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Fundamental Review

Determination of Nitric Oxide in Biological Samples

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Abstract. NO plays an important role in the regulation of physiological and pathophysiological mechanisms, and quite a lot of investigations have been focused on this kind of special molecule. It is difficult to achieve reliable ex vivo NO measurements with the existing analytical methods, and developing a reliable in vivo monitoring method for NO is still an urgent task. In this review, the techniques utilized for the determination of endogenous NO formed by enzymatic action and its reactions with other biological substances found in living organisms are discussed with respect to applications both in vivo or in vitro. In addition, various NO (micro)sensors and trap probes based on different principles are presented with their respective advantages and limitations. Finally, an NO monitoring system based on the combination of microdialysis sampling and chemiluminescence is introduced which is considered to be a prospective method for in vivo monitoring.

Key words: Nitric oxide; nitric oxide synthase; NO determination; chemical and biosensors; biological sample assay chemiluminescence.

As a widespread intracellular and intercellular second messenger in the human body, nitric oxide (NO) plays an important role in the regulation of diverse physiological and pathophysiological mechanisms of the cardiovascular system, the central and peripheral nervous system, and the immune system. The biological roles of NO have been associated with numerous clinical disorders (e.g. asthma, rheumatoid arthritis, multiple sclerosis, tuberculosis and Alzheimer's disease). Its physiological effects are attributed to a very small, freely diffusible, and potentially highly reactive radical. Recently, quite a lot of research in medicine, pharmacology, toxicology, biochemistry and so on have been focused on NO. NO can exhibit both protective action and destructive action [1–10]. Much progress has been made in understanding the action of NO in the human body, but it must be realized that the knowledge in this field is still quite poor. Numerous difficulties are still encountered concerning the detection of NO due to the following:

– The half-life time of NO is very short, implying that the steady-state concentration of NO in the organism quite low. This makes it very hard to achieve accurate monitoring of NO in vivo. Up to now, no ''perfect'' method exists. Studies on the variation of NO concentrations in biological fluids form the basis for understanding the effects of NO in different functions of the organism.

– As a free radical, the effect, or the related damage to the organism, is greatly disturbed by the following reactive species: (*a*) Free radicals such as the hydroxy radical (OH$^\bullet$) and superoxide anions (O$_2^{\bullet-}$), all of them being present in living organism and able to react with NO and to generate other kinds of reactive compounds. (*b*) Oxidant materials such as hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$), and

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antioxidant materials such as ascorbic acid, cysteine, NADPH, NADH and glutathione. (*c*) Some enzymes or proteins such as superoxide dismutase (SOD), catalase, hemoglobin and cytochrome c. (*d*) Certain metal ions, such as Fe^{2+}, Fe^{3+}, Cu^{2+} and Ca^{2+}. All the above species can react with NO directly or indirectly and enhance or quench the effect of NO by forming derived chemical species, which will result in a very complicated situation.

– As a second messenger, its effect is also disturbed by other neurotransmitters, such as acetylcholine, dopamine. Therefore, it is difficult to separate the contribution of NO from that of other species.

In order to understand the NO activity, it seems we have to know every detail of the mechanisms of operation of the human body. Quite a lot of progress concerning NO has been made so far, but there is still a need for much more research so as to fully understand the role this special molecule plays in the human body; thus the development of a reliable method for NO determination is still in urgent demand as pointed out or implied by numerous papers and many reviews about the progress of analytical techniques relating to in vivo and ex-vivo NO assays of biological interest, i.e. monitoring changes of NO production [11], establishment of physiological functions of NO [12, 14, 17, 19], evaluation of its toxicity [13, 18], analysis of samples [20], and determination of its kinetic or other properties [15, 16].

Properties and Function of Nitric Oxide

Investigating the properties and function of NO can provide important information with respect to developing methods for determination of NO [21–28].

Physical Properties of NO

NO is a colorless gas. The solid and liquid forms exhibit a pale blue color. The solubility of NO is 1.9 mM in aqueous solution at 1 atm pressure, and NO has been reported to diffuse at a rate of 50 μm per second in a single direction in biological systems. A simple rule of thumb for the solubility and transport of NO is that these properties are similar to those of dioxygen [29].

Chemical Properties of Nitric Oxide

One of the principal reasons that the chemical biology can be categorized into direct and indirect effects is due to the fundamental chemical properties of NO. It has an odd number of electrons with the unpaired electron in a $n_\pi{}^*$ antibonding orbital; hence, it is a stable radical. NO does not react rapidly with most biological substances (which is in contrast to oxygen radicals such as HO·). Since the in vivo lifetime of NO is relatively short (less than 10 sec), only the faster direct reactions of NO, (such as those with metal centers or other radicals), are likely to be important [30].

Direct Effects of NO

Direct effects involve the interaction of NO with the chemical or biological target.

(*a*) *Nitric oxide reactivity with metals*: reactions with metal centers are critical to understand the bioregulatory behavior of NO and why this molecule can serve as a signaling agent. The reaction of NO with some transition metal complexes results in the formation of metal-nitrosyl adducts. NO can react with ferrous ion to form iron nitrosyl. Heme-containing proteins are important in the biology of NO, i.e. the reaction between NO and heme cofactors within the protein are important in the regulation of guanylate cyclase activity. NO has been shown to bind to the heme moiety of this protein, thereby stimulating the conversion of GTP to cGMP.

(*b*) *The interaction of NO with cytochrome P_{450}*: several studies have shown that NO inhibits mammalian P_{450} which is thought to regulate the hormone metabolism and decrease the drug metabolism in the liver under infectious conditions.

(*c*) The direct reaction of NO with metallo-proteins is a major in vivo control mechanism of NO concentrations. NO reacts with oxy-hemoglobin (HbO_2) or oxy-myoglobin to form nitrate and met-hemoglobin (MetHb) or met-myoglobin. This reaction has been proposed as a key mechanism to control NO in vivo:

$$HbO_2 + NO \rightarrow MetHb + NO_3{}^-$$
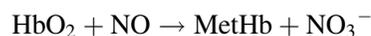
The reaction of ferryl heme proteins with NO is important to understand the mechanism of protection against reactive oxygen species by NO [30].

Reaction with Radicals

Rapid reactions with other radical species are the rule since NO has an unpaired electron. For

example, NO reacts with hydroxy radicals according to

$$NO + HO\cdot \rightarrow HNO_2$$
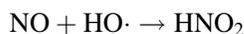
and with NO_2 to form N_2O_3:

$$NO + NO_2 \rightarrow N_2O_3$$

NO also reacts with alkyl-, alkoxy-, and alkylperoxide radicals according to the following reaction:

$$ROO\cdot + NO \rightarrow ROONO$$
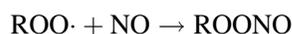
The reaction between NO and superoxide to form peroxynitrite has received considerable attention in the literature both as a potentially deleterious reaction and as a detoxication mechanism for reactive oxygen species (ROS):

$$NO + O_2^- \rightarrow OONO^-$$
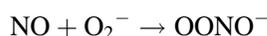
Indeed, the reaction of O_2^- with NO is 5 times faster than the decomposition of superoxide by superoxide dismutase. Peroxynitrite is relatively unreactive in alkaline solution and slowly dismutates to nitrite and oxygen. It reacts with sulfhydryl compounds, which results in the formation of disulfides at a rate constant of $10^3 \, M^{-1} \, sec.^{-1}$. Protonation of peroxynitrite gives the neutral HOONO species which can oxidize various biological substrates in competition with isomerization to nitrate:

$$OONO^- \Leftrightarrow HOONO$$

$$HOONO + biomolecule \rightarrow oxidized\ biomolecule$$

$$HOONO \rightarrow NO_3^-$$
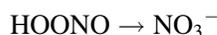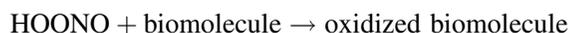
Despite the facile reactions discussed above, NO does not react with all radicals [31].

