produced from the Saville reaction. Peak 3 from this sample represents a mercury-stable N-nitroso compound and the difference in peak area from sample 2 and 3 can be quantified as the amount of RSNO in the sample (Fig. 8A). This technique is based on the mercuric chloride-induced cleavage of the RSNOs to form nitrite (Saville reaction). This method was first described independently by Feelisch et al. [71] and Gladwin et al. [72]. The method can quantify as little as 100 fmol bound NO [71]. A recent validation of this assay confirms the utility of this assay compared with more recent methods utilizing copper, cysteine, and carbon monoxide (3C assay) [77].

There are also assays validated that do not require any preincubation or chemical derivatization and therefore avoid the possibility of mislabeling certain nitroso species. The 3C assay offers specific RSNO detection without any sample pretreat-



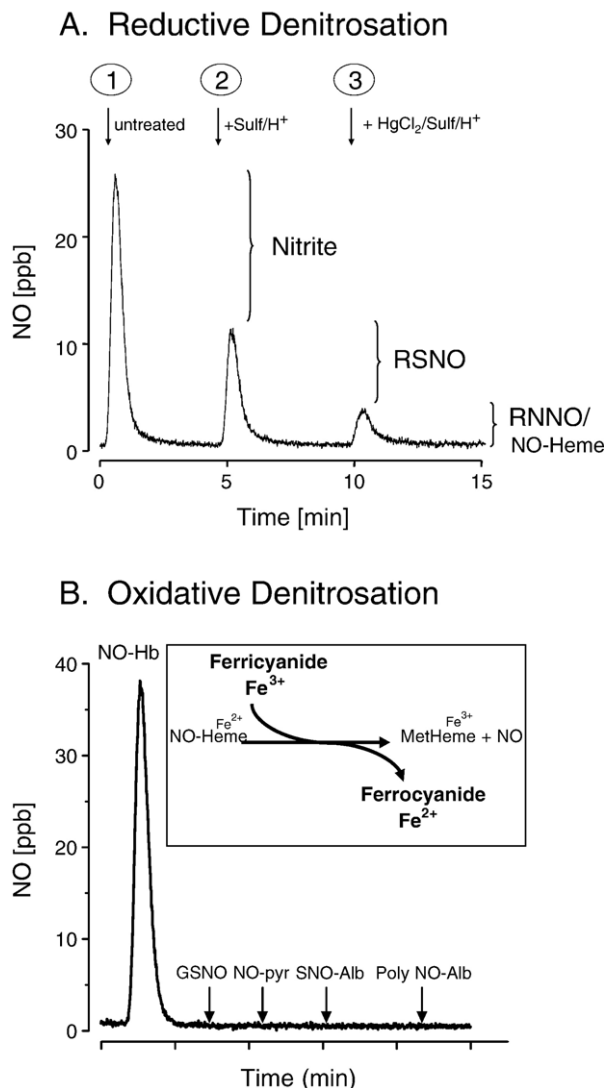


Fig. 8. (A) Chemiluminescence detection of nitrite, RSNO, and RNNO in reductive denitrosation assay by sample preincubation with group-specific chemical reagents. Subtraction of peak areas allows detection of nitrite and RSNOs. (B) Chemiluminescent detection of nitrosyl heme species using oxidative denitrosation solution of ferricyanide. This method is specific for NO-heme products with no cross-reactivity with RSNOs (GSNO or SNO-albumin), or RNNO (NO-pyrrolidine and *N*-nitrosoalbumin).

ment [78]. This method is based on transnitrosation from the endogenous RSNOs to cysteine present in the reaction chamber which can then be reduced by copper chloride to yield NO. The presence of CO serves to block all heme groups which would otherwise lead to autocapture of the released NO. Another means by which one can probe for RSNOs is through the use of high-intensity UV light to cleave the S–NO bond, termed photolysis. To distinguish FeNOs from SNOs in photolysis-based assays, inorganic or organic mercury (Hg) is added (in minimal excess over thiol at pH 7.4 to avoid protein precipitation or denaturation) [79]. Hg displaces NO from thiols in the form of nitrite, which goes largely undetected. Proteins are then desalted in physiological buffers to remove low-molecular-weight reactants. SNO levels are taken as the loss of signal caused by Hg. All SNOs are reactive to Hg, whereas FeNO is

unreactive [79]. Photolysis yields are largely independent of the chemical reactivities of FeNOs and SNOs.

