Analytical Chemistry of Nitric Oxide

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Abstract

Nitric oxide (NO) is the focus of intense research primarily because of its wide-ranging biological and physiological actions. To understand its origin, activity, and regulation, accurate and precise measurement techniques are needed. Unfortunately, analytical assays for monitoring NO are challenged by NO's unique chemical and physical properties, including its reactivity, rapid diffusion, and short half-life. Moreover, NO concentrations may span the picomolar-to-micromolar range in physiological milieus, requiring techniques with wide dynamic response ranges. Despite such challenges, many analytical techniques have emerged for the detection of NO. Herein, we review the most common spectroscopic and electrochemical methods, with a focus on the underlying mechanism of each technique and on approaches that have been coupled with modern analytical measurement tools to create novel NO sensors.

1. INTRODUCTION

Prior to the late 1980s, nitric oxide (NO) was known primarily as an environmental pollutant. In 1987, Ignarro et al. (1) and Furchgott (2) independently confirmed NO's role in mammalian physiology, determining that NO was the long-elusive endothelium-derived relaxing factor responsible for vasodilation and blood pressure regulation. Since these discoveries, a great deal of research has unraveled the multifaceted roles of NO. Indeed, NO plays key physiological roles in the cardiovascular (3) and nervous (4, 5) systems, as an endogenously produced antimicrobial agent (6), and as a signaling molecule capable of modulating cytokine production (7) in the immune response (8) and in wound healing (9). Endogenously, NO is produced by a class of heme-containing enzymes known as NO synthases (10, 11). In light of NO's pharmacological potential, several chemical NO donors have been developed to spontaneously generate NO (12, 13), allowing for further study.

The intense interest in NO research and the need to characterize chemical NO donors demand analytical techniques that can accurately and precisely quantify NO concentrations and rates of production. The unique chemical properties of NO make this a challenging task. For example, NO is highly reactive and is rapidly scavenged by endogenous compounds including oxygen (O_2), heme proteins (e.g., hemoglobin), thiols (e.g., cysteine residues, glutathione), and other free radicals (e.g., superoxide) (14–18). Nitric oxide's short half-life (on the order of seconds) also complicates its detection. Moreover, NO's actions are mediated by a broad range of concentrations (typically in the picomolar-to-micromolar range, i.e., spanning approximately six orders of magnitude) (14, 19), necessitating techniques with wide linear response ranges. The rapid diffusion of NO and its transient nature further require analytical methods for spatial detection that have rapid response times (20).

Fortunately, the same chemical properties that make monitoring NO challenging also allow a broad range of analytical techniques to be employed for its detection. The primary methods for detecting NO include absorbance-, fluorescence-, and chemiluminescence-based approaches, electron paramagnetic resonance (EPR), and electrochemistry. This review details the underlying mechanisms of these techniques, focusing on the applicable chemistry and analytical figures of merit such as detection limit, linear range, response time, and selectivity for NO over common interferents. We devote special attention to newly developed assays and approaches for which NO-detection techniques have either been coupled with modern analytical measurement tools [e.g., high-performance liquid chromatography (HPLC)] or exploited to create novel analytical sensors. We offer examples that illustrate both standard and new applications of each technique. The majority of this review focuses on spectroscopic and electrochemical NO-detection methods, which are summarized in **Table 1**.

DIFFICULTIES COMPARING NITRIC OXIDE DETECTION TECHNIQUES

The different analytical methods for measuring nitric oxide (NO) have resulted in inconsistent reporting of limits of detection (LOD), which range from concentration (e.g., micromolar or nanomolar amounts) to absolute amount (e.g., picomoles). Such inconsistencies arise in part because NO is measured both as a gas (in parts per billion or parts per million) and as a dissolved species (in molar units). We have made every effort to maintain consistency to allow comparison between techniques.

Table 1 Summary of nitric oxide detection techniques with associated limits of detection and detection ranges[1]

Class	Method	Technique	Limit of detection	Detection range	Reference(s)
Spectroscopic	Absorbance	Hemoglobin assay	1.3–2.8 nM	NR	22
		Hemoglobin assay (microdialysis)	7 nM	NR	29
		Tt Y140F bacterial heme protein	300 nM	30 μM	30
		Cytochrome *c′*–doped xerogel	1 ppm	25 ppm	31
		Cu(II) eriochrome cyanine R complex	0.23 ppm	6 ppm	25
		Griess assay	0.5 μM	NR	37
		Griess assay (flow-injection analysis)	50 nM	10 μM	54
		Griess assay (microgas analysis system)	7 ppb	50 ppm	55
	Fluorescence	FNOTC	Nanomolar range	5 μM	58
		Cu(II) fluorescein complex	5 nM	NR	60
		Diaminofluoresceins	5 nM	NR	63
		TMDABODIPY with HPLC	20 pM	800 nM	69
		Cytochrome *c′*–based probe	20 μM	1 mM	73
		Oregon Green 488 gold colloid probe	20 μM	1 mM	74
		Oregon Green–labeled Cytochrome *c′*	8 μM	1 mM	75
	Chemiluminescence	Ozone-based (solution)	Nanomolar-to-picomolar range	Millimolar range	76, 77
		Ozone-based (gas)	0.5 ppb	500 ppm	77
		Luminol-based	100 fM	1 nM	83
		Luminol (polypropylene hollow fiber membranes)	0.3 ppb	≥90 ppb	84
		Luminol (fiber optic)	1.3 μM	40 μM	85
	EPR	Fe-dithiocarbamate spin trap	6 pmol	NR	106
Electrochemical	Permselective	Shibuki chloroprene rubber membrane	Nanomolar range	3 μM	116
		Platinized Pt electrode with PTFE membrane	1 nM	≥350 nM	132
		Aminoalkoxysilane membrane	25 nM	15 μM	115
		Fluoroalkoxysilane membrane	83 pM	4 μM	135
	Electrocatalytic	Semiconducting Ni porphyrin	10 nM	300 μM	108
	Other	Hemoglobin-based electrode	20 pM	5 μM	137
		Dual NO/CO sensor	1 nM	Micromolar range[2]	139

[1]Abbreviations: CO, carbon monoxide; EPR, electron paramagnetic resonance spectroscopy; FNOCT, fluorescent nitric oxide cheletropic trap; HPLC, high-performance liquid chromatography; NO, nitric oxide; NR, not reported; PTFE, polytetrafluoroethylene; TMDABODIPY, 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)difluoroboradiaza-*s*-indacene.

[2]For NO.

2. SPECTROSCOPIC METHODS

2.1. Absorbance-Based Measurements

Absorbance-based methods for the detection of NO benefit from simple, affordable instrumentation and conceptually straightforward analysis procedures. As such, these methods are widely used for detecting NO, especially in biological systems.