Nitric Oxide Synthase (NOS)

Nitric oxide (NO) is synthesized in vivo from L-arginine by nitric oxide synthases (NOS) [32–35] which occur in three different isoforms. The properties of immobilized NOS showed differences compared to native NOS. For instance, the activity of immobilized NOS is independent of NADPH and calmodulin but Ca^{2+} is needed; it also shows much higher stability in operation and during storage than the free NOS. The activity of immobilized enzyme does not show any evident decrease in six months; on the other hand, the free NOS, which is stored under the same condi-

Table 1. Formation and reaction pathways of NO in biological systems

Enzymatic formation of NO in biological systems

$$L\text{-Arginine} \xrightarrow{NOS, cofactors} L\text{-citrulline} + NO$$

Reactions of NO in biological fluids in the presence of oxygen

$$NO + O_2(or\ O_2Hb) \longrightarrow NO_x \quad \begin{matrix} RNOS-species \\ [NO_2, N_2O_3, NO_2^-, NO_3^-, ONOO^-] \end{matrix}$$

Mechanism of formation of RNOS species (NO_x)

$$2NO^\bullet + O_2 \longrightarrow 2NO_2$$
$$NO^\bullet + NO_2 \longrightarrow N_2O_3$$
$$N_2O_3 + H_2O \longrightarrow NO_2^-$$
$$NO^\bullet + O_2^- \longrightarrow ONO_2^- \longrightarrow NO_3^-$$
$$NO^\bullet + O_2Hb \longrightarrow NO_3^-$$
$$NO_2^- + O_2Hb \longrightarrow NO_3^-$$

Reactions of RNOS species in biological systems

Substrates with $k_s/k_H \gg 10^3$

Fe(II) heme proteins
Fe(II) complexes
Cysteine
Glutathione
Ascorbate
Amino salicylic acid
Dopamine
Aromatic amines
k_s = kinetic constant for reaction with the substrate
k_H = kinetic constant of hydrolysis to nitrite

tions, loses about 60% activity after three months [36].

The generation and the reaction pathways of NO in vivo are summarized in Table 1.

Analysis of Nitric Oxide

Rapid and accurate determination of NO in biological matrices is needed to unravel its action. Usually, the following three main methods are used to detect NO. The first is based on the chemiluminescence reaction of NO with ozone or the luminol-H_2O_2 system. The second is dependent on the conversion of oxyhemoglobin to methemoglobin upon reaction with nitric oxide, followed by subsequent absorbance measurements with electron spin resonance (ESR) or by spectrophotometry. The third is based on either a fiber-optic or amperometric sensor, which, in turn, is based on the above or some other reaction principle [37, 39–42].

Other methods include (a) the use of bio-chemical, spectrophotometric or fluorimetric methods to detect the substrates of NO in the course of metabolism or (b) indirect methods via the determination of NOS [38, 43], cGMP and related substrates such as nitrite anion (NO_2^-), peroxynitrate ($ONOO^-$) [44]. However, these methods can not be used for monitoring in vivo, since they are not able to detect NO directly. The sensitivity of this assay is poor and is affected by numerous interfering compounds. All of these methods have their advantages and disadvantages. The dynamic detection of NO in vivo can provide important evidence for the biological function of NO. NO is stable in the gas phase but not in liquid phase. The following paragraphs give an overview of methods for NO determination and applications [45–115].

Electrochemical Assays for NO

These are based on the electrochemical oxidation (mostly) or reduction of NO on solid electrodes. If the current generated during NO oxidation is linearly proportional to the concentration, the oxidation (or reduction) current can be used as the analytical signal [75]. The majority of the electrode sensors are based on the electrooxidation process of NO since the electroreduction meets O_2 interference. The electrode oxidation sensors are usually classified as either direct detection or catalytic electrooxidation detection [112].

(*a*) The porphyrinic type is a catalytic electrooxidation sensor. It is based on the electrochemical oxidation of nitric oxide on a conductive polymeric porphyrine layer [39, 112, 37]. Using the porphyrinic sensor, NO is oxidized on a polymeric metalloporphyrin (n-type semiconductor) on which the oxidation reaction occurs at 630 mV versus the saturated calomel electrode (SCE), 270 mV lower than the potential required for the respective metal or carbon electrodes. The current (analytical signal) efficiency for the reaction is high, even at pH 7.4. The sensor was used for NO monitoring in dorsal horn of spinal cord of rat [37], in biological materials [39], and for the measurement of NO release of a single cell in situ [50].

(*b*) The Clark-type electrode, a direct electrooxidation sensor, is based on the oxygen probe (Clark electrode) and operates in the amperometric mode. The Clark-type electrode sensor consists of a platinum wire as the working electrode (anode) and a silver wire as the counter-electrode (cathode). The electrodes are mounted in a capillary tube filled with a sodium chloride/hydrochlorodrochloric acid solution separated from the sample solution by a gas-permeable membrane [75, 76, 112]. A constant potential of 0.9 V is applied, and the current (analytical signal) that results from the electrochemical oxidation of nitric oxide on the platinum anode is measured. Several improvements of this pseudo-type Clark electrode have been made by other researchers using carbon, glassy carbon, platinum or gold electrodes covered by selected types of membranes to make them selective. The selectivity with all these electrode sensors was further improved by using multi-layered Nafion film. However, the gain in selectivity is at the cost of sensitivity, and reported sensitivities are determined at concentrations higher than physiological relevant ranges [112]. A commercial ISO-NOP (Shibucki's-prototype electrode implemented with gas permeable NO-selective membrane) distributed by World Precision Instruments with the aid of an isolated NO meter (ISO-NO Mark II), made by the same company, was used to measure the NO generated by the vascular endothelium. Direct measurements of NO release induced by acetylcholine and bradykinin from the surgically prepared saphenous veins were compared with the control veins [57].

(*c*) An NO-sensitive catalytic electrooxidation electrode coated with the Ni(TMPP) complex by electrochemical oxidation (anodization) was proposed for enhanced sensitivity of the carbon fiber electrodes. Furthermore, by covering the coated electrode with Nafion film, better selectivity for NO is obtained [76].

These methods based on the use of porphyrine and Nafion film display good specificity, but the stability is not good enough for reproducible detection; furthermore, the sensitivity is still not high enough for in vivo monitoring.

Several other catalytic electrode oxidation microsensors have been prepared using different modifiers with Nafion film protection for electrocatalytic oxidation [112]; however, the lowest limit of detection among the catalytic electrooxidation sensors reported lies with the use of a porphyrinic microsensor operated in differential

pulse voltametry (DPV) mode or differential pulse amperometry (DPA) mode [112].

(*d*) An NO-resistivity sensor has recently been proposed using carbon fiber electrodes connected with polymerized metal organic material (Co-Salen) that changes resistivity with adsorption of NO; this device is still under investigation but it seems promising since its function is reversible [97].

