Published in final edited form as:

Free Radic Biol Med. 2007 September 1; 43(5): 645–657.

Methods to Detect Nitric Oxide and its Metabolites in Biological Samples

Nathan S. Bryan¹ and Matthew B. Grisham²

¹*Institute of Molecular Medicine, The University of Texas-Houston Health Sciences Center, Houston, TX 77030, USA*

²*Department of Molecular and Cellular Physiology, LSU Health Sciences Center, Shreveport, LA*

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 is 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 regards 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.

Keywords

nitrite; nitrate; nitrosothiols; methods; chemiluminescence; nitrotyrosine

Introduction

Nitric oxide (NO) is a diatomic free radical that is extremely short lived in biological systems (less than 1 second 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 (NO_2^-) and nitrate (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

Corresponding author: Matthew B. Grisham, Ph.D., Department of Molecular and Cellular Physiology, LSU Health Sciences Center, 1501 Kings Highway, Shreveport, LA. 71130, 318-675-6021 (phone), 413-280-9325 (fax), mgrish@lsuhsc.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 NO_2 , N_2O_3 and NO_2^- represent nitrogen dioxide, dinitrogen trioxide and nitrite, respectively. It should be noted that N_2O_3 is a potent nitrosating agent by virtue of its ability to generate the nitrosonium ion (NO^+). NO and nitrite are rapidly oxidized to nitrate in whole blood. The half life of NO_2^- in human blood is about 110 seconds [11]. Nitrate on the other hand has a circulating half life of 5-8 hours [12,13]. Although the mechanisms by which NO and NO_2^- are converted to NO_3^- *in vivo* are not entirely clear, there are several possibilities.

One mechanism, proposed by Ignarro *et al.*, suggests that the NO_2^- derived from NO autoxidation is rapidly converted to NO_3^- via its oxidation by certain oxyhemoproteins (P- Fe^{2+}O_2) such as oxyhemoglobin or oxymyoglobin [14]:

or

It should be noted however, that these investigators used large concentrations of NO (300 μM) which will rapidly autooxidize to NO_2^- . Although the authors suggested that the NO_2^- would

in turn react with the hemoproteins, this reaction is quite slow, requiring 2-3 hr. A second, possibly more reasonable explanation for the presence of predominately 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 NO_3^- before it has an opportunity to autoxidize to 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 $\text{ONOO}^-/\text{ONOOH}$ may play an important role in several patho-physiological situations, the question of whether $\text{ONOO}^-/\text{ONOOH}$ 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 bio-organic molecules. It is thought that ONOOH when protonated, exists as pair of the caged radicals NO_2 and hydroxyl radical ($\text{OH}^\cdot$). Furthermore, generation of both O_2^- and NO in the presence of physiological concentrations of $\text{CO}_2/\text{HCO}_3^-$ will produce the potent nitrating species nitrosoperoxycarbonate 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 demonstrate that 3NT may be generated by multiple pathways suggesting that the presence of 3NT is not necessarily a specific “footprint” for ONOO⁻ and/or ONOOCO₂⁻ formation *in vivo* (Figure 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 NO₂ (Figure 1) [31-34]:

It has been shown that myeloperoxidase (MPO) as well as other peroxidases (and heme/hemoproteins) will mediate this reaction. Co-localization of MPO (or eosinophil peroxidase) with 3NT during inflammation strongly indicates 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) possess the potential to catalyze the H₂O₂-dependent oxidation of nitrite to yield 3NT [36]. In addition to heme or hemoprotein-mediated NT formation, the MPO-derived oxidant hypochlorous acid (HOCl) will interact with nitrite to yield the potent nitrating agent nitryl chloride (ClNO₂) 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⁻, ONCOOO₂⁻, NO₂ and ClNO₂.

The 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 has 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 are 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 (200-600nM) with a half life in whole blood of 110 seconds [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.