2.1.1. Metalloprotein-based assays. First reported by Haussmann & Werringloer (21) as a method for the quantitative detection of NO, the hemoglobin assay involves the reaction of oxyhemoglobin (HbO_2) and NO to produce methemoglobin (MetHb) and nitrate (NO_3^-) with a concomitant spectral shift:

$$\text{Hemoglobin-Fe(II)-}O_2 + NO \rightarrow \text{Hemoglobin-Fe(III)} + NO_3^-.$$

The progress of this reaction may be monitored spectrophotometrically by measuring the absorbance at 401 nm (MetHb) and at 415–421 nm (HbO_2). This analytical technique has been reviewed in detail by Noack et al. (22) and Murphy & Noack (23). The reaction is nearly diffusion limited (23, 24) and is 26 times faster than the reaction between NO and O_2 (22), allowing measurement of NO in the presence of dissolved O_2. Interference from other nitrogen oxides is not observed. Each experiment requires a separate calibration that may be conducted either via oxidation of HbO_2 with potassium ferricyanide, with an NO donor that generates a known quantity of NO, or by addition of a saturated NO solution. Quantitative results are obtained by monitoring the difference in absorbance between 401 and 411 nm. Control solutions of HbO_2 alone must be run simultaneously to account for HbO_2 autoxidation. The theoretical detection limit is 1.3–2.8 nM (22, 23). Interferences may arise from fluctuations in temperature, the presence of other heme-containing proteins, or changes in pH, each of which may influence the absorbance spectrum of MetHb. The presence of other redox-active species may also interfere with measurement, as oxidation of HbO_2 and/or reduction of MetHb can affect the assay (22). A primary drawback of this technique is the need for fresh HbO_2 reagent (23), which is obtained via time-consuming oxygenation of hemoglobin followed by chromatographic purification (25).

The hemoglobin absorbance–based technique has been used to detect NO released from exogenous NO donors (23), cultured endothelial cells (26), platelets (27), and tissue (28, 29). For example, Zhang et al. (29) coupled the hemoglobin assay with intracerebral microdialysis to measure NO levels in rat brain. The authors inserted cellulose membrane coaxial microdialysis probes (molecular weight cutoff of 5000 Da) into the hippocampus of anesthetized rats and perfused the probes with Krebs buffer containing 10 μM HbO_2. Spectral shifts in the collected microdialysate indicated increased NO production in the brain after administration of kainic acid, an excitotoxin responsible for increased NO production (29). Autoxidation of hemoglobin was minimized by maintaining the perfusate at 0°C prior to entry into the microdialysis probe. The limit of detection (LOD) for the assay was 7 nM (29). With minor modifications, microdialysis coupled with the hemoglobin assay may be useful for monitoring NO levels in a range of tissues and organs.

Recently, Boon & Marletta (30) reported a new metalloprotein–based assay for detecting NO using a mutant of *Tt* H-NOX, a bacterial heme protein with significant homology to guanylate cyclase. The researchers introduced a point mutation (Y140F) that significantly decreased the affinity of the protein for O_2 while maintaining high affinity for NO, thus improving its use under aerobic conditions. The protein, *Tt* Y140F, was found to be a cumulative trap of NO, allowing measurement of NO by monitoring the change in absorbance at 424 nm. Based on the change in extinction coefficient of the protein at 424 nm with and without bound NO, the concentration

range over which NO could be detected was 300 nM to 30 μM. The authors employed the *Tt* Y140F assay to determine the amount of NO released from murine inducible NO synthase. Notably, the *Tt* Y140F was stable at temperatures up to 70°C and tolerated salt and buffer (30).

Heme-containing proteins other than hemoglobin are also suitable for quantitative NO detection based on absorbance changes after reaction with NO. For example, Aylott et al. (31) created an optical NO sensor by trapping cytochrome *c* in a 5-μm-thick tetramethylorthosilicate (TMOS) xerogel spin-coated on a glass substrate. The protein-doped xerogel film was affixed to a gas flow-cell normal to the light path of a spectrophotometer. Absorbance measurements indicated that NO binding caused a shift in λ_{max} from 406 to 414 nm. The sensor generated a linear response to NO from 1 to 25 ppm, with an LOD of 1 ppm and a relative standard deviation of less than 1% at 10 ppm. Although no interference was observed from O_2, nitrogen gas (N_2), or carbon monoxide (CO), introduction of nitrogen dioxide (NO_2) at 10–1000 ppm did result in an interfering signal (31).

In a similar manner, Lan et al. (32) developed optical sensors for NO based on manganese myoglobin (MnMb) encapsulated in a TMOS-xerogel matrix. Specificity for NO over O_2 was achieved by employing MnMb, which, unlike native myoglobin, binds NO but not O_2 (33). The MnMb-doped TMOS xerogels were placed in aerobic solutions containing NO generated either enzymatically from NO synthase or from *S*-nitroso-*N*-acetylpenicillamine, a small-molecule NO donor. Absorption spectra recorded before and after exposure to NO indicated a change in λ_{max} from 468 nm [Mn(III)Mb] to 424 nm [Mn(II)MbNO]. Although the sensitivity and LOD of the sensor were not reported, the technique was found to be specific for NO over both nitrite (NO_2^-) and NO_3^-, but not nitroxyl or NO_2 (32).

2.1.2. Other absorbance assays with metal-based indicators. Metal-based indicators other than metalloproteins have also received attention due to their potential for NO detection. For example, Dacres & Narayanaswamy (25, 34, 35) have developed optical NO sensors based on absorbance changes of Cu(II) complexes upon exposure to NO. Initial studies focused on a copper–eriochrome cyanine R complex [Cu(II)-ECR], which in the absence of NO exhibited a λ_{max} of 445 nm in buffer. Upon exposure to NO, the absorbance at 445 nm decreased, with a concomitant increase in absorbance at 569 nm due to reduction of the complex from Cu(II)-ECR to Cu(I)-ECR (25). To use this finding to develop an optical sensor for NO, the authors immobilized the Cu(II)-ECR complex in a silicone rubber membrane and cast it as a film into a gas flow-cell equipped with a fiber optic–based spectrometer. The immobilized complex exhibited spectral characteristics different from those observed in solution: NO exposure resulted in a λ_{max} shift from 580 to 610 nm, and there was a decrease in absorbance. Nevertheless, the optical sensor proved suitable for monitoring NO gas with an LOD of 0.23 ppm NO and a linear response up to 6 ppm (25). The authors have since developed an improved sensor by immobilizing the Cu(II)-ECR complex in an anion-exchange membrane (35). Although the LOD for the new immobilization strategy improved to 0.848 ppm NO, the sensitivity increased almost fivefold above that of the silicone rubber–based sensor. The response of the anion-exchange sensor was also reversible and selective for NO over O_2, NO_2, and CO (35).

2.1.3. Diazotization assay (Griess reaction). One of the most common methods for detecting NO from a wide variety of samples and matrices is the diazotization assay, also known as the Griess assay. First described by Griess in 1864 (36), the diazotization assay actually measures NO_2^-. Fortunately, NO's reactivity results in the formation of NO_2^- in oxygenated media via the

following reactions:

$$2NO + O_2 \rightarrow 2NO_2,$$
$$NO + NO_2 \rightarrow N_2O_3, \quad \text{and}$$
$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+.$$

Review articles describing the procedures for detecting NO via the Griess reaction and the assay's application to biological samples are available (37, 38). Nevertheless, we provide a brief description of the fundamentals of this NO-measurement technique.