Colorimetric Assays for NO

Distinct color changes are caused by NO in solutions of ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) or SULF/NEDD (sulfanilamide)/(N-(1-naphthyl)-ethylene-diamine dihydro chloride), known as the Griess method. The assays based on these changes are useful for tests in laboratory experiments involving NO dosing or administration. The ferrocyanide oxidation method can be used with NO concentrations of $>25\,\mu M$, whereas ABTS can be used at NO concentrations as low as $2\,\mu M$. The oxidative methods are useful; however, interference from reductants may in some cases limit their use. On the other hand, the Griess method is not subject to this interference problem and is relatively accurate at levels lower than $5\,\mu M$ of NO. These methods are relatively simple and can be performed with a UV-vis spectrophotometer [77]. In addition, a variety of different spectrophotometric methods have been developed to measure NO directly, as in the case of the reaction of NO with oxyhemoglobin to produce methemoglobin [54] or indirectly via the Griess reaction.

The Griess reaction is a diazotation reaction of sulfanilamide and further condensation of the produced diazonium salt with naphthyl-ethylene diamine hydrochloride. It is a two-step reaction, and interference may come only from compounds of sample matrix capable of entering the diazotation reaction step, such as NO/O_2^- intermediates, antioxidants, and nitrosating compounds or the condensation reaction step. Azide, ascorbic acid, cysteine glutathione, dithiothreitol, mercaptoethanol interfere at concentrations as low as $100\,\mu M$. Compounds such as alkylamines, most sugars, lipids, and aminoacids except those containing sulfhydryl groups do not interfere. Intermediates in Fe(II)/peroxide reaction do not interfere with SULF/NEDD to increase the absorption at 496 nM, nor do other NO_x species [99]. Indirect methods of analysis with the Griess reaction are common practice in biological sample assays.

Indirect Detection of NO by Measuring Related Substrates

Indirect determination procedures of NO include the following:

(*a*) Determination of the selectivity of reactive NO species on various substrates. A method for determining the effectiveness of different compounds in scavenging these intermediates in the NO/O_2 reaction has been described [79].

(*b*) Quantitation of nitrate, nitrite, and nitrosating agents [80, 47]. Quantitation of nitrate and nitrite is based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthylethylendiamine and sulfanilamide. NO-related nitrosations arise from either N_2O_3 or N_2O_4, although N_2O_3 predominates in physiological systems. Both N_2O_3 and N_2O_4 react rapidly with water, amines, and other nucleophiles that may be present in the system, and the steady state concentrations of these intermediates are therefore low. Their net nitrosating potential, however, can be readily estimated by quantitating the nitrosation products. Morpholine is often used as the nitrosable amine because its relatively high pK_a assures a significant concentration of free amine at physiological pH. Quantitation is obtained by GC-TEA (gas chromatography-thermal energy analyzers) or by GC-MS (gas chromatography-mass spectrometry) [80].

(*c*) Analysis of nitrate and nitrite using nitrate reductase and the Griess reaction. The Griess reaction turned out to be an extremely durable experimental tool. When used in conjunction with a nitrate reductase assay, it is sensitive, accurate, inexpensive, and accessible to all laboratories for measuring nitrite and nitrate in tissue culture systems and in biological fluids. When properly prepared and used, nitrate reductases are highly effective analytical tools [81].

(*d*) Analysis of nitrate, nitrite, and nitric oxide synthase activity by an automated flow injection (FI) technique. An automated FI technique for nitrate and nitrite analysis in the brain and its application in the determination of NOS activity were described. It is dependent on the principle of the Griess reaction for diazonium ion [82].

(*e*) Microtiter plate assay for determining the kinetics of nitric oxide synthase enzymatic action. NO

production is monitored via the rate of oxidation of ferroheme by NO, resulting in a progressive increase in A_{450}. NO assay by using ferroheme capture can be used to quantify NO production from any source. This method was used in the determination of nitric oxide synthase activity in crude and purified enzyme preparations [83].

(f) The Griess method is demonstrated for the determination of NO after deproteinization and reduction of nitrates in the presence of NADPH-sensitive reductase in human body fluids (serum, urine, and CSF) [100]. The Griess reaction was used also for the determination of nitrite released by macrophages as indicator of NO production in an investigation of its anti-leishamanial activity [73].

(g) The Griess reaction has also been used as a standardization method for the evaluation of biomarker capability to accurately measure the NO released in vivo, i.e. biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG) determination in urinary excretions of rats after chronic NO blockade using ELISA assay [67]. Generally, such indirect NO-selective assays have poor sensitivity with much interference and can detect NO in vitro with complicated sample preparation.

Other indirect methods for measurement of NO generated during the enzymatic action of NOS have been applied as follows:

(a) Liquid scintillation counting to measure the radio-isotope emission change of the ^{14}C or ^{3}H-isotope labeled substrates for measurements of L-arginine conversion and/or L-citrulline formation after isolation of L-citrulline by passing the reaction mixture through cation exchange resin. The method was used for measurement of NO synthase activity in biological tissues [43] and in peritoneal biopsies [65].

(b) Pre-column derivatization with o-pthalaldehyde (OPA) HPLC-assay with fluorescence detection of L-hydroxyarginine (L-NHA) (a by-product of the NO synthase enzymatic activity treated with lipopolysaccharide (LPS)) after extraction from the incubation mixture of NOS-related basic amino acids by cation exchange resin; mass spectrometric determination was made possible by pre-column derivatization of the basic amino acids through treatment with HCl acid in methanol and pentafluoropropionic acid anhydride to form the pentafluoropropionyl methyl ester derivatives. The derivatives were then sent through HPLC-MS apparatus for separation and detection/identification of eluted derivatives [38].

(c) A modified capillary electrophoresis method for the determination of nitric oxide correlated nitrate in several homogenates is also described [71]. The nitrate level of the tissue homogenates of control and L-NAME treated organs (heart, brain, kidney, stomach, lung, testis, and liver) were monitored, and it was found that the method is precise and accurate. The method was used at concentrations of the 10^{-5} region, and the %RSD was concentration-dependent and around 0.2.

(d) Confocal laser scanning microscopy combined with histochemistry for NADPH diaphorase (marker for neurons containing NO synthase) revealed that the number of NADPH-d-positive neurons in the cerebral cortex and striatum of old rats is significantly reduced compared to that in young rats [63].

(e) A photoacoustic spectroscopic technique equipped with a laser light source for the determination and monitoring of endogenous nitric oxide is reported [74]. The method was applied to fruits [74].

The majority of the above methods can detect NO only indirectly; they are still far away from an in vivo monitoring sensor, but provide important information for NO-mediated biological reactions and in general, and the sensitivity based on indirect methods is not high.

Fluorimetric Probe Assays

In attempts to enhance the sensitivity of measuring NO generated under physiological conditions or NO_2^- under acidic conditions, several fluorimetric methods have been developed that exploit the ability of NO to produce N-nitrosating agents. A number of these methods employed the use of the aromatic diamino compound 2,3-diaminonaphthalene (DAN) as an indicator of NO formation. The relatively nonfluorescent DAN reacts rapidly with the NO-derived N-nitrosating agent to yield the highly fluorescent product 2,3-naphthotriazole (NAT) [78].

DAN fluorescent probe detection was also used with an HPLC separation technique to monitor brain NO_x outflow through microdialysis procedure. The nitrate anion in microdialysate was converted to nitrite

by enzymatic reduction, which – when acidified – turns into an N_2O_3 species that reacts with the DAN probe and produces fluorescent triazole-derivatives; the triazole-derivative is then separated from the other constituents of the sample through HPLC elution and is detected by its fluorescence emission [55]. A detection limit of less than 1 nM is claimed when interfering compounds are absent.