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 air equilibrated isotonic solution of PBS containing 10mM 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 multi-compartment analysis it is absolutely critical that the investigator become very efficient at the procedure before any data be 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 seconds) 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 minute. 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 Lux) 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 upon 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 be analyzed for nitrite content which can interfere with analysis of the biological samples and samples be 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 upon 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 NO_2^- and NO_3^- in blood. In addition to blood, urinary levels of NO_2^- and 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 non-invasively systemic NO metabolism. As mentioned previously, it should be remembered that urinary (or plasma) NO_2^- and 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 hr fast will reduce plasma 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 NO_3^- and NO_2^- . This method requires that NO_3^- first be reduced to NO_2^- and then NO_2^- determined by the Griess reaction as shown in Figure 2. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autooxidation 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 NO_3^- and NO_2^- in extracellular fluids, we have found that enzymatic reduction of NO_3^- to NO_2^- using a commercially available preparation of nitrate reductase to be a satisfactory method. *Aspergillus* nitrate reductase is highly efficient at reducing small amounts of NO_3^- to 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 1mM potassium ferricyanide [48]. A known volume of premixed Griess reagent is added to each incubation mixture, 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 (Figure 3). The two resolved peaks then mix with the Griess reagent inline to form the classical diazo compound which can be detected spectrophotometrically. This system allows

for easy sample preparation, 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 Hg^{2+} and 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 $1\text{nM} \times 100\mu\text{l}$ 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 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 N_2O_3 generated from acidified nitrite (nitrous acid) or from the interaction of NO with oxygen (equations 1-3) to yield the highly fluorescent product 2,3-naphthotriazole (Figure 4). This assay offers the additional advantages of specificity, sensitivity, and versatility. This assay is capable of detecting as little as 10-30 nM (ie 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 (Figure 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 NO_2 it can first oxidize the diamino complex to an aromatic radical which then undergoes radical-radical coupling 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 limit 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 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 (RSNOs) 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 (ie 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 general behavior for any given protein. The detection of RSNO may utilize the Saville reaction in which mercuric chloride displaces the NO^+ (Figure 6). The resulting NO_2^- generated from the spontaneous decomposition of NO^+ in the presence of O_2 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 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.

The colorimetric reaction utilizes the same chemistry as described previously in which the nitrosating specie nitrosonium (NO^+) generated from the interaction between RSNO and mercuric chloride interacts with sulfanilamide to form a diazonium ion (Figure 6). The resulting diazonium salt then reacts with naphthylethylenediamine to form the colored azo complex (Figure 6). The fluorometric assay is based on the reaction of DAN with NO^+ liberated from RSNO following mercuric chloride addition to yield a primary nitrosamine which is converted rapidly to a fluorescent triazole (Figure 7). The colorimetric assay has a detection range of 0.5-100 μM , 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 μM 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. Upon 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 ~15ml of 1N 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, RNNOs 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 between nitrite, RSNO and RNNOs is by the use of group-specific reagents in the pretreatment of the samples prior to injection. Three aliquots of the same sample are taken separately. (1) The first aliquot is directly injected in the reaction vessel. The peak represents the sum of nitrite, RSNO and RNNOs compounds (Figure 8A). The second aliquot is pre-incubated with acidified sulfanilamide (0.5% final) for 10 minutes and then injected. Peak (2) below from this sample represents RSNO and RNNOs compounds since sulfanilamide specifically removes nitrite (Figure 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 minutes followed by 10 minutes sulfanilamide to quench nitrite produced from Saville reaction. The 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 (Figure 8A). This technique is based on the mercuric chloride induce cleavage of the RSNOs to form nitrite (Saville Reaction). This method was first described independently by Feelisch [71] and Gladwin [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 pre-incubation or chemical derivatization and therefore avoid the possibility of mislabeling certain nitroso species. The 3C assay offers specific RSNO detection without any sample pretreatment [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 0.05M 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 (Figure 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 μ M) [42]. However samples containing high concentrations (>10 μ M) 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 the physiological nitrite concentrations. At 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 Antifoam SE-15 from Sigma as it is very effective at eliminating foaming without quenching NO signals from the sample.

Quantification of 3-Nitrotyrosine

As mentioned 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^- and/or, ONOOCO_2^- , NO_2 derived from NO autoxidation or via the H_2O_2 -dependent, hemoprotein-catalyzed oxidation of NO_2^- and/or ClNO_2 produce from the reaction between HOCl and nitrite. 3-nitrotyrosine (3NT) represents a post-translational 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 (eg 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 post translational 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” analytic 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 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. Recognizing and understanding the inherent characteristics of a given protein and the mechanisms of detection as well as limitations of any methodology is 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 bio-molecules 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 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 yield 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.