The reaction developed by Griess involved reacting NO_2^- with sulfanilic acid and α-naphthylamine under acidic conditions to yield an azo dye, whose concentration could then be used as an indirect indicator of NO_2^- (and NO) concentration in the sample (38). The assay was later modified (39) by substituting sulfaniliamide (SA) and *N*-(1-naphthyl)ethylenediamine (NED) for sulfanilic acid and α-naphthylamine, respectively. This modified assay, which is the procedure widely used today, resulted in greater sensitivity and reproducibility as well as more rapid analysis times (38). As shown in **Figure 1**, this modern version of the Griess reaction involves first reacting NO_2^- with SA under acidic conditions to form a diazonium salt intermediate (37, 38). The diazonium salt intermediate is then coupled to NED to form the stable water-soluble azo dye ($\lambda_{max} \approx 540$ nm). The NO_2^- concentration is determined by comparing the absorbance

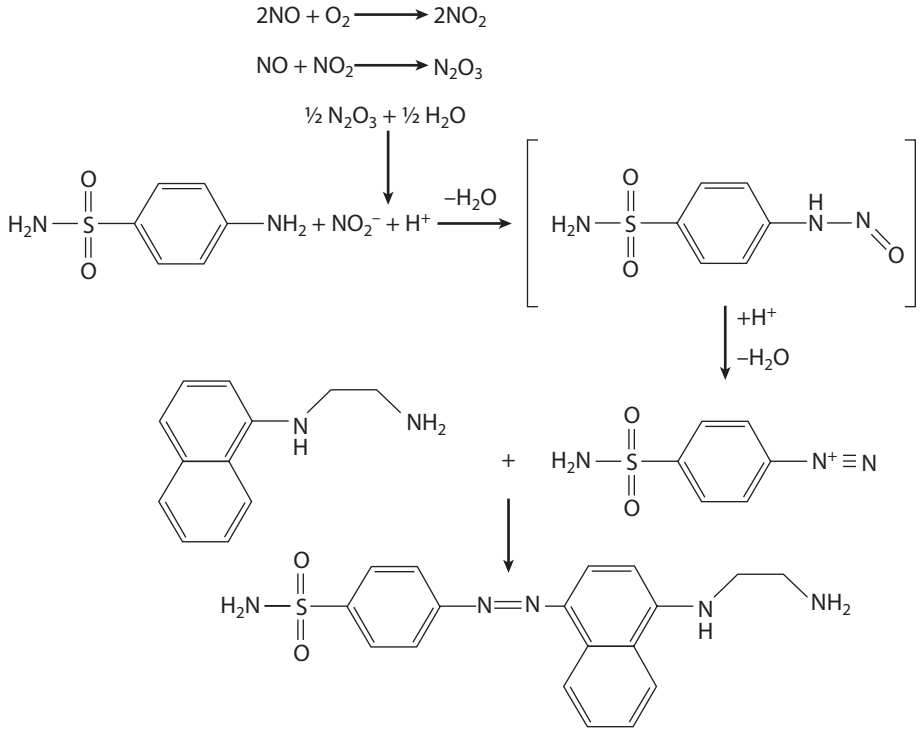

Figure 1

The most commonly employed diazotization reaction (also known as the Griess assay). Under aerobic conditions, nitric oxide (NO) reacts to form nitrite (NO_2^-), which reacts with sulfanilic acid to form a diazonium salt intermediate. The diazonium salt is then coupled to *N*-(1-naphthyl)ethylenediamine to form the stable water-soluble azo dye ($\lambda_{max} \approx 540$ nm).

of the azo dye solution to a calibration curve prepared with known concentrations of NO_2^-. The LOD for NO_2^- via the Griess assay is approximately 0.5 μM (37). Because the Griess assay detects a by-product of NO (i.e., NO_2^-), it is not suitable for monitoring NO in real time. Furthermore, careful control experiments must be performed to distinguish basal NO_2^- levels from those that arise from actual changes in NO concentration.

Variations in the assay exist due to the multistep nature of the reaction. In some cases, the sample is incubated with hydrochloric acid (HCl) and SA for 10 min, followed by addition of NED and further incubation (30 min) before measuring the absorbance (40). One can also add the sample directly to an acidic solution containing both SA and NED. However, most commercially available kits require the sample to be mixed with SA, then for NED to be added immediately. This has been reported to be the most sensitive method of NO_2^- determination (37).

In addition to NO_2^-, NO may also react in oxygenated solutions to yield NO_3^- via the following reactions:

$$2NO + O_2 \rightarrow 2NO_2,$$
$$2NO_2 \rightarrow N_2O_4, \quad \text{and}$$
$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+.$$

As a competing reaction that lowers the amount of NO_2^- produced from NO, NO_3^- formation represents a significant interference for quantitative determination of NO via the Griess assay. Fortunately, the reactions that culminate in NO_3^- production are slower than those leading to NO_2^- (40); thus, NO_2^- is considered the major NO by-product in oxygenated solutions. Nevertheless, because the Griess assay does not detect NO_3^-, it may be necessary to first reduce any present NO_3^- to NO_2^- for complete analysis. Methods for NO_3^- reduction (to NO_2^-) include treatment with chemical reductants such as cadmium, zinc, and vanadium chloride. Enzymatic reduction may also be employed via NO_3^- reductase obtained from bacteria (41). However, the enzymatic approach requires NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) as a cofactor; this compound has been shown to interfere with the Griess assay (42).

As a common method for measuring NO in biological systems, applications of the Griess assay are too numerous to discuss in detail here. Rather, we direct the interested reader to a recent review describing applications of the Griess assay for elucidating the mechanisms of NO's action in physiology (38) and to research articles that describe using the Griess assay to measure NO_2^- as a proxy for NO in urine (43), cerebrospinal fluid (44), plasma (45), serum (46), and blood (47). The Griess assay has also been used to quantify solution NO release from NO donors such as linsidomine (48), sodium nitroprusside (49), poly(ethylene glycol)-lysine dendrimers (50), and self-assembling diazeniumdiolate-based nanofiber gels (51).

Analytical applications of the Griess assay continue to expand with the development of new instrumentation for measuring NO based on the Griess reaction. Several reports describe absorbance-based flow-injection analysis systems for the measurement of NO via the Griess reaction (52–54). For example, Higuchi & Motomizu (54) describe a system in which the carrier solution (with sample) first passes through an online copperized cadmium reduction column to reduce NO_3^- to NO_2^-. The NO_2^- then reacts with SA and NED in a heated (50°C) reaction column. Using this system, the authors determined NO_2^- levels in urine, serum, plasma, and cell culture samples. A 50-nM LOD was reported, with linear calibration responses up to 10 μM NO_2^-.

Likewise, Toda et al. (55) developed new instrumentation for monitoring gaseous NO via the Griess reaction. They developed a microgas-analysis system that operates by passing a gas-absorbing solution (3% aqueous triethanolamine) through a microchannel scrubber formed on

polydimethylsiloxane coated with a porous poly(tetrafluoroethylene) (PTFE) membrane. After collecting sample from the scrubber, the gas-absorbing solution was reacted with Griess reagent and was passed through a reaction coil maintained at 50°C. The solution was then introduced to a 1-cm-pathlength detector with a 525-nm LED and a high-sensitivity photodiode. The LOD of the NO-microanalysis system was 7 ppb, with a linear response to 1 ppm, although NO concentrations as high as 50 ppm may be determined with a multipoint calibration. Such Griess assay–based instruments may prove useful for both environmental monitoring and medical applications (55).

2.2. Fluorescence-Based Measurements

A number of fluorescence-based probes have been developed for the detection of NO, and a recent review (56) highlights the mechanisms by which they operate and their utility in biological applications. Fluorescence-based probes are particularly useful for monitoring the spatial and temporal aspects of NO production. Most probes exhibit little or no fluorescence until they react with a by-product of NO decomposition (e.g., N_2O_3) upon which time they become highly fluorescent. Exceptions to this mechanism of action are fluorescent nitric oxide cheletropic traps (FNOCTs). FNOCTs are *o*-quinodimethane derivatives that react directly with NO to produce a nitroxide radical product with significantly different fluorescence properties (e.g., intensity and/or wavelength) from those of the FNOCT. Because their fluorescence does not depend on a by-product of NO decomposition, FNOCTs can unambiguously indicate the presence of NO (56, 57). Meineke et al. (58) employed FNOCTs both to measure NO release from cultured macrophages and to image intracellular NO production by endothelial cells via fluorescence microscopy. Similar fluorescent probes that react directly with NO include metal-based compounds synthesized by Lim & Lippard (59). Such probes include Co(II), Fe(II), Ru(II), Rh(II), and Cu(II) compounds that are directly sensitive to NO, not to an oxidation by-product (59). The detection limit of the Cu(II)-fluorescein complexes is roughly 5 nM (60), and these complexes are suitable for visualizing NO production from both human neuroblastoma cells and murine macrophages (61).