The thermolysis of NO-related compounds found in the blood samples converts them into nitrates, and after enzymatic reduction to NO_x-species and further reaction with DAN fluorescent probe reaction, they are detected fluorimetrically [44].

*Determination of Nitric Oxide by Electron
Spin Resonance (ESR)*

ESR or Electron Paramagnetic Resonance (EPR) based methods have been considered reliable tools with high sensitivity and selectivity, and various methods using different spin-trap probes have been proposed [102, 111] based on the following principles [84]:

(*a*) NO can react with a number of intercellular heme- and non-heme targets to form ESR-active nitrosyl complexes. These complexes have characteristic ESR spectra that can be detected at ambient and low temperatures. Iron dithiocarbamates can be added as exogenous traps to detect intracellular and extracellular formation of NO. Nitronyl nitroxides react with NO to form imino nitroxides. This transformation can be monitored with ESR continuously. Nitronyl nitroxides can, therefore, be used to efficiently antagonize other NO trapping reactions in biological systems under physiological conditions. The synthesized chelotropic spin-traps [109] are thermally stable, water soluble, and react with NO and NO_2 to form stable nitroxides; the reactions can be used to detect NO in hydrophilic and hydrophobic compartments of different types of cells. Nitric oxide causes physical broadening of cell membranes, forming stable lipid-soluble nitroxides, and ESR can be used to calculate the diffusion coefficients of NO in biological membranes. ESR can also provide quantitative measurements of NO production using standard signals from commercially available spin-standards [84].

(*b*) Ferrylhemoglobin (ferrylHb) and ferrylmyoglobin (ferrylMb), the high oxidation state hemoproteins and analogs of peroxidases formed by interactions of methemoproteins with hydroperoxides, are known to promote peroxidative reactions. Since NO inhibits these oxidations by reducing ferryl-hemoproteins, the interaction of NO with ferryl-hemoproteins can be detected by ESR [85, 45, 46]. The formation of S-nitroso-hemoglobin (SNO-Hb) mediated with heme-redox and preferential reactivity in β-subunits was found to take place after NO-release in vivo. The experimental procedure was based on monitoring the progress of reaction by UV-Vis spectroscopy and deconvolution of the spectra through computer software to find the contribution from met–Fe(II)NO- and Fe(III)NO-Hb and by sampling the reaction mixture at selected times and analyzing either by ESR-spectroscopy or directly for nitrite and nitrosothiols by Griess and Saville assays, respectively [115].

(*c*) 3,5-Dibromo-4-nitrosobenzenesulfonate (DBNBS) can trap radicals derived from the reaction related to nitric oxide. The NO-dependent radical formation in the human platelet system using DBNBS, and some basic reaction between DBNBS and various NO-related compounds can be assessed to identify DBNBS spin adducts by ESR [86].

(*d*) Stable organic radical nitronyl nitroxide derivatives, namely 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) and its derivatives (PTIOs), possess a specific scavenging action against NO released in both in vitro and in vivo systems. Evaluation of the scavenging ability was confirmed in biological systems using a TMA-PTIO spin-trap probe [110]. The reaction of PTIOs with NO changes the ESR signal, and the amount of NO can be quantitated by measuring the change of ESR signals [87].

(*e*) Several distinct dithiocarbamate ions, such as N,N-diethyl dithiocarbamate (DETC) [49, 103, 104] and N-methyl D-glucamine dithiocarbamate (MGD) [104–108], are used as ligands for the iron complex spin-trap probe. The complex [Fe(II)(DETC)$_2$] is not particularly soluble in water while the complex [Fe(II)(MGD)$_2$] is readily soluble. The obtained ESR signal consists of a $g_\parallel = 2.02$ and $g_\perp = 2.04$ with unresolved hyperfine structure in axial symmetry (frozen solutions) or an isotropic triplet at $g_{av} = 2.03$ at 37 °C. ESR spectroscopy using a [Fe(II)(DETC)$_2$] spin-trap probe was applied to on-line detection of NO formation in

aqueous phases [49]. An improved version that increases the solubility of the [Fe(II)(DETC)$_2$] spin-trap probe using albumin and stabilizes the Fe(II) oxidation state by sodium thiosulfate gave a significantly lower detection limit [103]. Ex-vivo detection of NO with samples taken from several regions of the brain was obtained [104]. The detection sensitivity was increased significantly with the use of a [Fe(II)(MGD)$_2$] spin-trap probe, and measurements of NO release from nitroprusside NO donor were accurate [105]; it was further used for continuous monitoring of NO formation ex-vivo and in vivo of the rat liver [106, 107] and for quantitation of NO levels in heart after experimental myocardial ischemia [108].

All in all, the above methods can provide adequate sensitivity and specificity in NO detection, but they cannot be used for NO monitoring in vivo directly, and they are mostly limited by expensive instrumentation, time-consuming sample preparation, and complicated operation.

Determination of NO by Using Chemiluminescence Assays

The chemiluminescence (CL) assay is considered to be the most useful method because of its high sensitivity and its capability for real-time monitoring of NO. The CL detection methods proposed are based on the following reactions of NO:

The reaction of NO with ozone (O_3) yields excited-state nitrogen dioxide (NO$_2$*) which emits a photon on conversion to NO$_2$ (ground state), with the photon emission being quantitated by chemiluminescence detection [59].

$$NO + O_3 \rightarrow NO_2{}^* + O_2, \quad NO_2{}^* \rightarrow NO_2 + light.$$
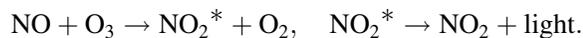
The above CL-based assay for direct measurement of NO is rapid and reliable. The method was applied to the determination of NO in whole blood samples through quantitative reduction of nitrite and nitrate to NO using V(III) at 98 °C [64] or by thermolysis of NO-related compounds to nitrate, enzymatic reduction to NO and CL detection or fluorescence detection [44]. The ozone CL reaction was utilized for monitoring NO formation and its release by endothelial cells [114], for NO formation by NO synthase activity, for the quantitation of NO produced in assays of denitrifying enzymes [48], for NO production during the biotransformation of nitrovasodilator agents including glyceryl trinitrate and sodium nitroprusside, and for

NO production during photochemically induced degradation of sodium nitroprusside and streptozotocin. The CL assay is also used to ensure that the desired NO dosage is warranted by inhalation in the treatment of persistent pulmonary hypertension of the newborn and the adult respiratory distress syndrome [88], as well as for the determination of exhaled NO levels, since NO is associated with pulmonary inflammation in asthma cases [66, 68, 69], and nasal NO-measurements [70]. This method has very high sensitivity, but it can detect NO in gas phase only. As a result, it is not suitable for NO in vivo monitoring, which usually involves an aqueous phase. Furthermore, it is prone to certain interferences, and the reproducibility is strongly affected by the ozone gas stream, since it is difficult to control a stable, repeatable gas stream from ozone generators. However, headspace gas analysis techniques can be combined with CL detection to provide NO detection with samples that are relatively rich in NO [88].

