Method Article

Quantitative determination of nitric oxide from tissue samples using liquid chromatography—Mass spectrometry

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\textbf{A B S T R A C T}

Ever since it was found to mediate the endothelium-dependent dilation of blood vessels, nitric oxide (NO) has generated enormous research interest throughout the biological sciences. Over thirty years of research has identified NO as a ubiquitous and versatile regulatory factor utilized by both vertebrates and invertebrates. The short lifetime and low concentration of NO make quantitation difficult. Here we report a method for measuring NO using the selective reaction with 2- (4- carboxyphenyl)- 4, 5- dihydro- 4, 4, 5, 5- tetramethyl-1H- imidazolyl- 1- oxy- 3- oxide (carboxy-PTIO) to form carboxy-PTI. We used tandem mass spectrometry to verify the validity of this reaction, and liquid chromatography – mass spectrometry to quantitate the amount of carboxy-PTI formed. Using diethylamine nonoate as a NO donor we demonstrate this method can quantify NO concentrations with a detection limit of 5 nM. We successfully determined the amount of NO generated endogenously by frog heart/aorta when stimulated by carbachol, a non-selective acetylcholine receptor agonist. Based on these results, we suggest that this technique can be useful for the quantitative determination of NO in biological samples.

- We report a method to measure NO by reacting it with carboxy-PTIO to form carboxy-PTI.
- The carboxy-PTI is quantified by liquid chromatography mass spectrometry (LCMS).
- This method can quantitate NO concentrations ranging from 5 nM to 1 μM

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Specifications table

| Subject Area:                      | Neuroscience                      |
|-----------------------------------|-----------------------------------|
| More specific subject area:       | Measuring Nitric Oxide            |
| Method name:                      | Quantifying Nitric Oxide in Biological Samples using Liquid Chromatography – Mass Spectrometry (LCMS) |
| Name and reference of original method: | NA                                |
| Resource availability:            | NA                                |

Method details

Background

Over the last three decades, the molecule nitric oxide (NO) has captured a great deal of interest in many biological and biochemical fields such as neuroscience, physiology, and microbiology. NO was initially revealed to mediate acetylcholine (ACh) induced arterial smooth muscle relaxation [1–4]. After many years of intensive research, NO has been shown to be a ubiquitous and versatile regulator of great variety of physiological and pathological processes such as neurotransmission, immune response, and bacterial stress-response [5–8]. In a typical pathway, endogenous NO is generated by an enzyme nitric oxide synthase (NOS) using L-arginine, NADPH, and oxygen. Once NO is produced, it diffuses across the cell membrane to reach its target [8,9]. There is considerable interest in developing methods for determining levels of NO in biological samples with high specificity and sensitivity.

Quantitative determination of NO in biological samples is particularly challenging given its short lifespan and low concentration in biological systems – generally estimated to be on the order of seconds and nanomolar respectively [10,11]. Several tools to detect NO in biological systems have been developed and reviewed [12–14]. Many of the commonly utilized methods suffer from some disadvantages [15,16]. Table 1 summarizes the major methods for detecting NO. The first method to be widely used for quantifying NO was the Griess test, in which NO$_2^-$, a natural autoxidation product of NO, is derivatized to a purple azo dye that can be quantified using spectrophotometry [13]. However, the NO autoxidation process is subject to interference in biological systems, as NO and its natural derivatives (e.g., NO$_2$, N$_2$O$_3$, NO$_2^-$) are readily trapped by biomolecules such as heme, glutathione, sulfhydryl, and unsaturated fatty acids [10,17,18]. Thus, NO$_2^-$, the analyte of the Griess test, may not represent the NO concentration. One method to increase the accuracy of the Griess test is to use 2- (4- carboxyphenyl)- 4, 5- dihydro- 4, 4, 5, 5- tetramethyl- 1H- imidazolyl- 1- oxy- 3- oxide (carboxy-PTIO) to rapidly convert NO to NO$_2$, which is then transformed to NO$_2^-$, by reacting with carboxy-PTIO or a combination of NO and H$_2$O [10,17,18].