Diaminofluoresceins (DAFs), first described by Kojima et al. (62, 63) in 1998, are among the most widely employed NO-sensitive fluorescent probes because of their utility for cellular imaging. Upon reaction with a by-product of NO (i.e., N_2O_3), the fluorescence intensity of the DAF probes increases by approximately 100-fold (63). In addition to having a visible excitation wavelength similar to that of fluorescein (an advantage for cellular applications), a diacetate-derivatized form of DAF (DAF-2 DA) allows for efficient uptake by cells. Hydrolysis of the acetate bonds by intracellular esterases results in a trapped nonpermeable form of the probe (DAF-2) (56, 63). The DAF probes show no fluorescence in the presence of common interferents for NO such as NO_2^-, NO_3^-, hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), and they have a detection limit as low as 5 nM (63). DAF probes have been used to quantify changes in NO production in stimulated perfused rabbit hearts (64) and to image NO production in living zebrafish (65).

DAF probes have also been coupled with analytical techniques such as flow cytometry for analysis of NO production by cells. For example, Stirjdom et al. (66) used DAF-2 DA and flow cytometry to monitor intracellular NO in cardiomyocytes either treated with diazeniumdiolate-modified diethylamine (DEA/NO), an NO donor, or subjected to simulated ischemia (hypoxia). In a similar study, Havenga et al. (67) combined DAF-2 DA with flow cytometry to measure NO in human lung and liver epithelial cells. The authors compared the amount of NO assayed from the DAF/flow-cytometry experiment to NO measured both by citrulline assay [i.e., measuring L-arginine conversion (68)] and by reducing NO_2^- and NO_3 to NO, followed by chemiluminescent NO detection (see Section 2.3). Good correlation was observed between the assays, thereby validating the DAF/flow-cytometry approach with established methods of cellular NO detection.

Notably, incubation of cells with higher concentrations of DAF-2 DA (250 μM) increased the sensitivity of the assay. However, DAF-2 DA concentrations higher than 10 μM have been found to be toxic to some cells (67).

Fluorescent dyes have also been coupled with both HPLC and capillary electrophoresis (CE) for the sensitive detection of NO. For example, Huang et al. (69) developed a technique in which NO was reacted with 1,3,5,7-tetramethyl-8-(3′,4′-diaminophenyl)difluoroboradiaza-*s*-indacene (TMDABODIPY) prior to injection onto a nonpolar octadecylsilane (C_{18}) HPLC column. Using fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 498/507 nm), baseline separation between reacted and unreacted TMDABODIPY was observed within 4 min, with a linear response to NO and an LOD of 0.8–800 nM and 20 pM, respectively. This method was used to measure NO in serum of patients with ischemic cardiocerebrovascular disease (69). In an improvement over the original procedure, a solid-phase microextraction method was employed to preconcentrate the TMDABODIPY-derivatized NO prior to HPLC analysis (70). Use of a poly(methacrylic acid–ethylene glycol dimethacrylate) monolithic extraction column improved the LOD to 2 pM and allowed for the measurement of NO in rat heart, kidney, and liver samples (70).

CE has been employed to improve the selectivity of DAF probes for NO over common intracellular interferents (71). Intracellular ascorbic acid is known to reduce levels of N_2O_3, the NO reaction by-product that generates the fluorescent form of DAF (63). Moreover, dehydroascorbic acid reacts with DAF to yield a product with fluorescence properties similar to those of the desired DAF reaction product with N_2O_3 (72). To minimize the interfering effects of both intracellular interferents, Kim et al. (71) employed ascorbate oxidase to catalyze the oxidation of ascorbic acid to dehydroascorbic acid and water, thus removing the N_2O_3 scavenger (i.e., ascorbic acid). CE was then employed to separate the DAF–N_2O_3 reaction product from the fluorescent DAF–dehydroascorbic acid product. The CE method allowed for the successful measurement of NO from *Aplysia californica* metacerebral cells, effectively separating the DAF–N_2O_3 reaction product from the unidentified neuron-specific interferents.

The inherent fluorescence of select proteins is also sensitive to NO. To exploit this property, Barker et al. (73) used the fluorescence attenuation of cytochrome *c′* upon NO binding to create NO-sensitive optical sensors. Upon NO binding, the fluorescence intensity of *Chromatium vinosum* cytochrome *c′* decreased and exhibited a ∼10-nm blueshift ($\lambda_{excitation}$ = 530 nm). Sensors were fabricated by photopolymerizing a 9:1 (w/w) (acrylamide):(*N*,*N*-methylenebisacrylamide) solution with *C. vinosum* cytochrome *c′* onto multimode optical fibers. Using 10-mW 514.5-nm Ar^+ laser excitation, a linear response to NO was observed at concentrations up to 1 mM. Following an irreversible response thought to arise from NO's reaction with the polymer matrix (73), the authors coated the sensor with a layer of gold colloid, onto which cytochrome *c′* was adsorbed via protein amine groups. The sensors exhibited full reversibility with an LOD of 20 μM and a linear response to 1 mM, with a second linear response region higher than 1 mM. A response time of ∼0.9 s was reported, with minimal interference from NO_2^-, NO_3^-, O_2, N_2, or ascorbic acid (73).

In addition to protein-based sensors, optical NO sensors have also been developed based on synthetic NO-sensitive fluorescent probes. For example, Barker & Kopelman (74) fabricated fluorescence-based NO sensors by immobilizing 4-carboxy-2′,7′-difluorofluorescein (Oregon Green® 488) dye onto the 50-nm gold colloid prior to coating the optical fibers. Although Oregon Green 488 in solution is insensitive to NO, when immobilized on gold its fluorescence intensity is attenuated upon NO coadsorption to the gold surface. Carboxylate-modified polystyrene fluorescent microspheres (40 nm in diameter), which are insensitive to NO, were also incorporated as part of the sensor, allowing the ratiometric measurement of NO by normalizing the change in Oregon Green 488's fluorescence intensity to that of reference microspheres. The

analytical response of such sensors was characterized by a 20-μM NO LOD, a linear response up to 1 mM NO, and a <0.25-s response time (0–100% response). Although no interference was observed from NO_2^- (1 M), NO_3^- (1 M), 100% O_2, or H^+ at pH > 6, slight (≤ 4% signal change) interference was observed from H_2O_2 (18 μM), superoxide (3 μM), and $ONOO^-$ (6 μM). The Oregon Green 488/colloidal gold sensors were used to monitor NO release from resting and stimulated murine macrophages by placing the sensor ∼0.5 cm from cultured cells. An increase in NO production (from < 20 to 190 ± 70 μM) was measured upon stimulation of the macrophages with interferon-γ and lipopolysaccharide (74). Similar fluorescence-based NO sensors were also fabricated by labeling cytochrome c' with Oregon Green 488 prior to attaching the protein/label conjugate to gold colloid–coated fibers that were coimmobilized with reference polystyrene microspheres (75). The analytical response of the dual protein/dye sensor was similar to that of the previously developed protein- and dye-only sensors; however, the latter demonstrated an improved LOD (8 μM NO) (75).