The reaction of NO with H_2O_2 to form peroxynitrite (ONOO$^-$), a stronger oxidizing species than H_2O_2 [51, 53], has been linked to the CL reaction of luminol with peroxynitrite. This reaction forms the basis for the detection of NO production in biological samples:

$$ONOO^- + luminol \rightarrow light.$$
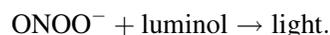
The luminol-H_2O_2 system is fairly specific to NO in that other nitrogen-containing compounds (organic nitrite, organic nitrate, and thio-nitroso compounds) or endothelium-derived compounds do not interfere. This method is characterized by high sensitivity and is applicable to aqueous phases. However, selectivity is dramatically affected by some reductants. If implemented with another selective tool, this method will allow significantly improved assays.

Newly Developed NO Detection Methods

Several novel methods based on electrode or CL sensors and on spectrophotometers with multichannel detectors to measure spectral changes at more than one wavelengths were also designed.

(a)

(1) Nitric oxide can be electrocatalytically reduced at electrodes modified with electropolymerized films of [Cr(v-tpy)$_2$]$^{3+}$. Upon further modification of the electrodes with a thin film of Nafion (to prevent interferences from anions, especially nitrite), these elec-

trodes can be employed as NO sensors in solution with submicromolar detection limits and fast response. Preliminary studies of cellular NO release from *Rhodobacter sphaeroides* bacterial cells yielded very good results [52]. The above Nafion-coated electrode still encountered biological interferences, and mostly it was strongly interfered by the intrinsic voltage and current of the whole body; thus in vivo monitoring is rather difficult.

(2) A high spatial resolution electrode microsensor was developed based on the Shibucki electrode probe for the measurement of cellular NO formation in vitro and in vivo [58].

(3) A Cu–Pt microparticle CME anode microsensor [60] was constructed to measure NO release from hippocampus of rat. Real-time continuous measurements of NO production in rat heart were obtained by the use of the above electrode microsensor [62], and changes of physiological NO level were induced and monitored using NO release inhibitors.

(4) A new tool for O_2^-/NO_x using a suitable enzymatic electrode sensor. The electrode is a Shibucki type including a membrane part for the immobilization of enzyme that converts the superoxide to hydrogen peroxide. The NO reacts with superoxide, causing the response of the combined electrode to be decreased [89].

(b) A fiber-optic sensor for NO was constructed by placing a small amount of an internal reagent solution at the tip of a fiber-optic bundle with a piece of gas-permeable membrane. Nitric oxide diffuses across the membrane into this internal solution, where a chemiluminescence reaction between nitric oxide, hydrogen peroxide, and luminol takes place. The resulting light intensity is related to the concentration of nitric oxide in the sample [53]. This method has the big advantage of excluding potential interferences by using a pore-size-limited membrane, but NO diffusion efficiency was limited as well, and sensitivity is not high enough for in vivo monitoring.

(c)

(1) A sensitive spectrophotometric assay was developed for the simultaneous quantification of NO and O_2^- in aqueous solution that is based on the NO-induced oxidation of oxyhemoglobin (oxyHb) to methemoglobin and the O_2^--mediated reduction of ferricytochrome c. to ferrocytochrome c. Using a photodiode array photometer, spectral changes of either reaction are analyzed, and appropriate wavelengths are identified for simultaneous monitoring of absorbance changes in the individual reactions [54]. The method was tested for accuracy using 3-morpholinosydnonimine (SNAP) NO-donor and was applied in cultured endothelial cells and isolated aortic tissue NO measurements using Ca-ionophore and NADH for stimulation of NO and O_2^- formation, respectively [54]. This method is reliable, but cannot be used for in vivo monitoring; furthermore, its sensitivity is not high enough.

(2) A flow-injection/spectrophotometric method for NO was used for on-line detection of NO_3^- and NO_2^-, after reduction with Cu–Cd micro-column and formation of the $[Fe(II)(SCN)(NO)]^+$ complex [101].

Recently, a series of new fiber-optic chemical or biosensors and new, more effective fluorescent probes for NO have been developed with high sensitivity:

(a) A fiber optic NO sensor based on a gold-adsorbed fluorophore was developed. This sensor is composed of a fluorescein derivative dye attached to colloidal gold. The fluorescein dye rearranges as nitric oxide adsorbs onto gold, inducing a decrease in the fluorescence intensity of the dye [42]. Also, a similar fiber-optic NO biosensor was developed by attaching cytochromes c′ to optic fibers covered by colloidal gold [40]. The conformational alterations in cytochrome c′ induced by adsorbed NO binding to gold can be monitored via the fluorescence of the hemoprotein at 630 to 640 nm. Earlier, fiber-optic sensors using cytochrome c′ were prepared using sol-gel immobilization, and the NO release detection was based on shifts of the Soret absorbance peak (400 nm) of the metal porphyrine [56]. The above fluorescent probe sensors suffer from interference by potential biological fluids, and the surface fouling brings about poor stability.

Last year a new fiber-optic sensor was reported for measuring NO in the gas phase using the colorimetric reaction between Cu(II) complex of

Eriochrome cyanine R (ECR) and NO in phosphate buffer (pH = 7.4). The sensor consists of the Cu(II) complex incorporated into a silicone rubber membrane that is attached on the optic fiber. The changes of NO content in the gas phase were detected by Vis-spectrometry through changes of absorbance. A detection limit of 0.277 ppm is reported [113].

(b)

(1) The diaminofluoresceins (DAFs) have been designed and synthesized for the detection and imaging of NO. The fluorescent chemical transformation of DAFs is based on the reactivity of the aromatic vicinal diamines with NO in the presence of dioxygen. The N-nitrosation of DAFs, yielding the highly green-fluorescent triazole form, offers the advantages of specificity, sensitivity, and a simple protocol for direct detection of NO [41]. This method has recently been criticized due to severe interference from Ca^{2+}. In fact, it will be difficult to use it for NO in vivo monitoring in the presence of Ca^{2+} [90]. Furthermore, a new method was proposed for the simultaneous measurement of intracellular Ca^{2+} and NO [61] using DAF-2-DA and Fura-2 as fluorescent probes for NO and Ca^{2+}, respectively, and a photomultiplier or CCD camera for recording the signals generated in porcine endothelial cell cultures. DAF-FM (3-amino-4-(N-methylamino)-2′-7′-difluoro-fluorescein was used to detect the spontaneous and substance P-induced NO-release from isolated porcine coronary arteries, and to obtain fluorescence images of cultured smooth muscle cells of the rat urinary bladder. For quantitative measurement of NO, the triazole derivative was determined after RP-HPLC elution and fluorescence detection [72]. A linear fluorescence intensity between 2–200 nM is reported.

(2) A new ratiometric direct assay for NO based on a novel signal switching mechanism using a [Fe(II)(Mmc-cyclam)]-(fluorescamine-PROXYL) adduct fluorescence probe is proposed for NO determination in vivo [98].

(3) A new fluorescent probe for NO was developed that is based on difluoroboradiaza-s-indacene fluorophore (TMDABODIPY) with high photo-

stability, wide range pH-stability, and strong autofluorescence and trapping NO capacity in the presence of O_2 forming weak fluorescent triazole. The method gives a linear calibration in the range of 0.01–0.40 μM, and a detection limit of 2.5 nM. The data were obtained using S-nitroso cysteine as NO releasing agent [96].

(c) A novel nitroxyl spin-probe (refered to as DHLA-TEMPO and being a conjugate of 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl with dihydrolipoic acid) was synthesized and proposed for the evaluation of O_2^-, NO, $ONOO^-$ by inhibition of ESR signal [95b]. Nitroxyl probes are mainly used for trapping free radicals, including reactive oxygen and nitrogen species. Recently, in vivo ESR spectroscopy was developed, and it enabled non-invasive in vivo measurements of radicals at all levels in animals. ESR spectrometer, coupled to computed tomography and implemented with spectral-spatial imaging provides the means for imaging the reactive oxygen and nitrogen species in living organisms. Nitroxyl probes that pass through the blood-brain barrier have also been synthesized for successful non-invasive localization of free radicals in brain tissue [95a].