Tissue NO-heme adducts can be determined in parallel by injection of aliquots of tissue homogenates into a solution of 0.05 M ferricyanide in PBS at pH 7.5 and 37 °C. This method, which represents a modification of a recent assay used for determination of the iron nitrosyl content of RBCs [72], employs one-electron oxidation rather than reduction to achieve denitrosylation (Fig. 8B). Released NO is quantified by gas-phase chemiluminescence [71]. Extensive validation experiments were performed with nitrosyl hemoglobin (NOHb), nitrosylated catalase, and a variety of biological samples spiked with NOHb standards [42]. The denitrosylation mechanism involves oxidation of the heme iron “underneath” the ligand, which—owing to the weaker NO affinity of ferric over ferrous heme [80]—is associated with a release of NO into the gas phase. No cross-reactivity was observed with nitrite, *S*-nitrosoglutathione (GSNO), *S*-nitrosoalbumin, and different RNNOs (NO-pyrrolidine) (all tested at 1–100 μM) [42]. However, samples containing high concentrations (>10 μM) of nitrite are not compatible with this assay [Bryan, unpublished observations]. Under the anaerobic conditions (100% nitrogen) used in this assay, high concentrations of nitrite can be reduced to NO by the biological sample and thus interfere with the selectivity of NO-heme determination. However, this method appears to be specific for NO-heme compounds at physiological nitrite concentrations. Under the conditions specified above, which were optimized for ferricyanide concentration, pH, and reaction temperature, recoveries for nitrosyl-hemoglobin in spiked tissue homogenates ranged from 96 to 101%. Experiments performed with this technique indicated that tissue nitrosyl species are rather unstable, necessitating the analysis of samples to be carried out within 1 min after tissue homogenization. This assay requires the addition of antifoam prior to sample injection to prevent foaming of protein-containing samples in the purge vessel. However, some antifoam solutions can quench the NO signal [Bryan unpublished observations]. We recommend Anti-foam SE-15 from Sigma as it is very effective at eliminating foaming without quenching NO signals from the sample.

### Quantification of 3-nitrotyrosine

As noted previously, reactive nitrogen oxide species with potent oxidizing and nitrating activity may be generated by a variety of different reactions during certain pathophysiological situations and include ONOO<sup>−</sup> and/or ONOOCO<sub>2</sub><sup>−</sup>, NO<sub>2</sub> derived from NO autoxidation or via the H<sub>2</sub>O<sub>2</sub>-dependent, hemo-protein-catalyzed oxidation of NO<sub>2</sub><sup>−</sup>, and/or ClNO<sub>2</sub> produced from the reaction between HOCl and nitrite. 3-Nitrotyrosine represents a posttranslational modification of proteins and peptides that play important roles in cell signaling, protein turnover, and cell proliferation to mention just a few. Because 3NT has been shown to correlate with certain disease states (e.g., inflammatory and cardiovascular diseases), the ability to specifically quantify the trace amounts of this nitrated amino acid in proteins and peptides would allow for quantification of nitrative stress in biological tissues as well ascertain the importance of nitrated

proteins as a prognostic marker of disease. Several different methods have been proposed; however, only a relative few provide the specificity and sensitivity needed to quantify this posttranslational modification in biological samples. The most widely used methods to detect 3NT have been the immunohistochemical techniques that utilize antibodies specific for the nitrotyrosine residue. A major limitation with the use of immunohistochemistry to quantify 3NT in cells is the lack of any study that quantitatively compares results obtained from immunohistochemistry with those generated using “gold standard” analytical methods such as mass spectrometry (MS) [81]. In an attempt to enhance the specificity for detection of 3NT, methods such as HPLC combined with UV absorption [82], electrochemical detection [83], and fluorescence spectroscopy [84] have been developed. As with all analytical techniques certain limitations are known to exist. For example, the techniques described above require the derivatization of samples and do not allow for the simultaneous monitoring of artifactual formation of 3NT during sample preparation. In much the same way gas chromatography (GC)–MS has also been utilized to quantify both free and protein-bound 3NT; however, samples must be derivatized, thereby increasing the potential for artifactual generation of additional metabolites including 3NT. However, recent work by Mani et al. has utilized radiolabeled internal standards to monitor and correct for spurious nitration during sample preparation [85]. More recently, liquid chromatography (LC)–MS has been introduced to enhance both sensitivity and specificity for the quantification of 3NT [86,87]. These methods use the very expensive triple quadrupole MS instrumentation. Obviously, the availability of these types of techniques is limited by the cost. The recent development of HPLC with online electrospray ionization (ESI) tandem MS on a benchtop ion-trap MS provides for the rapid, sensitive, and specific method for the detection of trace amounts of free and protein-bound 3NT in biological samples [88]. Using isotopically labeled internal standards with this new technique offers the advantage of being able to determine and correct for artifactual generation of 3NT during sample preparation. Sensitivity has been determined to be in the femtomolar ( $10^{-12}$  molar) range [88].