2.3. Chemiluminescence

Two types of chemiluminescent reactions have been employed to selectively detect NO. The first approach is based on the reaction of NO with ozone (O_3) in a reaction cell to produce excited-state nitrogen dioxide (NO_2^*), which emits a photon upon relaxation to the ground state:

$$NO + O_3 \rightarrow NO_2^* + O_2,$$
$$NO_2^* \rightarrow NO_2 + h\nu.$$

As described by Bates (76), the O_3-based chemiluminescent reaction is highly specific for NO, although NO_2^- and NO_3^- may be measured upon their reduction to NO. The emitted light is measured with a photomultiplier tube (PMT) with an intensity proportional to the amount of NO in the reaction cell. Although interferences may arise due to chemiluminescence from the reaction of O_3 with species such as ethylene hydrocarbons, sulfur compounds, and carbonyls, the wavelength of chemiluminescence effectively discriminates among such interferents. Indeed, ethylene reaction with O_3 results in chemiluminescence at ∼440–470 nm, whereas sulfur compounds result in chemiluminescence at <400 nm. In contrast, the NO/O_3 reaction emits light at wavelengths greater than 600 nm, allowing the use of a simple filter to remove undesirable signal and to impart complete selectivity for NO (76). The most commonly employed O_3-based chemiluminescence NO detectors (**Figure 2**) use an inert gas (e.g., Ar or N_2) to deoxygenate the sample solution and carry gaseous NO into a reaction cell, where it reacts with O_3 generated from O_2 either directly from air or from a dedicated oxygen tank. The major advantage of employing a carrier gas is that the most troublesome interferents (e.g., NO_2^- and NO_3^-) are not transferred from the sample vessel to the reaction cell; thus, NO selectivity is enhanced. However, this measurement mode is susceptible to changes in the flow rate of the carrier gas and is only suitable for detecting gaseous NO; therefore, this method is incompatible with many biological experiments (76). The LOD for solution-based samples is in the nanomolar-to-picomolar range (depending on the sample volume) (76), with a linear response to the millimolar range (77). For gas samples, the detection limit is 0.5 ppb, and the analysis limit is up to ∼500 ppm (77). The analysis range for solution shows excellent sensitivity, and near-real-time monitoring of NO has resulted in widespread use of chemiluminescence for NO detection. Indeed, several reports have described the use of O_3-based chemiluminescence for measuring NO from NO-releasing materials such as silica nanoparticles (78), nitrosothiol-modified dendrimers (79), and xerogel polymers (80), as well as from biological samples such as human arterioles (81) and exhaled breath (82).

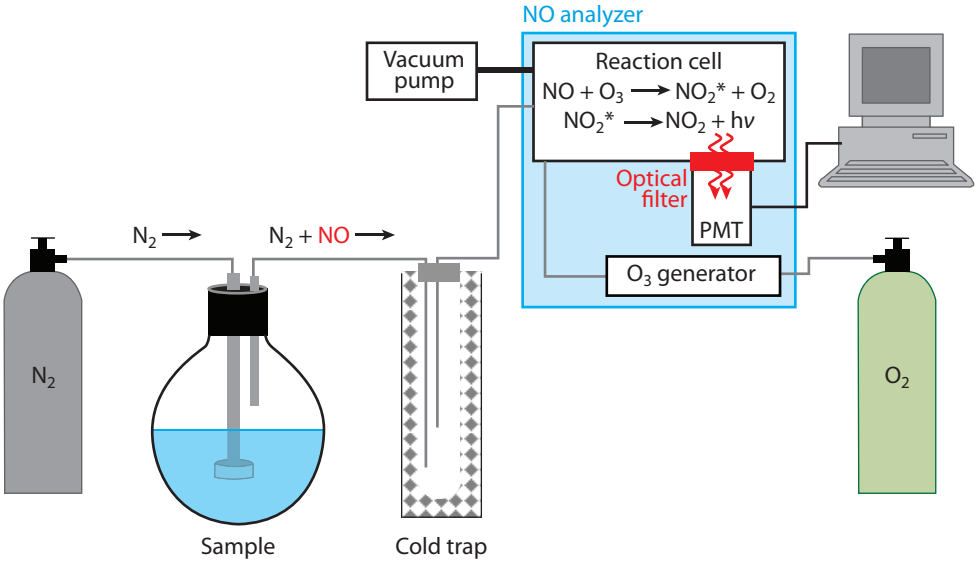

Figure 2

Schematic diagram of a chemiluminescence-based nitric oxide (NO) analyzer. An inert gas [e.g., nitrogen (N_2)] is used both to deoxygenate sample buffer and to carry NO from the sample flask through a cold trap (to remove water vapor) into a reaction cell within the NO analyzer. In the reaction cell, NO reacts with ozone (O_3) to form excited-state nitrogen dioxide (NO_2*), which emits a photon (i.e., chemiluminescence) upon its relaxation to the ground state (NO_2). Emitted light passes through an optical filter and is detected by a photomultiplier tube (PMT).

The second method of chemiluminescent NO detection, first described by Kikuchi et al. (83), is based on the reaction of NO with H_2O_2 to form $ONOO^-$, which subsequently reacts with luminol to yield characteristic chemiluminescence. In the authors' initial assay, samples containing NO were added to luminol solution (30 μM) premixed with H_2O_2 (10 mM). A linear calibration curve was obtained for NO concentrations from 100 fM to 1 nM. Possible interferents such as NO_2^- and NO_3^- gave no chemiluminescent signal, but the addition of NO_2 resulted in a small signal (<5% of the signal obtained from NO) (83). Coupled with a flow-cell-type detector, the authors used luminol chemiluminescence to measure NO from a perfused rat kidney and to correlate NO levels with arterial perfusion pressure (83).

The luminol/H_2O_2 chemiluminescence approach has also been employed to create analytical sensors for NO. For example, Robinson et al. (84) developed a chemiluminescence-based technique for measuring NO in exhaled breath. Porous polypropylene hollow fiber membranes were used to channel gaseous NO into a solution of luminol and H_2O_2, from which chemiluminescence was detected with a miniature PMT. The instrument had an LOD of 0.3 ppb. The authors suggested that the device may be useful for monitoring NO as a diagnostic marker for asthma or respiratory infections (84). In similar work, Zhou & Arnold (85) developed a luminol-based chemiluminescence fiber optic sensor for measuring NO in solution. A solution containing luminol and H_2O_2 was trapped at the end of a fiber optic probe with a gas-permeable membrane (nonvulcanized matte-matte silicone sheeting). Light emission from the chemiluminescent reaction was transferred through the fiber to a PMT for detection. The optical sensor had an LOD of 1.3 μM NO, a linear response to 40 μM, and a response time of 10 to 17 s. Physiological substances that reduced ambient oxygen increased the response of the sensor by inhibiting the oxidation of NO. Both thiols and CO also significantly interfered with the sensor's response to NO (85).

2.4. Electron Paramagnetic Resonance Spectroscopy

EPR spectroscopy (also known as electron spin resonance spectroscopy) has proven particularly useful for detecting NO in complex biological matrices such as tissues and organs. Radical species with unpaired electrons such as NO are readily observed in magnetic fields based on the absorption of electromagnetic radiation in the microwave region that induces resonance between parallel and antiparallel orientations relative to the applied magnetic field (86). The EPR signal is typically reported as the first derivative of the absorption spectrum. Modern EPR instruments fix the microwave frequency and scan the magnetic field strength until resonance between the parallel and antiparallel states is reached and a signal is observed. As with nuclear magnetic resonance spectroscopy, atoms with a magnetic moment that neighbor the atom with the unpaired electron influence the EPR signal via hyperfine coupling. Both qualitative and quantitative information can be obtained from EPR.