The basic principles of the analytical methods mentioned above are summarized in Table 2 with some brief remarks.

Generally, all the above methods have their own advantages and limitations. Limitations include the lack of appropriate sensitivity or specificity to interference from factors commonly present in biological fluids. Specifically the reproducible Griess method allows for standardization but is an indirect method and unsuitable for kinetic studies, and it has a poor detection limit; the oxyhemoglobin spectrophotometric assay is time-consuming since the reagent is not commercially available and its preparation needs time and appropriate care; the ESR spectroscopy spin-trap techniques have poor detection limits (1 μM) and because of the heavy apparatus are unsuitable for routine work; the ozone CL assay is very useful for applications related to NO exhaled gas, but not for effective NO detection in biological fluids; the electrochemical sensors that are promising for in vivo and in-situ measurements under physiological and patho-physiological conditions have not found wide application because of the inherent fouling problem; finally, the fluorescent probes offer low detection limits, but

Table 2. Reactions for NO determination in biological fluids


A. *Electrochemical*

One-electron oxidation:

$$NO \xrightarrow{\text{Anode}(0.63\,V)} NO^+ + e$$
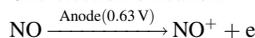
Porphyrinic sensor: (Malinski and Taha) polymeric porphyrine on carbon electrode at 630 mV (vs SCE) [75, 39, 50]

(a) *Microsensor*: chemically modified carbon fiber electrode (CME) by electropolymerization of Ni(II) tetrakis(3-methoxy-4-hydroxy-phenyl)porphyrin (NiTMHPP) or by the Ni(II) tetra-N-methyl pyridiniumporphyrin chloride, coated with Nafion membrane [75]
(b) *Integrated sensor*: implemented with gas-permeable membrane
(c) *New integrated sensor*: improved integrated sensor [37]
(d) *Gas-integrated sensor*: modified for gaseous samples

Cu–Pt microparticles coated CF electrode [60, 62]

Two-electron oxidation:

$$2NO + 4OH^- \xrightarrow{\text{Anode}(0.9\,V)} NO_3^- + 2H^+ + 4e$$
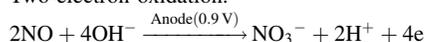
*Clark-type probe* (Shibuki): Pt-anode (800–900 mV), Ag/AgCl cathode [75]

(a) *Integrated Clark type probe* [76]: implemented with gas permeable membrane electrode
(b) *Shibuki type probe* [89]: implemented with enzymatic (superoxide dismutase) sensor

Generation of $O_2^{\bullet-}$: $Xanthine + H_2O + O_2 \xrightarrow{\text{XO,pH}=10.2} Uric.acid + 2H^+ + O_2^{\bullet-}$

Enzymatic reaction: $2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{Superoxide.Dismutase}} H_2O_2 + O_2 \ldots \ldots$

Electrode reaction: $H_2O_2 \xrightarrow{\text{Pt-electrode}(0.7\,V)} O_2 + 2H^+ + 2e \ldots \ldots \ldots \ldots$
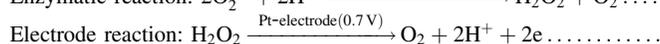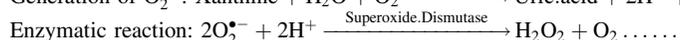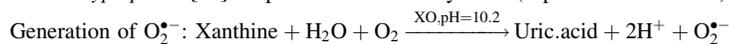
(Based on the modulation of the probe response towards H_2O_2 due to NO that scavenges the superoxide radicals.
(c) *Shibuki type probe* [58]: modified for high spatial resolution for in vivo and in vitro measurements of NO in isolated cells

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Two-electron reduction (assumed)

$$2NO + 2e \xrightarrow{\text{Cathode}(-0.9\,V)} N_2O_2^{2-}$$
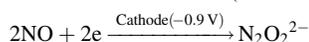
1. *CF|[Cr(v-tpy)₂]³⁺|Nafion-sensor.* [52]: glassy carbon fiber-cathode, Pt-wire counter electrode (potential vs Ag/AgCl saturated electrode) made of Chemically Modified Carbon Fiber Electrode (CM-CFE) by electropolymerized films of vinylterpyridine complex of chromium, $[Cr(v\text{-}tpy)_2]^{3+}$, and coated with nafion
2. *Co-Salen polymer resistivity-sensor* using microelectrode) [97]
   Co-salen-polymer + NO $\longrightarrow$ NO-Co-salen-polymer

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B. *Colorimetric/spectrophotometric absorptiometry*

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1. *(ABTS) chromogenic reaction*: [77, 99]
   $ABTS^{2-} + \overset{\bullet}{N}O \xrightarrow{O_2,-e} [\text{oxidized.ABTS}^{2-} - NO]^+$
   ABTS = 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)
2. *Griess reaction* (A_{543}) [77, 82, 99, 47, 73]:
   $4NO + O_2 + 2H_2O \longrightarrow 4NO_2^- + 4H^+$
   $NO_3^- \xrightarrow{\text{Reduction(chemical/enzymatic)}} NO_2^-$
   Sulfanilamide + NO_2^- + HX $\longrightarrow$ sulfanilamide.diazo.salt + $2H_2O$
   Sulfanilamide.diazo.salt + NEED $\longrightarrow$ sulfanilamide-NEED adduct + 3HCl
   NEED = N-(1-naphthyl)-ethylene-diamine dihydro chloride
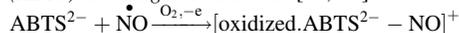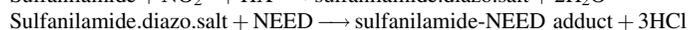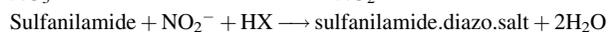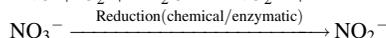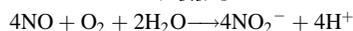
   (a) *Batch method* [100]
   (b) *Flow Injection method* [82]
3. *Ferrocyanide reaction*:
   $K_4[Fe(CN)_6] + NO \xrightarrow{O_2} K_3[Fe(CN)_5(NO)] + KCN$
   $NO + Fe(II) \xrightarrow{SCN^-,H^+} [Fe(SCN)(NO)]^+$

   (a) *Flow injection method* [101]
4. *Hb or Mb reaction* (A_{434}):
   $Fe(II) - Hb(Mb) \xrightarrow{NO_x} Fe(III) - Hb(Mb)$

   (a) *Batch method*
   (b) *Titer plate* (A_{405}) [83]: implemented with fiber-optic conduits.


Table 2 (*continued*)

(Used for kinetic analysis).