### Understanding the controversy of NO methodology

The tri-iodide CLD assay is of general utility for quantifying the total amount of NO in a given biological compartment in a high-throughput fashion. However, it along with all methodologies has its limitations and remains a topic of intense discussion and controversy [77,89–92] specifically regarding its efficacy and specificity for detecting and quantifying RSNOs in hemoglobin and red cells. Although hemoglobin may be considered an archetypical model protein system, other proteins may be much simpler in terms of NO reactivity and stability. Concerns have been raised regarding the validity of the tri-iodide assay on the following grounds: Only 2 proteins have been studied (SNO-alb, SNO-Hb). The *in vitro* data on SNO-Alb and SNO-Hb suggest that tri-iodide does not capture the general behavior of SNO or FeNO [89]. SNO-Alb and SNO-Hb

levels in plasma and RBCs are entirely different by tri-iodide vs. other methods of detection. The reagents used in the tri-iodide do not exhibit well-validated chemistry, and chemistry that is known is incompatible with general SNO and FeNO reactivity [89]. Tri-iodide has not been validated against an intracellular SNO or FeNO pool. Since GSNO and SNO-Alb do not contribute significantly to the intracellular SNO pool other standards should be considered for any intracellular system. With that stated, Recognizing and understanding the inherent characteristics of a given protein and the mechanisms of detection as well as limitations of any methodology are critical for the subsequent interpretation of the data generated by any given method. The best methodology is one which recapitulates physiology to the extent possible. This is often impractical since most biological samples are extracted from their normal environment and then processed for analysis. There are several important parameters one must consider when detecting and quantifying any NO-related species: (1) What is the proper “physiological” standard by which the instruments are calibrated? (2) Do steady-state levels of NO-modified biomolecules remain stable from time and means of sampling to analytical detection? (3) Do “stabilization solutions” actually stabilize the endogenous species of interest without artifactual formation of another NO species, i.e., intramolecular shuffle of  $\text{NO}^+$  from thiol to heme or vice versa? (4) Does the analytical system preserve protein allostery/reactivity and yield sufficient recovery in complex biological matrices? These considerations appear to be at the heart of the controversy and discussions regarding NO analytical techniques and are beyond the scope of this review but warrant special attention when considering any analytical method. It is the responsibility of each investigator to validate the method in his/her system to ensure that there is accurate quantification and labeling of NO products/metabolites within a protein or tissue homogenate.

Many investigators use simple low-molecular-weight RSNOs such as (*S*-nitrosoglutathione (GSNO) as calibration standards which typically give 100% yield in many analytical systems. However, this same recovery may not sustain for complex proteins in biological matrices. The chemical means by which reductive denitrosation occurs, either by the use of mercury or cysteine for transnitrosation in the 3C assay or chemically in the tri-iodide assay, the RSNO must be accessible to the probing molecule or condition. This is difficult to predict in tertiary protein structure whereby there may be endogenous SNO in hydrophobic compartments within the protein. Under denaturing conditions, one would assume that all RSNOs would be accessible to the surrounding chemical environment or probing molecule. However, denaturing conditions may destabilize the RSNO and lead to decomposition before it can be detected, leading to an underestimation in the amount of SNO in the sample. Therefore in order to accurately detect and implicate SNO in modulating a particular protein function, one must have an appreciation for the inherent characteristics of a particular SNO protein. In synthesizing protein RSNOs as standards, it is important to consider the type of RSNO hybrid synthesized with respect to the ratio of NO molecules bound to total thiols within the protein. Common protocols for synthesizing SNO standards

use acidified nitrite which may denature proteins and form SNO hybrids that may not be physiologically relevant and furthermore give different yields in the analytical system. This is particularly important if one is interested in a single protein. Then extreme care must be taken to understand the particular reactivity and stability of SNO within the protein of interest and recapitulate conditions specific for that protein. Given the lack of consensus on a unified method for determining RSNOs in red blood cells, one is well served to utilize more than one method and to never become enamored with any single method.

On the other hand, gross determination of total amount of NO bound in cells or tissue homogenates normalized to total protein can be quantified using any of these assays with the understanding of its limitations. This often times provides the first indication on changes in NO biochemistry in vivo which then justifies further characterization and isolation of a particular protein or enzyme. Many of the methodologies discussed in this review including the tri-iodide can provide that type of analytical information provided there is an open revelation on the inherent limitations for the methods employed.

### Concluding remarks

The research field of NO is frequently compared with a field rich in analytical mines and pitfalls but continues to be a field rich in discoveries. Irrespective of the analytical methodology, researchers should make every attempt to recognize, appreciate, and eliminate the pitfalls and utilize the method that best fits their needs. It is our hope that the methods described in this review will provide the reader with a helpful overview of some of the most commonly used techniques to detect and quantify NO products/metabolites in biological samples and to recognize the inherent limitations of any given method.

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