Acknowledgements

DK43785 (MBG), DK64023 (MBG) and the Center of Excellence for Arthritis and Rheumatology.

References

1. Kelm M. Nitric oxide metabolism and breakdown. *Biochimica et Biophysica Acta* 1999;1411:273–289. [PubMed: 10320663]
2. Bryan NS. Nitrite in nitric oxide biology: Cause or consequence? A systems-based review. *Free Radic Biol Med* 2006;41(5):691–701. [PubMed: 16895789]
3. Pelletier MM, et al. The measurement of blood and plasma nitrite by chemiluminescence: pitfalls and solutions. *Free Radic Biol Med* 2006;41(4):541–8. [PubMed: 16863986]
4. Rassaf T, Feelisch M, Kelm M. Circulating NO pool: assessment of nitrite and nitroso species in blood and tissues. *Free Radic Biol Med* 2004;36(4):413–22.
5. Tsikas D. Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. *Free Radic Res* 2005;39(8):797–815. [PubMed: 16036360]
6. Yang BK, et al. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Radic Res* 2003;37(1):1–10. [PubMed: 12653211]
7. Moncada S, Palmer RMJ, Higgs A. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 1991;43(2):109–142. [PubMed: 1852778]
8. Yoshida K, et al. Biotransformation of nitric oxide, nitrite and nitrate. *Int Arch Occup Environ Health* 1983;52:103–115. [PubMed: 6629503]
9. Grube, R., et al. The Biology of Nitric Oxide. In: Moncada, S., et al., editors. *Enzymology, Biochemistry, and Immunology*. Portland Press; London: 1994. p. 201–204.
10. Kelm, M., et al. The Biology of nitric oxide. In: Moncada, S., et al., editors. *Physiological and Clinical Aspects*. Portland Press; London: 1992. p. 319–322.
11. Kelm M. Nitric oxide metabolism and breakdown. *Biochim Biophys Acta* 1999;1411:273–289. [PubMed: 10320663]
12. Tannenbaum SR. Nitrate and nitrite: origin in humans. *Science* 1994;205:1333–1335.
13. Kelm, M.; Yoshida, K. Metabolic Fate of Nitric Oxide and Related N-oxides. In: Feelisch, M.; Stamler, JS., editors. *Methods in Nitric Oxide Research*. John Wiley and Sons; Chichester: 1996. p. 47–58.
14. Ignarro LJ, et al. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A* 1993;90(17):8103–7. [PubMed: 7690141]
15. Rhodes P, et al. The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. *Biochem Biophys Res Commun* 1995;209:590–596.
16. Zweier JL, et al. Enzyme-independent formation of nitric oxide in biological tissues. *Nature Medicine* 1995;1(8):804–809.
17. Walters CL, Casselden RJ, Taylor AM. Nitrite metabolism by skeletal muscle mitochondria in relation to haem pigments. *Biochim Biophys Acta* 1967;143:310–318. [PubMed: 4292888]
18. Reutov VP, Sorokina EG. NO-synthase and nitrite-reductase components of nitric oxide cycle. *Biochemistry (Mosc)* 1998;63(7):874–884. [PubMed: 9721340]
19. Kozlov AV, Staniek K, Nohl H. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett* 1999;454:127–130. [PubMed: 10413109]
20. Nohl H, et al. Mitochondria recycle nitrite back to the bioregulator nitric monoxide. *Acta Biochim Pol* 2000;47:913–921. [PubMed: 11996114]
21. Tischner R, Planchet E, Kaiser WM. Mitochondrial electron transport as a source for nitric oxide in the unicellular green algae *Chlorella sorokiniana*. *FEBS Lett* 2004;576:151–155. [PubMed: 15474028]