(c) *Fiber optic sesnsor* based on cytochrome c′ probe [56]

5. *OxyHb (OxyMb) reaction*:

Sensitive spectrophotometric assay of $NO/O_2^{\bullet -}$ with the aid of diode array spectrophotometer and ferricytochrome $c(Fe^{3+})$ [54] according to reaction

$HbO_2(MbO_2) + NO \longrightarrow metHb(Mb) + NO_3^-$
$ferricytochrome\ c(Fe^{3+}) + O_2^{\bullet -} \longrightarrow ferrocytochrome\ c(Fe^{2+}) + O_2$

C. *Fluorimetric*

---

1. *Fluorescence of triazoles formed by the action of NO on o-diamino aromatic compounds*

   (a) *Diaminonaphthalene (DAN) probe-*reaction [78]:

   $Diaminonaphthalene \xrightarrow{NO_x} Diazoaminonapthalene\ salt \longrightarrow Triazole$
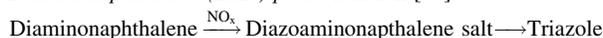
   (b) *Diaminofluorescein (DAF) imaging-reaction* [41, 61, 72, 90]:

   $Diaminofluorescein + NO + O_2 \longrightarrow fluorescein\ triazole$

   (c) *Cytochrome c′* [40]
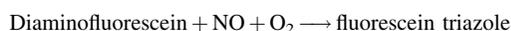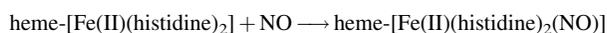
   $heme\text{-}[Fe(II)(histidine)_2] + NO \longrightarrow heme\text{-}[Fe(II)(histidine)_2(NO)]$
   (The Fe(II) of the product is pentacoordinated)

   (d) *TMDABODIPY probe-reaction* ($\lambda_{ex} = 500$, $\lambda_{em} = 510\,nm$) [96]:
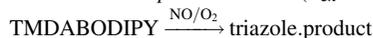
   $TMDABODIPY \xrightarrow{NO/O_2} triazole.product$
   (TMDABODIPY = 1,3,5,7-tetramethyl-8-(3,4-diaminophenyl)-difluoroboradiaza-s-indacene)

2. *Optochemical Sensors*

   (a) *Hemoproteins on polyacrylamide-coated optic fibers* [40]
   (Based on shifts of the Soret absorbance peak at 400 nm of the metal porphyrin).

   (b) *Fluorescein derivatives attached on gold-coated optic-fibers* [42]
   (Based on the decrease of fluorescence emission at 517 nm by NO adsorbed on gold).

   (c) *Cytochrome c′ attached on gold-coated optic-fibers* (monitor at 630–640 nm) [40]
   (Based on the energy transfer between hemoprotein and the fluorescent dye).

   (d) *An adduct of fluorescent Mmc-cyclam iron complex with fluorescamine-PROXYL sensor* [98].
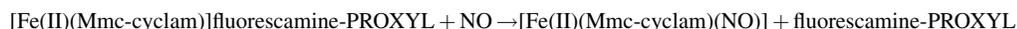
   $[Fe(II)(Mmc\text{-}cyclam)]fluorescamine\text{-}PROXYL + NO \rightarrow [Fe(II)(Mmc\text{-}cyclam)(NO)] + fluorescamine\text{-}PROXYL$
   fluorescamine-PROXYL = Tetramethylpyrrolidin-N-oxid-fluorescein derivative [Fe(II)(Mmc-cyclam)] = methoxycumarine Fe(II) heme-complex
   ($\lambda_{ex}([Fe(II)(Mmc\text{-}cyclam)]) = 360\,nm$, $\lambda_{em}(Mmc\text{-}cyclam) = 410\,nm$, $\lambda_{em}(fluorescamine\text{-}PROXYL) = 470\,nm$):

   (e) *Fiber-optic sensor for gas-phase NO monitoring* [113]

   $Cu(II)(ECR)_2^{2+} + ROH + NO \longrightarrow Cu(I)(ECR)_2^+ + RONO + H^+$
   (Based on the reduction of the Cu complex and then relevant change in absorbance spectrum)

D. *Electron spin resonance*

---

1. *Formation of Nitrosyl complexes with Fe(II) compounds* [84, 111]

   (a) *Heme-compounds* [45, 46, 102]
   $[>N - Fe(L)_4]^{2+} + NO^{\bullet} \longrightarrow [>N - Fe(L)_4 - NO]^{2+}$
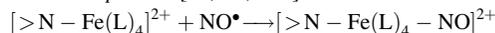
   (b) *Non-heme compounds*
   $Fe(II) + 2L^- + 2NO^{\bullet} \longrightarrow [Fe(NO)_2(L)_2]$

   (c) *Dithiocarbates (DETCs)* [49, 104, 103, 105–108, 102]
   $Fe(II) + NO^{\bullet} + 2DETC^- \longrightarrow [Fe(NO)(DETC)_2]$
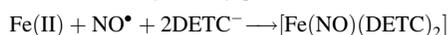
2. *Formation of Imino nitroxide (INO) compounds by reaction with Nitronyl nitroxide (N–NO) compounds* [84, 87, 85]
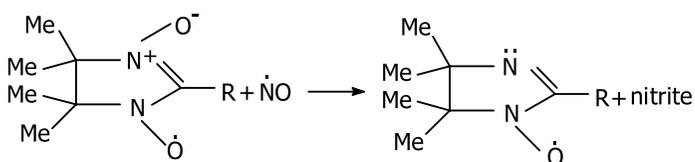
Nitronyl nitroxide (NNO)   Imino nitroxide (INO)

Table 2 (*continued*)

(a) *PTIOs-reaction* [87, 110]

PTIOs + NO $\longrightarrow$ PTIs + NO$_2$
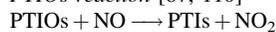
PTIOs = derivatives of 2-phenyl-4,4,5,5-tetramethylimidazoline-1oxyl-3-oxide.

PTIs = derivatives of 2-phenyl-4,4,5,5-tetramethylimidazoline-1oxyl.

3. *Nitroxide chelotrops (NOCTs) of NO to form nitroxide adducts* [84, 109]

$$R = H, SO_3^-, CO_2^-$$
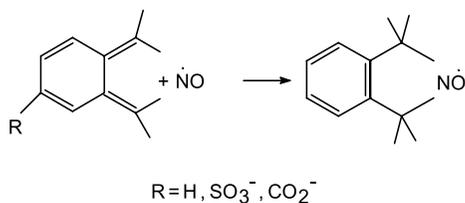
4. *Dibromo-nitroso-benzene sulfonate (DBNBS)-reaction* [86]
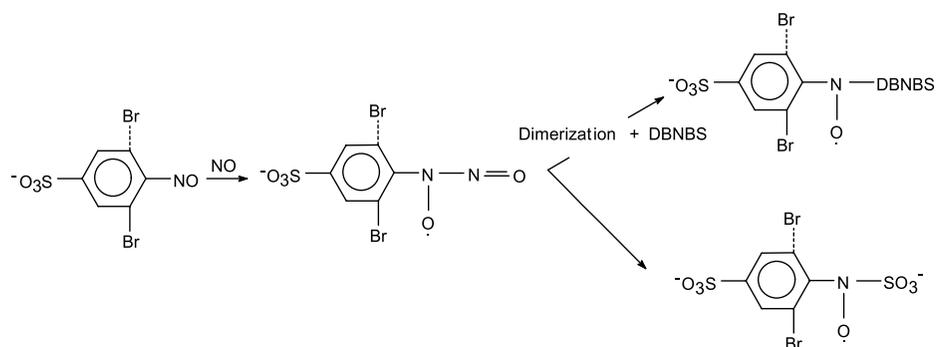
E. *Indirect methods of analysis (NO-substrate determination)*