Several fluorescence-based methods have also been developed to measure NO concentration with high sensitivity and high spatial and temporal resolution [28]. One common method uses the fluorescent probe 4,5-Diaminofluorescein (DAF-2) from the family of diaminofluoresceins [19,29]. Modification of DAF-2 into the diester form, DAF-FM, which allows it to be indiscriminately delivered into the cytoplasm of cells, has further increased its usefulness [19]. However, just like the Griess test, DAF-2 does not target NO but rather autoxidation products such as N$_2$O$_3$ [19]. The DAF-2 probe also responds to common metabolites such as dehydroascorbic acid and ascorbic acid [30]. As a result, this method suffers from false-positive signals. Similar limitations have been reported for other fluorescence-based probes [29,31]. Recently a new fluorescent probe, FP-NO, has been described which exploits the restriction of rotation of an N-N single bond in coumarin when NO binds, dramatically
increasing its fluorescence [32]. This method is reported to have a detection limit of 47.6 nM; however, it has undergone minimal testing in living organisms [32].

Another class of NO detection methods utilizes amperometric microelectrodes [15,21–25]. This method has extremely high spatial and temporal precision. Over the past few years, it has also seen significant improvements in its sensitivity and specificity [21]. Currently, it is capable of detecting nanomolar changes in NO concentration over milliseconds. Clearly, NO electrodes are a powerful quantification tool given the right expertise to fabricate, calibrate, and use them [25,33].

A method that uses chemoluminescence to detect NO has recently been described [20]. In this method, soluble guanylate cyclase converts GTP to cGMP and pyrophosphate (PP\(_3\)) when activated by NO. The PP\(_3\) is then converted to ATP and the ATP is detected by a luciferin-luciferase assay. This method is reported to be two orders of magnitude more sensitive than using DAF-FM [20].

A technique to measure NO that uses an o-phenylenediamine moiety to trap NO by forming a benzotriazole group using tandem mass spectrometry (MS/MS) to quantify the resulting product was reported [26]. However, it has been noted that the lack of a separation step could cause possible interference in the mass spectra by molecules other than the analyte [27]. Here we present a liquid chromatography-mass spectrometry (LCMS) based NO-quantitation method by reacting carboxy-PTIO with NO to form carboxy-PTI and employ LCMS to detect and quantify carboxy-PTI. In biological studies, carboxy-PTI is often referred to as an NO scavenger, because it rapidly reacts with NO and prevents the diffusion of the gas molecule. The radical-specificity of carboxy-PTIO means this technique is free from interference by intracellular components such as O\(_2^-\) [34]. Our results suggest this straightforward method has excellent precision and accuracy when detecting NO released from the NO donor, diethylamine nonoate (DEANO). DEANO belongs to the nonoate family of NO donors that readily dissociate in an aqueous solution, and compared to other nonoates, DEANO has a relatively high yield of 1.5 NO molecules per DEANO. Just as other nonoates, the decomposition rate of DEANO is low at high pH (>8) and rapid in acidic solutions [35,36], a useful characteristic for the titration experiments of this paper. We show that our technique can detect NO liberated from as little as 5 nM DEANO when titrated with 100 nM carboxy-PTIO. We also demonstrate that this is sufficient sensitivity to detect NO release from a single frog heart/aorta stimulated by the acetylcholine receptor agonist carbachol.

### Table 1
Methods used for detecting nitric oxide.

| Method                | Analyte | Detection limit | Advantages                                      | Disadvantages                                      | References |
|-----------------------|---------|-----------------|-------------------------------------------------|---------------------------------------------------|------------|
| Griess test           | NO\(_2^-\) | \(\mu\)M | Easy to use                                     | Low sensitivity; not directly measuring NO         | [13]       |
| (spectrophotometry)   |         |                 |                                                 |                                                   |            |
| DAF-2 (fluorescence)  | NO metabolites | nM | Easy to use; high spatial, temporal resolution | Not directly measuring NO                         | [19]       |
| Luciferin-luciferase  | NO      | pM              | High sensitivity                                | Specialized instrumentation                       | [20]       |
| (luminescence)        |         |                 |                                                 |                                                   |            |
| NO electrodes         | NO      | nM              | High spatial, temporal resolution               | Specialized fabrication and instrumentation       | [15,21–25]|
| (electrochemical)     |         |                 |                                                 | Contamination due to the lack of sample separation; low spatial, temporal resolution | [26,27]   |
| ESI-MS/MS             | NO      | nM              | Instrument common in academic institutes        |                                                   |            |
| LCMS                  | NO      | nM              | Using a common instrument and a frequently used reagent in NO research | Low spatial, temporal resolution | This paper |