As a free radical with an unpaired electron, NO is readily monitored via EPR spectroscopy. Because EPR is only sensitive to free radical species, EPR as an NO-measurement method is highly selective over common interferents such as NO_2^- and NO_3^-. However, because NO is characterized by a short half-life and because it rapidly reacts to form nonparamagnetic species, it is necessary to "trap" NO and convert it to a more stable radical species for monitoring. "Spin trapping" involves reacting a free radical with a spin trap reagent to form a stable adduct that may be monitored via EPR:

$$R^* + ST \rightarrow R\text{-}SA^*,$$

where R* is the radical to be monitored, ST is the spin trap, and R-SA* is the stable radical trap adduct (86). A review by Kleschyov et al. (86) describes the measurement of NO via spin trapping and EPR. Both endogenous and exogenous spin traps exist for NO detection, and both have been employed for monitoring NO in biological applications (86). In the following sections, we discuss representative examples of EPR and spin traps for monitoring NO and the information obtainable from such analyses.

2.4.1. Endogenous spin traps. Of the endogenous NO spin traps, hemoglobin has proven particularly useful. Although the major reaction products of HbO_2 and NO are MetHb and NO_3^- (the basis of the hemoglobin assay for NO described above in Section 2.1.1), approximately 10% of the HbO_2 is converted to a stable HbNO adduct. Dikalov & Fink (87) reported the use of HbNO to monitor NO via EPR in both whole blood and erythrocytes. They performed calibration by incubating washed erythrocytes with known amounts of either NO_2^- or diazeniumdiolate NO donor. This EPR technique was then employed to monitor HbNO levels in mice fed low- and high-NO_2^-/NO_3 diets. In a similar study, Hall et al. (88) used ex vivo EPR analysis of HbNO and MetHb (also paramagnetic) combined with Griess analysis to conclude that hyperthermia in rats results in increased production of NO at levels similar to those observed during septic shock and endotoxin administration.

Alternative endogenous spin traps for NO include other metalloproteins in tissue such as MnMb, cytochrome *c*, catalase, and cyclooxygenase (86). Although NO-heme complexes are paramagnetic and are detectable by EPR, O_2- and CO-heme complexes are diamagnetic and thus are not observable by EPR, representing yet another aspect of EPR selectivity for NO (89). Using heme proteins as endogenous spin traps, Kuppusamy et al. (90) employed EPR to both measure and image NO generation in mice subjected to cardiopulmonary arrest. As expected, increased NO production was observed in the mice suffering cardiopulmonary arrest. The authors determined the mechanism of NO production by administering ^{15}N-labeled nitrite (i.e., $^{15}NO_2^-$) to the mice

(as NO derived from $^{15}NO_2^-$ may be distinguished from enzymatically generated NO via EPR analysis). The majority of NO was produced nonenzymatically from the acidic reduction of tissue NO_2^-. Double integration of the EPR signal from individual organs allowed the location of NO production to be determined, with the most NO produced in the lungs and heart. Whole-body imaging obtained from EPR spectra confirmed the spatial distribution of NO production in the lungs, heart, and liver (90).

2.4.2. Exogenous spin traps. The most common exogenous NO spin traps are iron-dithiocarbamates, which have proven particularly useful for monitoring NO in cells and tissues (86, 91) including animal tumors (92), kidneys (93), mouse organs (94, 95), and plants (96). As with endogenous heme protein spin traps, iron-dithiocarbamates also allow imaging of NO production in live animals (97). Using iron-dithiocarbamates to facilitate EPR detection of NO has been the subject of several reviews (98, 99). This approach exploits NO's high affinity for the Fe center of the spin trap. The general structure of iron-dithiocarbamates is $Fe(S_2CN\text{-}RR')_2$, where R and R′ can be methyl-, ethyl-, glucamine-, sarcosine- (86, 97, 100–102), or amino-acid substituents such as proline or serine (103–105). The identity of the substituents dictates the regions in animals or tissues in which NO may be most readily detected. For example, the lipophilic Fe-diethyldithiocarbamate accumulates in hydrophobic membranes and therefore is suitable for detecting NO in tissue. In contrast, the more hydrophilic Fe-*N*-methyl-D-glucamine dithiocarbamate remains in the extracellular fluid and thus is more useful for measuring NO outside cells (86). The detection limit of NO using exogenous spin traps is roughly 6 pmol [from a study using rat thoracic aorta sample (106)].

3. ELECTROCHEMICAL TECHNIQUES

Electrochemical sensors are likely the most commonly employed analytical method for monitoring NO in physiology/biology due to their specific inherent advantages, which include real-time monitoring, amenability to miniaturization, and the ability to enhance selectivity and sensitivity via electrode modification and/or applied potential (107). Moreover, electrochemical detection of NO affords excellent spatial resolution, with extraordinarily low LOD. Indeed, porphyrinic-based NO microsensors have been used to detect as little as 10^{-20} mol of NO in single cells (108, 109). Although electrochemical reduction of NO may be employed for NO detection (107, 110–113), it is often plagued by the threat of interference from O_2, whose reduction occurs more readily than that of NO (107, 114). As a result, most electrochemical NO sensors are based on the oxidation of NO to NO_2^-. Unfortunately, the relatively high working potential required to oxidize NO [+0.7 to 0.9 V versus Ag/AgCl, depending on the type of electrode (115)] may lead to interference from other redox-active species that are common to physiological milieus, such as NO_2^-, ascorbic acid, uric acid, dopamine, and CO. Thus, bare electrodes are often modified to reduce interference and to facilitate selective detection of NO. The two primary modifications are (*a*) coating the electrode with a membrane that selectively allows the diffusion of gases while excluding other interferents and (*b*) modifying the electrode by immobilization of an electrocatalytic compound such that the potential necessary to oxidize NO is lowered compared to that of the interferents.

The Shibuki NO sensor is an early example of a membrane-coated electrochemical NO sensor (116). As shown in **Figure 3*a***, a Teflon©-coated platinum (Pt) working electrode and a Pt reference electrode were placed in a glass micropipette filled with NaCl/HCl solution prior to sealing one open end of the pipette with NO-permeable chloroprene rubber. NO was detected via oxidation at +0.8 to +0.9 V versus a Ag/AgCl reference electrode. The response of the sensor was linear from 1 to 3 μM NO, with a sensitivity of 28.3 ± 4.4 pA μM^{-1}. Shibuki (116) first used the sensor