22. Hunter CJ, et al. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. *Nat Med* 2004;10:1122–1127. [PubMed: 15361865]
23. Cosby K, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature Medicine* 2003;9:1498–1505.
24. Li H, et al. Characterization of the effects of oxygen on xanthine oxidase-mediated nitric oxide formation. *J Biol Chem* 2004;279:16939–16946. [PubMed: 14766900]
25. Alikulov ZA, L'vov NP, Kretovich VL. Nitrate and nitrite reductase activity of milk xanthine oxidase. *Biokhimiia* 1980;45(9):1714–1718. [PubMed: 6894704]
26. Webb A, et al. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci USA* 2004;101:13683–13688. [PubMed: 15347817]
27. Lundberg JO, et al. Nitrate, bacteria and human health. *Nat Rev Microbiol* 2004;2(7):593–602. [PubMed: 15197394]
28. Lundberg JO, et al. Urinary nitrite: more than a marker of infection. *Urology* 1997;50(2):189–191. [PubMed: 9255286]
29. Lundberg JO, Weitzberg E. NO generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol* 2005;25(5):915–22.
30. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996;271(5 Pt 1):C1424–37. [PubMed: 8944624]
31. Eiserich JP, et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 1998;391(6665):393–7. [PubMed: 9450756]
32. Eiserich JP, et al. Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J Biol Chem* 1996;271(32):19199–208. [PubMed: 8702599]
33. Eiserich JP, et al. Molecular mechanisms of damage by excess nitrogen oxides: nitration of tyrosine by gas-phase cigarette smoke. *FEBS Lett* 1994;353(1):53–6. [PubMed: 7926022]
34. Thomas DD, et al. Protein nitration is mediated by heme and free metals through Fenton-type chemistry: an alternative to the NO/O₂- reaction. *Proc Natl Acad Sci U S A* 2002;99(20):12691–6. [PubMed: 12226478]
35. Brennan ML, et al. A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 2002;277(20):17415–27. [PubMed: 11877405]
36. Kilinc K, et al. Myoglobin-catalyzed tyrosine nitration: no need for peroxynitrite. *Biochem Biophys Res Commun* 2001;285(2):273–6. [PubMed: 11444837]
37. Bryan NS, et al. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol* 2005;1(5):290–7. [PubMed: 16408059]
38. Kleinbongard P, et al. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radical Biology & Medicine* 2003;35(7):790–796. [PubMed: 14583343]
39. Lauer T, et al. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci USA* 2001;98(22):12814–12819. [PubMed: 11606734]
40. Shiva S, et al. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol* 2006;2(9):486–93. [PubMed: 16906150]
41. Gladwin MT, et al. The emerging biology of the nitrite anion. *Nat Chem Biol* 2005;1(6):308–14. [PubMed: 16408064]
42. Bryan NS, et al. Cellular Targets and Mechanisms of Nitros(yl)ation: An Insight into Their Nature and Kinetics in vivo. *Proc Natl Acad Sci USA* 2004;101(12):4308–4313. [PubMed: 15014175]
43. Larsen FJ, et al. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* 2006;355(26):2792–3. [PubMed: 17192551]
44. Marley R, et al. Formation of nanomolar concentrations of S-nitroso-albumin in human plasma by nitric oxide. *Free Radical Biology & Medicine* 2001;31(5):688–696. [PubMed: 11522454]