**Scheme 1.** Schematic representation of the reactions between NO and carboxy-PTIO. NO reacts with carboxy-PTIO to produce carboxy-PTI and NO$_2$ in Reaction 1. NO$_2$ then feeds into a cycle of Reactions 2 and 3, which consumes another molecule of NO. Both Reactions 2 and 3 ($k = 1.5 \times 10^7$ M$^{-1}$s$^{-1}$ and $2.3 \times 10^5$ M$^{-1}$s$^{-1}$, respectively) are much faster than Reaction 1 ($k = 6 \times 10^3$ M$^{-1}$s$^{-1}$) [19]. The cycle of Reactions 2 and 3 results in no loss of carboxy-PTIO or gain of carboxy-PTI. Together, these reactions result in an overall NO:carboxy-PTI stoichiometry between 1:1 and 2:1 [19].

**Scheme**

We demonstrate a method to measure nitric oxide (NO) in aqueous solutions by reaction with carboxy-PTIO. The derivatization reaction between NO and carboxy-PTIO is shown in Scheme 1. Previous studies have found that NO reacts specifically with carboxy-PTIO to produce carboxy-PTI and NO$_2$ much faster than the typical residence time (a few seconds) of NO in biological samples (Reaction 1) [10,18]. NO$_2$ is not the end product of this system as it rapidly oxidizes carboxy-PTIO to carboxy-PTIO$^+$ (Reaction 2). carboxy-PTIO$^+$ rapidly reacts with another molecule of NO to produce NO$_2^-$ and regenerates carboxy-PTIO (Reaction 3). This results in an overall NO:carboxy-PTI stoichiometry of $n:1$ (1<$n$<2) [18]. Thus, we quantitated the change in carboxy-PTI, the reaction product, as an indicator for the amount of NO in the solution.

**Determining optimal concentration of carboxy-PTIO**

To quantify the amount of carboxy-PTI in the solution, we used 10 $\mu$M tryptophan as an internal standard. The relative abundance of carboxy-PTI was calculated from the ratio of the intensity of the carboxy-PTI and the tryptophan (deaminated in the ionization process) peaks in the chromatogram (Fig. 1A, see Figure S1A for a total ion chromatograph). The carboxy-PTI peak identity was verified via collision induced dissociation (CID) (Figure S2). To determine the ranges of detection and linearity of this method, we titrated, in quadruplicate, 100 nM, 400 nM, and 4000 nM carboxy-PTIO with the pH-dependent NO donor DEANO and analyzed the reaction mixtures using LCMS (Fig. 1B). Each titration reaction of carboxy-PTIO with DEANO was carried out for at least 4 hours at pH 5.5 to ensure complete DEANO dissociation (reported $t_{1/2}$: 16 min at 25°C and pH 7.4) [37].
Fig. 1. Titration curves of 100 nM, 400 nM, and 4000 nM carboxy-PTIO with DEANO. A, Sample chromatograph of carboxy-PTI (m/z=263.14, upper panel) and tryptophan-NH$_2$ (m/z=188.06, lower panel). B, Normalized titration curves in water compared with readings from the Griess test. C-E, The regions of linearity for 100 nM (C), 400 nM (D) and 4000 nM (E) carboxy-PTIO are 5–90 nM, 100–900 nM and 40–1000 nM DEANO respectively. Griess test data are shown only when DEANO concentrations are within its range of linearity (i.e. D and E). Error bars represent ± 1 standard error of the mean.
The LCMS results show different ranges of linearity for all three titration curves. The regions of linearity for 100 nM (Fig. 1C), 400 nM (Fig. 1D) and 4000 nM (Fig. 1E) carboxy-PTIO are found to be 5–90 nM, 100–900 nM and 40–1000 nM DEANO respectively (see Table S1 for the regression parameters). As an independent confirmation of the technique and of the use of DEANO as an NO donor, we also analyzed most reaction mixtures using the modified Griess test as described in Amano and Noda [17]. The carboxy-PTIO oxidizes NO to NO$_2^-$ which reacts with the Griess reagent. A reference reading of the Griess test is shown for 400 nM and 4000 nM carboxy-PTIO. The region of linearity for DEANO concentrations in the 100 nM carboxy-PTIO titration series is below the detection limit of the Griess test, which is typically reported as 500 nM NO$_2^-$ [38]. We also found using the Griess test the DEANO-NO stoichiometry of dissociation is 1:1.46±0.01, close to the literature value of 1:1.5 (Figure S3) [35,37].