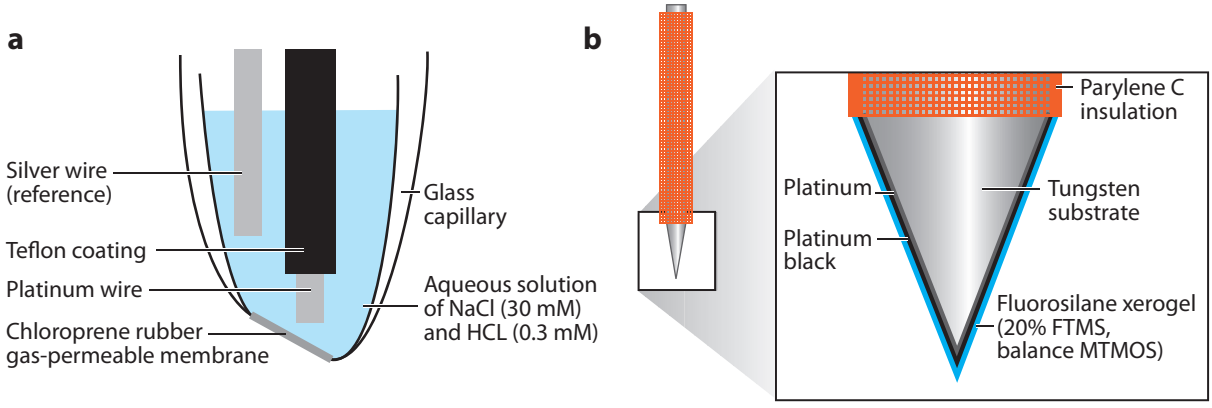

Figure 3

Diagrams of (*a*) a Shibuki-style nitric oxide (NO) sensor (116) and (*b*) a fluorosilane xerogel–coated microelectrode-based electrochemical NO sensor. Diagrams are not to scale. The external reference electrode is not depicted in panel *b*. Abbreviations: FTMS, (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane; MTMOS, methyltrimethoxysilane.

to monitor NO generation from sodium nitroprusside (a chemical NO donor) and from electrically stimulated rat cerebellar slices. Several permselective membranes have since been employed to fabricate electrochemical sensors with analytically useful selectivity. Examples of such membranes include cellulose acetate (117), collodion/polystyrene (118), polycarbazole (119), Nafion© (120), polyeugenol (121), polydimethylsiloxane (122), PTFE (123), and phenylenediamine (124).

The first use of an electrocatalytic modification to reduce the potential necessary to oxidize NO was reported by Malinski & Taha in 1992 (108). They applied a p-type semiconducting nickel porphyrin film to a thermally sharpened carbon fiber microelectrode prior to coating the electrode with Nafion. The oxidation potential of the sensor for NO was lowered to approximately +0.65 V, a clear indication of the catalytic properties of the Ni porphyrin (107, 108). The sensor had an LOD of 10 nM NO and responded linearly to NO up to 300 μM, allowing the determination of NO release from single porcine aorta endothelial cells (108).

Other approaches for electrocatalytically lowering the oxidation potential of NO include modifying electrodes with metalloporphyrins (125), metal phthalocyanines (126, 127), and other organometallic compounds with Ni, Fe, Co, Cu, and Mn centers (128–130). A review highlighting sensors fabricated using both permselective membranes and electrocatalytic modifications was published in 2003 (131). The advances discussed herein represent more recent work and new strategies designed to improve selectivity over interferents.

3.1. Platinization and Permselective Membranes for Enhancing the Response of Electrochemical Nitric Oxide Sensors

Many publications describe the use of polymeric sensor membranes to improve selectivity for NO over common interferents. For example, Lee et al. (132) designed an amperometric NO sensor using a platinized Pt electrode coated with a microporous PTFE gas-permeable membrane. The process of platinization has been employed since the nineteenth century (133) to improve electrochemical sensor response characteristics such as sensitivity and LOD by increasing surface area and enabling faster electron-transfer kinetics between the electrode and analyte (132). Lee et al. platinized Pt working electrodes in a 3% chloroplatinic acid solution and placed them with a coiled Ag wire reference electrode in a glass micropipette. A PTFE gas-permeable membrane

was affixed to the distal end of the sensor with an O-ring. The effect of platinization on the oxidation potential of NO was determined via linear sweep voltammetry. Electrode platinization was found to increase the sensor response (i.e., current) for both NO and NO_2^- approximately eightfold above that of unplatinized electrodes. Furthermore, the platinized electrodes enabled faster electron-transfer kinetics for both NO and NO_2^-, allowing the oxidation potential to be reduced to +0.75 V while maintaining the same sensitivity to NO as sensors operated at +0.9 V. Platinization also improved the lifetime of the sensor, as the platinized sensors exhibited no change in response (within ± 10%) after 3 days. However, the sensitivity of the nonplatinized sensors was reduced by approximately 50% after only 3 h. The response time of the sensor (i.e., the time required to reach 90% of steady-state response when NO was changed from 10 to 400 nM) was 14–25 s, whereas the LOD was 1 nM NO. Furthermore, the PTFE membrane resulted in complete selectivity over NO_2^- up to 10-mM NO_2^- concentrations (132). Unfortunately, ammonia (NH_3) was found to interfere with sensor response (134). To overcome this interference, the PTFE-based sensor was further modified by applying a Teflon AF® [2,2-bis(trifluoroethylene)-4,5-difluoro-1,3-dioxole] coating over the PTFE membrane. The Teflon AF layer resulted in a ∼1000-fold improvement in sensor selectivity for NO over NH_3 (134).

To reduce the size of the NO sensors, the Schoenfisch research group (115, 135) developed xerogel-based permselective membranes. Sol-gel-derived materials (i.e., xerogels) are formed via the hydrolysis and condensation of alkoxysilanes under relatively mild synthetic conditions. Such materials are characterized by unique chemical versatility based on precursor selection and the ability to adhere to many substrates (115). In the first report of xerogel-based electrochemical NO sensors, Shin et al. (115) employed amino- and alkyl-alkoxysilanes as xerogel precursors and an NO templating strategy following the formation of the xerogel. The optimum membrane composition, based on permeability to NO and selectivity to NO over NO_2^-, consisted of (aminoethylaminomethyl)-phenethyltrimethoxysilane [20% v/v, balance methyltrimethoxysilane (MTMOS)] doped with 17% (v/v to total silane) Nafion. The Nafion enhanced both permeability and selectivity for NO over NO_2^- and other interferents. The selectivity coefficients ($\log K_{NO,j}$) of the resulting NO sensor were -5.8, <-6, <-6, and <-6 for j = NO_2^-, ascorbic acid, uric acid, and acetaminophen, respectively. Other notable sensor-performance characteristics included a response time (i.e., $t_{95\%}$ 400–500 nM NO) of 9 s, an LOD of 25 nM, a sensitivity of 0.17 pA nM^{-1}, and a linear response to 15 μM (115). Notwithstanding, the membrane-conditioning steps prior to NO measurement were cumbersome.

To simplify the sensor-fabrication protocol, Shin et al. (135) replaced the amino-alkoxysilane precursor with fluoroalkoxysilane, a PTFE-like polymer precursor. In contrast to previously developed PTFE-based sensors (132, 134), the fluorinated xerogels provided a more straightforward and reproducible method for coating the working electrode. Indeed, a benefit of sol-gel chemistry is the ability to dip-coat thin layers of xerogel onto electrodes. The optimal membrane composition consisted of a ∼10-μm-thick (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17% FTMS, balance 20% MTMOS) xerogel film, resulting in a more hydrophobic membrane with a water contact angle of approximately 104°. The NO selectivity ($\log K_{NO,j}$) of sensors constructed with such membranes over common interferents was -1.33, -5.74, and -5.84 for j = CO, NO_2^-, and ammonia/ammonium, respectively, and <-6 for j = ascorbic acid, uric acid, acetaminophen, and dopamine (135). The NO permeability ($\Delta I_{xerogel}/\Delta I_{bare}$) of the fluorinated xerogel membrane was significantly enhanced compared to the aminosilane-based xerogel membranes (0.72 versus 0.1, respectively, for 10 μM NO) (115). As shown in **Figure 3*b***, NO microsensors were subsequently fabricated by coating platinized Pt/W microelectrodes with the optimized fluorinated xerogel membrane. The analytical performance of such sensors included an LOD of 83 pM NO, a response time of <3 s, a sensitivity of 7.91 pA nM^{-1}, and a linear

response to 4 μM. Furthermore, the sensors maintained >90% of their initial sensitivity after storage for 20 days in phosphate-buffered saline at room temperature. In total, the performance of the fluorosilane-based NO sensors surpassed that of many previously developed miniaturized NO sensors (135). Notably, the Ag/AgCl reference electrode was separate from the working electrode, in contrast to the larger Shibuki-based devices.