45. Rodriguez J, et al. Chemical nature of nitric oxide storage forms in rat vascular tissue. *Proc Natl Acad Sci USA* 2003;100:336–341. [PubMed: 12502793]
46. Daiber A, et al. A new pitfall in detecting biological end products of nitric oxide-nitration, nitros(yl)ation and nitrite/nitrate artefacts during freezing. *Nitric Oxide* 2003;9(1):44–52.
47. Van Den Berg L, Rose D. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions; the reciprocal system $\text{KH}_2\text{PO}_4\text{-Na}_2\text{-HPO}_4\text{-H}_2\text{O}$. *Arch Biochem Biophys* 1959;81(2):319–29. [PubMed: 13637993]
48. Grisham MB, Johnson GG, Lancaster JR Jr. Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* 1996;268:237–46. [PubMed: 8782590]
49. Granger DL, et al. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. *Methods Enzymol* 1996;268:142–51.
50. Dejam A, et al. Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* 2005;106(2):734–9. [PubMed: 15774613]
51. Miles AM, et al. Determination of nitric oxide using fluorescence spectroscopy. *Methods Enzymol* 1996;268:105–20. [PubMed: 8782577]
52. Kojima H, et al. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 1998;70(13):2446–53. [PubMed: 9666719]
53. Kojima H, et al. Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore. *Anal Chem* 2001;73(9):1967–73. [PubMed: 11354477]
54. Espey MG, et al. Distinction between nitrosating mechanisms within human cells and aqueous solution. *J Biol Chem* 2001;276(32):30085–91.
55. Espey MG, et al. Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proc Natl Acad Sci U S A* 2002;99(17):11127–32. [PubMed: 12177414]
56. Espey MG, et al. Ingress and reactive chemistry of nitroxyl-derived species within human cells. *Free Radic Biol Med* 2002;33(6):827–34. [PubMed: 12208370]
57. Jourdain D, et al. Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions in vivo. *J Biol Chem* 2001;276(31):28799–805. [PubMed: 11373284]
58. Rodriguez J, et al. Performance of diamino fluorophores for the localization of sources and targets of nitric oxide. *Free Radic Biol Med* 2005;38(3):356–68. [PubMed: 15629864]
59. Arnelo DR, Stamler JS. NO^+ , NO, and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* 1995;318(2):279–85. [PubMed: 7733655]
60. Stamler JS. S-nitrosothiols and the bioregulatory actions of nitrogen oxides through reactions with thiol groups. *Curr Top Microbiol Immunol* 1995;196:19–36. [PubMed: 7634823]
61. Stamler JS, et al. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A* 1992;89(1):444–8. [PubMed: 1346070]
62. Gow AJ, Buerk DG, Ischiropoulos H. A novel reaction mechanism for the formation of S-nitrosothiol in vivo. *J Biol Chem* 1997;272(5):2841–5. [PubMed: 9006926]
63. Jourdain D, Jourdain FL, Feelisch M. Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism. *J Biol Chem* 2003;278(18):15720–6. [PubMed: 12595536]
64. Wink DA, et al. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* 1993;6:23–27. [PubMed: 8448345]
65. Bryan NS, et al. Bound NO in human red blood cells: fact or artifact? *Nitric Oxide* 2004;10(4):221–228.
66. Saville B. A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst* 1958;83:670–72.
67. Wink DA. Detection of S-nitrosothiols by fluorometric and colorimetric methods. *Methods Enzymol* 1999;301:201–11.