**Determining accuracy and precision of the method**

Since these results indicate that 100 nM carboxy-PTIO gives the best sensitivity, we used this concentration of carboxy-PTIO to determine the method’s accuracy and precision. In the accuracy test, we prepared DEANO samples of 15 nM, 35 nM, and 50 nM and derivatized them with 100 nM carboxy-PTIO. The LCMS results give a carboxy-PTI relative abundance of the 50 nM sample that is 103±2% of the sum of the 15 nM and 35 nM samples after baseline correction (n=5 for each concentration). The relative standard deviation of 10 samples of 100 nM DEANO derivatized with 100 nM carboxy-PTIO was found to be 2.57%, demonstrating excellent precision.

**Testing the specificity of carboxy-PTIO**

As seen in Fig. 1B, 4000 nM carboxy-PTIO gives an unexpected non-zero normalized carboxy-PTI relative abundance. This suggests that carboxy-PTIO is unstable at a high concentration. To investigate the origin of carboxy-PTI production in the absence of a source for NO (termed auto-derivatization below), we first monitored the time-course of the auto-derivatization reaction at 4000 nM carboxy-PTIO (Fig. 2A). This reaction is significantly slower than the reported reaction between carboxy-PTIO and NO ($k = 6\cdot10^{-3}$ M$^{-1}$s$^{-1}$) [18]. Treating the solution with argon for 10 min significantly lowered the rate of this reaction, suggesting that carboxy-PTIO reacts with a dissolved gas (Fig. 2A, solid triangles). Interestingly, treating the solution with a low concentration (200 pM) of DEANO instead of argon slowed down the auto-derivatization rate to as well (Fig. 2A, empty circles), presumably because this gas reagent also reacts with DEANO, its nonoate moiety, or NO. Regardless of the exact origin of this phenomenon, the fact that 4000 nM carboxy-PTIO can produce carboxy-PTI in the absence of NO shows that carboxy-PTIO should not be used at this high concentration. Consequently, we think carboxy-PTIO is perhaps unsuitable for quantifying high concentrations of NO, a limitation that is unlikely applicable to biological systems (see below for an example).

Because our experiments used exclusively DEANO as the NO donor, an alternative interpretation of our results is that carboxy-PTIO reacts with DEANO instead of NO to produce carboxy-PTI. To test this possibility, we carried out, in triplicates, experiments using carboxy-PTIO and sulfo-nonoate (SulfoNO), which shares the same nonoate moiety with DEANO but releases N$_2$O instead of NO [35] (Fig. 2B). We found that 100 nM carboxy-PTIO does not react with SulfoNO when the concentration of the latter is as high as 8000 nM. Similarly, 100 nM carboxy-PTIO does not react with pre-dissociated DEANO, as in this case all released NO molecules have been converted to NO$_2^-$ (Fig. 2B). We therefore conclude that carboxy-PTIO reacts specifically with NO instead of nonoates or N$_2$O.