3.2. Electrocatalytic Sensor Modifications

In addition to developing permselective membranes, research has focused on modifying working electrodes electrocatalytically to facilitate more efficient oxidation of NO. Fabricating NO sensors modified with Ni- and Co-phthalocyanines has been of particular interest due to these sensors' improved selectivity for NO (126, 127). Notably, the conditions under which metallophthalocyanines are applied to electrodes greatly affect the analytical performance of the resulting sensor. Oni et al. (136) sought to understand the influence of pH, temperature, solution ionic strength, and the type of ions present in solution on the performance of the resulting sensor. The authors deposited Co(II) phthalocyanine onto glassy carbon electrodes by either electrodeposition or drop-casting. They then acquired differential pulse voltammetry data using a 40-μM NO solution. Although both electrode modification procedures reduced the potential required to generate an oxidation current, electrodeposition resulted in greater sensitivity (i.e., current) at a lower potential (~790 and ~900 mV versus Ag/AgCl reference for electrodeposition and drop-casting, respectively). It is likely that electrodeposition generates a more highly ordered layer compared to drop-casting, facilitating the electron-transfer process between adjacent Co(II) phthalocyanine molecules (136).

The effect of changing the pH of the metallophthalocyanine deposition solution has also been investigated. Ni(II) phthalocyanine tetrasulfonic acid tetrasodium salt deposited at pH 1, 4, 7, and 10 resulted in peak oxidation potentials for NO of 1040, 939, 822, and 868 mV (versus Ag/AgCl), respectively. Deposition pH also affected the sensitivity of the sensor, with the greatest peak current obtained from electrodes modified at pH 7. In a similar study, Hrbáč et al. (129) optimized the conditions under which Ni(II) tetrakis(3-methoxy-4-hydroxyphenyl) porphyrin was deposited onto carbon fiber electrodes. The optimized membrane-deposition procedure resulted in an NO sensor with an LOD of 1.5 nM and with high selectivity over NO_2^-, ascorbate, and dopamine (129).

3.3. Other Advances in Electrochemical Nitric Oxide Detection

The high affinity of NO for heme proteins has been coupled with electrochemical detection to create heme protein–based electrochemical NO sensors (137, 138). For example, Fan et al. (137) fabricated a hemoglobin-based NO biosensor that operated under aerobic conditions. They cast a mixture of hemoglobin and sodium montmorillonite onto the surface of a polished pyrolytic graphite disk electrode. Although the hemoglobin/montmorillonite film facilitated the reduction of O_2, the addition of NO to the solution attenuated the O_2-reduction current, shifting the reduction potential negative. The authors attributed this shift to competition between NO and O_2 for the heme site (137). Of analytical utility is the fact that the shift in potential was proportional to NO concentration. Furthermore, the sensor response to NO was linear to 5 μM NO, with an LOD of 20 pM. With respect to interferents, both ascorbate and NO_2^- induced negligible peak shifts (<4% of that by the same concentration of NO), whereas dopamine, uric acid, and epinephrine proved to be significant interferents (shifts >15%) (137).

Although electrochemical sensors are both sensitive and fast responding, development of sensors that can discriminate between NO and CO is still a critical need in the field of NO detection.

Because NO and CO have similar properties (e.g., size, hydrophobicity, reactivity with heme proteins, oxidation potential), NO sensors traditionally possess little or no selectivity over CO. In fact, the literature commonly overlooks the issue of selectivity over CO. To address this issue, Lee & Kim (139) developed an amperometric sensor for simultaneous detection of both NO and CO. To discriminate between NO and CO, the authors constructed a dual sensor with two working electrodes, each with different sensitivities for NO and CO based on differences in electrode size and surface modification. Two Pt disk working electrodes (25 and 250 μm in diameter) were housed behind a PTFE gas-permeable membrane following platinization. The 250-μm electrode was further modified with an electrochemically deposited layer of tin to enhance its sensitivity to CO. This modification had a negligible effect on the NO response. When coupled, the two electrodes allowed for quantification of both NO and CO. The NO/CO sensor was characterized by an LOD of ~1 and <5 nM for NO and CO, respectively, with linear responses up to micromolar levels for each analyte and with selectivity for NO and CO over ascorbic acid, uric acid, acetaminophen, and dopamine up to 500 μM and for NO_2^- up to 1 mM. The authors employed the sensor to simultaneously monitor NO and CO in mice kidneys (ex vivo) and found that basal levels of CO production were approximately seven times greater than levels of NO (139).

4. OTHER NO-DETECTION TECHNIQUES

The spectroscopic and electrochemical methods described above are by far the most commonly employed methods for measuring NO. Other less frequently employed approaches include mass spectrometry (140, 141), X-ray photoelectron spectroscopy (142), infrared (143–146) and ultraviolet (147–149) lasers, quartz crystal microbalance (150, 151), photoluminescent porous silicon (152) and cadmium selenide (153), gas chromatography (154, 155), and Raman spectroscopy (156). Space constraints do not permit us to describe these detection techniques in detail. However, these methods often consist of extensive instrumentation, thus inhibiting throughput and analysis by nonexperts.

SUMMARY POINTS

1. Detection of NO presents unique challenges to analytical chemists due to its rapid scavenging, high reactivity, and swift diffusion as well as its wide range of physiologically relevant concentrations.
2. The most commonly employed methods for measuring NO are based on spectroscopy and electrochemistry.
3. Absorbance-based methods (e.g., metalloprotein-based assays, Griess reaction) benefit from simple instrumentation and experimental procedures but suffer from the inability to detect NO in real time and at low concentrations.
4. Common NO-sensitive fluorescent probes are usually sensitive to a reactive by-product of NO oxidation (e.g., N_2O_3).
5. The two types of chemiluminescent reactions that exist for NO detection are based on NO or a by-product reacting with O_3 or luminol; both have low LOD (in the femtomolar-to-nanomolar range) and wide detection ranges (up to six orders of magnitude).
6. EPR spectroscopy coupled with both endogenous and exogenous NO spin traps provides a useful method of detecting and imaging NO production in biological samples both in vivo and ex vivo.

7. Sensitive and selective electrochemical NO sensors have been fabricated using permselective sensor membranes and/or electrocatalytic sensor modifications to reduce the potential necessary to oxidize NO.
8. The major challenge to constructing electrochemical NO sensors is reducing the signal generated by common interferents such as O_2, NO_2^-, ascorbic acid, uric acid, dopamine, and CO.

FUTURE ISSUES

1. Future research should focus on methods to correlate intracellular fluorescence from NO-sensitive fluorescent probes to an actual concentration of NO. Achieving this correlation will probably require combining several analytical approaches such as NO-sensitive fluorescent probes, confocal microscopy, and electrochemical NO measurements.
2. CO continues to pose a significant problem as an interferent for NO detection. Techniques for better discriminating between NO and CO are still needed, especially for electrochemical sensors.
3. Sensors capable of measuring NO simultaneously in all its forms, including reactive nitrogen oxide (rNO_x) species (e.g., NO, NO_2, N_2O_3, N_2O_4, $ONOO^-$), nitrosothiols, and metal-NO complexes, would improve the understanding of NO's role in physiology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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