68. Jourdain D, Gray L, Grisham MB. S-nitrosothiol formation in blood of lipopolysaccharide-treated rats. *Biochem Biophys Res Commun* 2000;273(1):22–6. [PubMed: 10873557]
69. Jourdain D, et al. Dynamic state of S-nitrosothiols in human plasma and whole blood. *Free Radic Biol Med* 2000;28(3):409–17. [PubMed: 10699753]
70. Samouilov A, Zweier JL. Development of chemiluminescence-based methods for specific quantitation of nitrosylated thiols. *Anal Biochem* 1998;258(2):322–30.
71. Feelisch M, et al. Concomitant S-, N-, and heme-nitrosylation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J* 2002;16:1775–1785. [PubMed: 12409320]
72. Gladwin MT, et al. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O₂/NO-linked allosteric function. *Journal of Biological Chemistry* 2002;277:27818–27828. [PubMed: 12023289]
73. Marley R, et al. A chemiluminescence-based assay for S-nitrosoalbumin and other plasma S-nitrosothiols. *Free Radic Res* 2000;32(1):1–9. [PubMed: 10625212]
74. Ewing JF, Janero DR. Specific S-nitrosothiol (thionitrite) quantification as solution nitrite after vanadium(III) reduction and ozone-chemiluminescent detection. *Free Radic Biol Med* 1998;25(45):621–8. [PubMed: 9741600]
75. Doctor A, Gaston B, Kim-Shapiro DB. Detecting physiologic fluctuations in the S-nitrosohemoglobin micropopulation: Tri-iodide versus 3C. *Blood* 2006;108(9):3225–6. 3226–7. [PubMed: 17057023] author reply
76. Ishibashi T, et al. NO(x) contamination in laboratory ware and effect of countermeasures. *Nitric Oxide* 2000;4(5):516–25. [PubMed: 11020340]
77. Wang X, et al. Measurement of nitric oxide levels in the red cell: validation of tri-iodide-based chemiluminescence with acid-sulfanilamide pretreatment. *J Biol Chem* 2006;281(37):26994–7002. [PubMed: 16845122]
78. Doctor A, et al. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O₂ gradients. *Proc Natl Acad Sci U S A* 2005;102(16):5709–14. [PubMed: 15824313]
79. McMahon TJ, Stamler JS. Concerted nitric oxide/oxygen delivery by hemoglobin. *Methods Enzymol* 1999;301:99–114. [PubMed: 9919558]
80. Sharma VS, et al. Reaction of nitric oxide with heme proteins and model compounds of hemoglobin. *Biochemistry* 1987;26(13):3837–43.
81. Schoneich C, Sharov VS. Mass spectrometry of protein modifications by reactive oxygen and nitrogen species. *Free Radic Biol Med* 2006;41(10):1507–20.
82. Crow JP, Ischiropoulos H. Detection and quantitation of nitrotyrosine residues in proteins: in vivo marker of peroxynitrite. *Methods Enzymol* 1996;269:185–94. [PubMed: 8791648]
83. Shigenaga MK, et al. Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc Natl Acad Sci U S A* 1997;94(7):3211–6. [PubMed: 9096372]
84. Kamisaki Y, et al. Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 1996;685(2):343–7.
85. Mani AR, et al. Nitration of endogenous para-hydroxyphenylacetic acid and the metabolism of nitrotyrosine. *Biochem J* 2003;374(Pt 2):521–7. [PubMed: 12797864]
86. Althaus JS, et al. LC-MS/MS detection of peroxynitrite-derived 3-nitrotyrosine in rat microvessels. *Free Radic Biol Med* 2000;29(11):1085–95. [PubMed: 11121715]
87. Yi D, et al. Quantification of 3-nitrotyrosine in biological tissues and fluids: generating valid results by eliminating artifactual formation. *J Am Soc Mass Spectrom* 2000;11(6):578–86.
88. Nicholls SJ, et al. Quantification of 3-nitrotyrosine levels using a benchtop ion trap mass spectrometry method. *Methods Enzymol* 2005;396:245–66. [PubMed: 16291237]
89. Hausladen A, et al. Assessment of nitric oxide signals by tri-iodide chemiluminescence. *Proc Natl Acad Sci U S A* 2007;104(7):2157–62. [PubMed: 17287342]
90. Rogers SC, et al. Detection of human red blood cell-bound nitric oxide. *J Biol Chem* 2005;280(29):26720–8. [PubMed: 15879596]
91. Stamler JS. S-nitrosothiols in the blood: roles, amounts, and methods of analysis. *Circ Res* 2004;94(4):414–7. [PubMed: 15001539]

92. Singel DJ, Stamler JS. Chemical Physiology of Blood Flow Regulation by Red Blood Cells: The role of nitric oxide and S-nitrosohemoglobin. *Annu Rev Physiol* 2005;67:99–145. [PubMed: 15709954]

Figure 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^- .

Figure 2. The Griess Reaction

The nitrosating agent dinitrogen trioxide (N_2O_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-naphthylethelene diamine to yield a colored diazo product that absorbs strongly at 540 nm.

Figure 3.

(Top) Schematic of ENO-20 method of detection for nitrite and nitrate. (bottom) Standard chromatogram of 10 pmol nitrite and nitrate injected into ENO-20 (100μl of 100nM solution of nitrite and nitrate). Sensitivity of 1nM for each anion with 100μl injection volume. No interference with protein or colored species.

Figure 4. Fluorometric Detection of Nitrite or NO Using Diaminonaphthalene

The nitrosating agent N_2O_3 generated from acidified nitrite or from the autoxidation of NO reacts with diaminonaphthalene (DAN) to yield the highly fluorescent product naphthotriazole (NAT).

Figure 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_x) nitrosate DAF-2 to yield its highly fluorescent product DAF-2 triazole.

Figure 6. Detection of S-nitrosothiols (RSNO) by the Saville Reaction

Liberation of the nitrosating agent nitrosonium (NO^+) by interaction of RSNO with Hg^{+2} 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.

Figure 7. Fluorometric Detection of RSNO

Liberation of NO^+ by interaction of RSNO with Hg^{+2} in the presence of the diaminonaphthylene (DAN) at pH 7.4 results in the formation of the fluorometric derivative naphthotriazole (NAT).

Figure 8.

(Panel A) Chemiluminescence detection of nitrite, RSNO, RNNO in reductive denitrosation assay by sample preincubation with group specific chemical reagents. Subtraction of peak areas allow detection of nitrite and RSNOs. (Panel 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-nitroso-albumin).