**Measuring NO generated by frog heart and aorta**

To determine the effectiveness of this method in physiological assays, we set up an experiment to quantitate the NO generated by the frog heart/aorta in response to the activation of acetylcholine receptors (AChRs). To control for any pH and ionic strength effects on the reaction between NO and carboxy-PTIO, we recreated the standard curve of DEANO titration in the frog Ringer solution (Fig. 3A). In all physiological experiments, a heart and approximately 0.5 cm of its connected aorta were...
Fig. 2. Testing the specificity of carboxy-PTIO for NO. A, At 400 nM, carboxy-PTIO undergoes a slow reaction to form carboxy-PTI (●). Treating the solution with argon (▼), 200 pM DEANO (●) or a combination of both (△) significantly attenuated the reaction. B, Substitution of either 8 μM or 100 nM DEANO (black bars) with SulfoNO (gray bars) of equal molarity does not produce any carboxy-PTI. Dissociated DEANO (dotted), at 8000 nM, also does not produce carboxy-PTI. White bar shows the baseline carboxy-PTI relative abundance in absence of NO donors. All reactions here use 100 nM carboxy-PTIO. Error bars represent ± 1 standard error of the mean. *P < 0.001, one-way ANOVA with Tukey posthoc test.
Fig. 3. Quantitation of NO in frog aorta and heart. A. The calibration curve from titration of carboxy-PTIO with DEANO in frog Ringer solution. Error bars represent ±1 standard error of the mean. B. NO was monitored using 100 nM carboxy-PTIO in the Ringer solution (see Materials and Methods) using the same method as the previous assays. Application of 0.5 mM carbachol (thick bar) at 60 min increases the NO production rate of the tissue sample to the equivalent of 44 nM DEANO per 30 min (solid circle). No increase in NO concentration is seen if the Ringer solution also contains 1 mM L-NAME, a nitric oxide synthase inhibitor (empty circle). The baseline NO is at the limit of detection. ***p<0.001, Student’s t-test. Error bars represent ±1 standard error of the mean. N = 3 animals per condition.
incubated in a series of tubes filled with 0.5 mL frog Ringer solutions containing 100 nM carboxy-PTIO, for 30 min. We then applied 0.5 mM carbachol, a non-selective AChR agonist, and determined the NO concentration in the solution. Carbachol produced the equivalent of 44 nM DEANO (Fig. 3B, see sample chromatograph in Figure S1B) [39]. Blocking nitric oxide synthase (NOS) by pre-incubating the heart in 1 mM L-NAME prevents carbachol-induced NO release, therefore affirming the release of NO upon AChR activation. We estimate that this corresponds to a NO release rate of 1.1 pmol/min into the extracellular medium by assuming a DEANO-NO stoichiometry of 1:1.46 (Figure S3) and an extracellular solution volume of 0.5 mL. These results provide new quantitative insights into the well-documented AChR-induced NOS activation in the frog heart/aorta endothelial cells and subsequent smooth muscle relaxation [40].

Conclusions

In this work, we demonstrate the feasibility of using carboxy-PTIO, a common laboratory reagent for the study of NO, and LCMS to effectively quantitate nitric oxide (NO) level in an aqueous solution. We found this technique to be precise, accurate, and sensitive. We successfully utilized this technique to quantitate the NO liberated from frog heart/aorta when stimulated by carbachol. Future work should include automating the process of drawing carboxy-PTI-containing samples in order to quantify NO at a higher temporal resolution. In addition, sample preprocessing and filtering will need to be incorporated in order to apply this method to other biological systems such as the plasma and the cerebral spinal fluid.

Materials and instrumentation

Chemicals (catalog number in parentheses)

DEANO (D5431), hydroxydiazenesulfonic acid 1-oxide disodium salt (SulfoNO, S8432) and L-Tryptophan (T0254) were purchased from Millipore-Sigma. The Griess reagent kit (G7921) and carboxy-PTIO (C7912) were obtained from Thermo-Fisher. NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 0665) and (2-Hydroxyethyl)trimethylammonium chloride carbamate (carbachol, 2810) were purchased from Tocris Bioscience.

Chemical preparation

All chemical stock solutions were stored as instructed. The Griess reagents were stored at −4 °C. Both DEANO (8 mM) and SulfoNO (10 mM) stock solutions were stored at pH 12.5 at −80 °C to minimize decomposition [28]. Aqueous stock solutions of carboxy-PTIO (40 mM), L-NAME (500 mM), and carbachol (10 mM) were stored at −20°C.

Instruments

A Waters Acquity (Milford, MA, United States) ultraperformance liquid chromatography (UPLC) system with a 2.1×50 mm C18 reverse-phase column (Waters) was used for separations. Standard parameters were used for both the LC and the electrospray ionization mass spectrometry (ESI-MS). The flow rate was 0.3 mL/min and the gradient was set to ramp from 95%/5% (A/B) to 50%/50% (A/B) over 5 min (A: H2O with 0.1% formic acid, B: acetonitrile with 0.1% formic acid). The column temperature was 40 °C. A Xevo quadrupole-time of flight (Q-Tof) ESI mass spectrometer (Waters, Milford, MA) was used in positive mode to detect and identify the LC peaks with an electrospray voltage of 3 kV, source temperature of 120 °C, desolvation gas flow of 700 L/h and desolvation temperature of 300°C. Leucine-enkephalin was used as a mass reference. LCMS data were analyzed using Masslynx.

Griess test results were analyzed using an Agilent 8453 diode array spectrophotometer (Ramsey, MN, United States).

We extracted the ion counts corresponding to the m/z ratios of monoprotonated carboxy-PTI ([M+H]+ = 263.14) and the internal control Tryptophan ([M-NH2]+ = 188.06) from the
chromatograph. Peaks corresponding to carboxy-PTI and tryptophan are consistently seen at $T = 3.2$ min and $T = 1.8$ min, respectively. We integrated the areas under the two peaks and calculated the ratio of the carboxy-PTI peak over the tryptophan-peak as the relative abundance of carboxy-PTI.

**Titration of carboxy-PTIO**

We chose DEANO as the NO donor in this study because the rate of its decomposition can be controlled via pH (fast in low pH and slow in basic conditions) [35,36], thereby preventing unwanted NO generation before carboxy-PTIO was added in. We suspect other sources of NO donors (e.g., S-nitrosothiols) would give similar results, provided that the rate of NO generation is precisely controlled. The titration series of carboxy-PTIO with DEANO was performed by diluting a stock solution of carboxy-PTIO (40 mM) water to the desired concentration (100, 400, 4000 nM) and titrating in cold DEANO (0°C, pH=12.5). The final volume and pH of the reaction mixture were 1 mL and 5.5 respectively. Because of the wide range of pH needed (5.5 to 12.5) for the precise control of DEANO decomposition, we used unbuffered water for the titration experiments.

Each reaction was allowed at least 4 h of reaction time at room temperature before tryptophan was added, at a final concentration of 10 μM, as an internal standard for the subsequent LCMS analysis. Because tryptophan readily dissociated in the MS, the -NH2 (m/z 188.06) peak was monitored. For the SulfoNO titrations, all procedures were exactly the same as described above, except DEANO was substituted with SulfoNO. Typically, all samples were analyzed within 48 h after preparation.

**Griess test**

Griess tests were performed using the procedure provided by the manufacturer. In brief, 300 μL sample, 2.6 mL dH2O, 50 μL N-(1-naphthyl)ethylenediamine dihydrochloride (1 mg/mL) and 50 μL 4-aminobenzenesulfonic acid (10 mg/mL) were mixed and allowed at least 20 min reaction time before being analyzed by monitoring absorption at 540 nm.

**Physiological assay**

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Grinnell College. Small (2-3 in.) frogs, species Rana pipiens, were obtained from Carolina Biological Supply (Burlington, North Carolina, United States) and housed at 4–8 °C before experiments. Prior to each experiment, the frog was first double-pithed as described in Lindgren and Laird [41]. The heart with its connected aorta (about 0.5 cm in length) was separated from the rest of the body and washed thoroughly with frog Ringer solution (112 mM NaCl, 3.2 mM KCl, 0.5 mM Na2HPO4, 2.7 mM CaCl2, 2.0 mM Tris, pH 7.2). Each washed heart was then incubated consecutively, for 30 min each, in 6 tubes which contained 100 nM carboxy-PTIO diluted in 0.5 mL frog Ringer solution. Solutions also contained carbachol, L-NAME, or both (as indicated). After the incubation was completed, tryptophan was added to a final concentration of 10 μM before the solutions were analyzed by LCMS. DEANO titration series were also performed in the Ringer solution using the same method as described above.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101412.

References

[1] R.F. Furchgott, J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, Nature 288 (1980) 373–376, doi:10.1038/288373a0.

[2] L.J. Ignarro, G.M. Buga, K.S. Wood, R.E. Byrns, G. Chaudhuri, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, Proc. Natl. Acad. Sci. 84 (1987) 9265–9269, doi:10.1073/pnas.84.24.9265.

[3] R.M.J. Palmer, A.G. Ferrige, S. Moncada, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, Nature 327 (1987) 524–526, doi:10.1038/32754a0.

[4] I. Fleming, R. Busse, NO: the primary EDRF, J. Mol. Cell Cardiol. 31 (1999) 5–14, doi:10.1016/jjmcc.1998.0839.

[5] E.M. Schuman, D.V. Madison, Nitric oxide and synaptic function, Annu. Rev. Neurosci. 17 (1994) 153–183, doi:10.1146

[6] J.Jr Taintor Hibbs, R.R. Vavrin, Z. Rachlin, Nitric oxide: a cytotoxic activated macrophage effector molecule, Biochem. Biophys. Res. Commun. 157 (1988) 79–94.

[7] I. Gusarov, E. Nuider, NO-mediated cytoprotection: Instant adaptation to oxidative stress in bacteria, PNAS 102 (2005) 13855–13860.

[8] S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43 (1991) 109–142.

[9] C. Nathan, Q. Xie, Nitric oxide synthases: roles, tolls, and controls. Cell 78 (1994) 915–918, doi:10.1016/0092-8674(94)

[10] J. Stamler, D. Singel, J. Loscalzo, Biochemistry of nitric oxide and its redox-activated forms, Science 258 (1992) 1898–1902, doi:10.1126/science.1281928.

[11] S. Archer, Measurement of nitric oxide in biological models, Faseb. J. 7 (1993) 349–360, doi:10.1096/fasebj.7.2.844041.

[12] P.N. Coneski, M.H. Schoenfisch, Nitric oxide release: part III. Measurement and reporting, Chem. Soc. Rev. 41 (2012) 3753–3758, doi:10.1039/c2cs15271a.

[13] E. Goshi, G. Zhou, Q. He, Nitric oxide detection methods in vitro and in vivo, Med. Gas. Res. 9 (2019) 0, doi:10.4032/

[14] M.N. Möller, N. Rios, M. Trujillo, R. Radi, A. Denicola, B. Alvarez, Detection and quantification of nitric oxide–derived oxidants in biological systems, J. Biol. Chem. 294 (2019) 14776–14802, doi:10.1074/jbc.rev118.006136.

[15] F. Bedioui, D. Quinton, S. Griveau, T. Nyokong, Designing molecular materials and strategies for the electrochemical detection of nitric oxide, superoxide and peroxynitrite in biological systems, Phys. Chem. Chem. Phys. 12 (2010) 9976–9988, doi:10.1039/c0cp00271b.

[16] R.A. Hunter, W.L. Storm, P.N. Coneski, M.H. Schoenfisch, Inaccuracies of nitric oxide measurement methods in biological media, Anal. Chem. 85 (2013) 1957–1963, doi:10.1021/ac303787p.

[17] F. Amano, T. Noda, Improved detection of nitric oxide radical (NO•) production in an activated macrophage culture with a radical scavenger, car-y PTIO, and Griess reagent, Febis Lett. 368 (1995) 425–428, doi:10.1016/0014-5793(95)00700-j.

[18] S. Goldstein, A. Russo, A. Samuni, Reactions of PTIO and carboxy-PTIO with NO, NO2, and O2 . “”, J. Biol. Chem. 278 (2003) 50949–50955, doi:10.1074/jbc.M308317200.

[19] H. Kojima, N. Nakatsuho, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins, Anal. Chem. 70 (1998), doi:10.1021/ac9801723.

[20] Y.Y. Woldman, T.D. Eubank, A.J. Mock, N.C. Stevens, S. Varadharaj, J. Turco, M.A. Gavrin, B.R. Branchini, V.V. Khramtsov, Detection of nitric oxide production in cell cultures by luciferin–luciferase chemiluminescence, Biochem. Biophys. Res. Commun. 465 (2015) 232–238, doi:10.1016/j.bbrc.2015.08.001.

[21] F. Bedioui, A. Ismail, S. Griveau, Electrochemical detection of nitric oxide and S-nitrosothiols in biological systems: past, present & future, Curr. Opin. Electrochem. 12 (2018) 42–50, doi:10.1016/j.coelc.2018.04.014.

[22] K. Ichimori, J. Ishida, M. Fukahori, H. Nakazawa, E. Murakami, Practical nitric oxide measurement employing a nitric oxide-selective electrode, Rev. Sci. Instrum. 65 (1994) 2714–2718.

[23] E.M. Hetrick, M.H. Schoenfisch, Analytical chemistry of nitric oxide, Annu. Rev. Anal. Chem. 2 (2009) 409–443, doi:10.1146

[24] M.D. Brown, M.H. Schoenfisch, Selective and sensor-compatible electrochemical nitric oxide sensors with a bilamina design, Acs Sens. 4 (2019) 1766–1773, doi:10.1021/acssensors.9b00170.

[25] B.J. Privett, J.H. Shin, M.H. Schoenfisch, Electrochemical nitric oxide sensors for physiological measurements, Chem. Soc. Rev. 39 (2010) 1925–1935, doi:10.1039/b919060h.

[26] Z. Shen, A. Webster, K.J. Welham, C.E. Dyer, J. Greenman, S.J. Haswell, Detection of nitric oxide in tissue samples by ESI-MS, Analyst 135 (2010) 302–305, doi:10.1039/b917643h.

[27] D. Tsigkas, B. Sandmann, B. Beckmann, Z. Shen, A. Webster, K.J. Welham, C.E. Dyer, J. Greenman, S.J. Haswell, Analysis of NO and its metabolites by mass spectrometry. Comment on “Detection of nitric oxide in tissue samples by ESI-MS “, Analyst 136 (2010) 407–410, doi:10.1039/b9cn0041a.

[28] T. Nagano, Practical methods for detection of nitric oxide, Luminescence 14 (1999) 283–290 https://doi.org/10.1021/sic15227a001.

[29] C.N. Hall, J. Garthwaite, What is the real physiological NO concentration in vivo? Nato Sci. 5 A Life Sci. 21 (2009) 92–103, doi:10.1007/jo

[30] X. Zhang, W.-S. Kim, N. Hatcher, K. Potgieter, L.L. Moroz, R. Gillette, J.V. Sweedler, Interfering with nitric oxide measurements 4,5-diaminofluorescein reacts with dehydroascorbic acid and ascorbic acid, J. Biol. Chem. 277 (2002) 48472–48478, doi:10.1074/jbc.m209130200.
[31] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects, Free Rad. Bio Med. 43 (2007) 995–1022, doi:10.1016/j.freeradbiomed.2007.06.026.

[32] Q. Han, J. Liu, Q. Meng, Y.-L. Wang, H. Feng, Z. Zhang, Z.P. Xu, R. Zhang, Turn-on fluorescence probe for nitric oxide detection and bioimaging in live cells and zebrafish, Acs Sens. 4 (2018) 309–316, doi:10.1021/acssensors.8b00776.

[33] M.D. Brown, M.H. Schoenfisch, Electrochemical nitric oxide sensors: principles of design and characterization, Chem. Rev. 119 (2019) 11551–11575, doi:10.1021/acs.chemrev.8b00797.

[34] G.Y. Ko, P.T. Kelly, Nitric oxide acts as a postsynaptic signaling molecule in calcium/calmodulin-induced synaptic potentiation in hippocampal CA1 pyramidal neurons, J. Neurosci. 19 (1999) 6784–6794, doi:10.1523/jneurosci.19-16-06784.1999.

[35] C.M. Maragos, D. Morley, D.A. Wink, T.M. Dunams, J.E. Saavedra, A. Hoffman, A.A. Bove, L. Isaac, J.A. Hrabie, L.K. Keefer, Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects, J. Med. Chem. 34 (1991) 3242–3247, doi:10.1021/jm00115a013.

[36] R. Ferrero, F. Rodríguez-Pascual, M.T. Miras-Portugal, M. Torres, Comparative effects of several nitric oxide donors on intracellular cyclic GMP levels in bovine chromaffin cells: correlation with nitric oxide production, Br. J. Pharmacol. 127 (1999) 779–787, doi:10.1038/sj.bjp.0702607.

[37] L.K. Keefer, R.W. Nims, K.M. Davies, D.A. Wink, NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms, Methods Enzymol. 268 (1996) 281–293, doi:10.1016/s0076-6879(96)8030-6.

[38] W.R. Tracey, J. Linden, M. Peach, R.A. Johns, Comparison of spectrophotometric and biological assays for nitric oxide (NO) and endothelium-derived relaxing factor (EDRF): nonspecificity of the diazotization reaction for NO and failure to detect EDRF, J. Pharm. Exp. Ther. 252 (1990) 922–928.

[39] D.A. Eberhard, R.W. Holz, Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms, J. Neurochem. 49 (1987) 1634–1643.

[40] G.E. Knight, G. Burnstock, The involvement of the endothelium in the relaxation of the leopard frog (Rana pipiens) aorta in response to acetylcholine, Br. J. Pharmacol. 118 (1996) 1518–1522, doi:10.1111/j.1476-5381.1996.tb15568.x.

[41] C.A. Lindgren, M.V. Laird, Nitroprusside inhibits neurotransmitter release at the frog neuromuscular junction, Neureport 5 (1994) 2205 https://doi.org/10.1097/00001756-199410270-00054.