Selectivity measurements of RNOS species with substrates

Reaction scheme for product formation analysis [79]
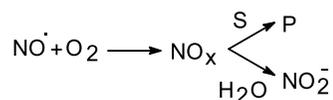
Reaction scheme for product quenching analysis [79]
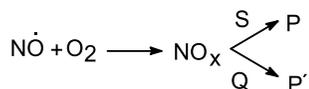
Reactions of RNOS species with substrates

1. *Diaminonaphthalene (DAN)-reaction* [79]

DAN $\xrightarrow{\text{NO}_x}$ TRI
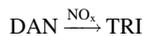
TRI = Triazole derivative

(a) *HPLC-NO$_x$ assay for microdialysate samples* [55]

(Based on DAN-reaction with N$_2$O$_3$ formed from nitrates according to enzymatic reduction reactions)

NO$_3^-$ + NADPH + H$^+$ $\xrightarrow{\text{Nitrate Reductase}}$ NO$_2^-$ + NADP$^+$ + H$_2$O

2. *Sulfhydryl-reaction* [81]

NO$_x$ + RSH $\longrightarrow$ RSNO

3. *Amine reaction* [81]

N$_2$O$_3$ + RNH$_2$ $\longrightarrow$ RNH(NO) + NO$_2^-$ + H$^+$

Table 2 (*continued*)

4. *Morpholine-reaction* [81]
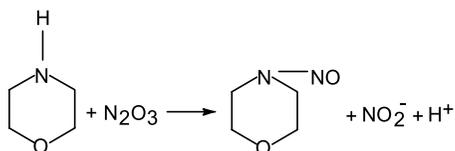
5. *Nitrate reductase-reaction* [81, 100]

$$NO_3{}^- \xrightarrow{\text{nitrate.reductase}} NO_2{}^- \xrightarrow{\text{Griess.Reagents}} \text{colored.product}$$
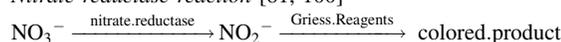
F. *Chemiluminescence*

1. *NO–O₃ CL-reaction* [88, 66, 64, 65, 70, 48, 69]

$$NO^{\bullet} + O_3 \longrightarrow \bullet NO_2{}^* + O_2 \longrightarrow \bullet NO_2 + O_2 + h\nu$$

2. *Luminol-Peroxynitrite CL reaction* [94]

$$NO^{\bullet} + O_2{}^{\bullet -} \longrightarrow ONOO^- \text{ (formation in biological systems)}$$
$$HONO + HOOH \longrightarrow HOONO + H_2O \text{ (preparation in the lab)}$$
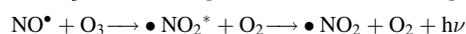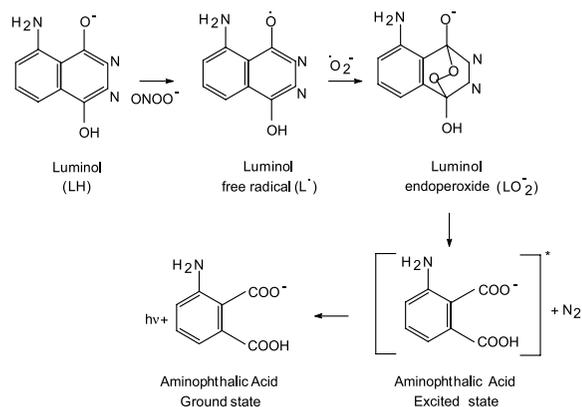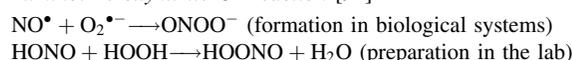
3. *Luminol-H₂O₂ CL reaction*. (Kikuchi) [51, 44]

$$H_2O_2 + NO \longrightarrow OONO^- + 2H^+ \xrightarrow{\text{luminol}} \text{aminophthalic acid} + h\nu$$
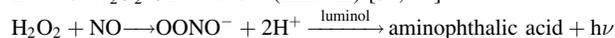
   (a) *Fiber-optic sensor* [53]
   (b) *FI/Luminol CL detection* implemented with NO trap [91]
       (Based on the NO release from its complex with porphyrine substances)
   (c) *FI/Luminol CL detection* implemented with composite membrane NO trap sampler [92].
       (Based on the principle of previous reference, but with composite membrane sampler for extra protection from interference)
   (d) *Microdialysis sampler/Luminol CL detection* [93].
       (Based on the principle of previous reference, but using a microdialysis system for measurements in vivo and real-time monitoring of NO).

the stability of the probes and/or products of reaction and the specificity is questioned in biological fluids for in vivo and in-situ monitoring of the endogenous released NO. Table 3 tabulates the analytical parameters of the various detection methods applied in NO determination as they are reported in the relevant published papers; the table is divided into sub-tables each associated with a particular class of detection methods followed by some remarks about their per-

formance. Up to now, there has been no perfect method that can quantify NO dynamically in vivo with high sensitivity and selectivity, even though much attention has been focused on this subject for a long time. The development of a new NO in vivo monitoring method is still an urgent demand.

Recently, a kind of on-line determination of NO by flow injection and based on porphyrine compounds combined with chemiluminescence detection was

Table 3. Analytical parameters of the assay methods used for NO measurements in biological samples

A. *Electrochemical sensors*

Sensor type	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
New integrated porphyrinic sensor* [75]	amperometric differential voltametry pulse	10^{-8} M–10^{-9} M	At 630 mV response free of O_2, CO, CO_2, O_2^-	10 nM–200 μM		H_2O_2, nitrite
Integrated Shibucki-style electrode** [76]	amperometric	10–8 M	non-specific	10–400 nM		O_2, NO_2, N_2O, CO, CO_2
Enzymatic Shibucki-style electrode*** [89]	amperometric	5 μM	specific	10–150 μM	4.2	not reported
Cu–Pt CME-CF sensor [62]	amperometric	30 nM	not reported	80 nM–4.8 μM		not reported
CF, Cr(v-tpy)$_2$]$^{3+}$, Nafion-cathode sensor [52][#]	voltametric	submicromolar	not reported	0.57 μA μM^{-1}		O_2
Co-salen-microsensor[##] [98]	resistivity	not investigated	not investigated	not investigated	not investigated	not reported

 * Disadvantages: fragile gas-permeable membrane, relatively slow response time, expensive, tedious calibration, mechanical stability, fouling, severe temp. dependence; the new type is improved and able to be used for in vivo measurements. ** Disadvantages: very slow response time, high temp. coefficient not linear calibration curves above 1 μM, fragility of membrane, current saturation, baseline drift, interference; it is used for real-time analysis in samples of low NO concentrations and for short periods of time. *** Response time 5 min., life-time 4 days; not tested for real samples. [#] The sensor has a fast response time; it is used for NO determination released from cells. [##] Under investigation.

B. *Colorimetric/spectrophotometric methods and sensors*

Method	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
Griess reaction	spectrophotometric FIA modified method* [82]	500 nM	good	0,1–1 μM	very good	not repoted
	classical spectrophotometric [95]	0.1–2 ppb [95] $\varepsilon_{495} = 12000$ M^{-1} [99] 25–39 μM in blood [100]	good	up to 250 ppb	0.5–2 [95] 0.01 [99] 1.3–2.2 [100]	
Fe(II) hemoprotein reaction	kinetic method with the use of titer plates and fiber-optic spectrophotometer** [83]	5 pmol min^{-1} in 100 μL reaction volume	non-specific	not reported	not reported	not reported
OxyHb(Mb) reaction*** [54]	spectrophotometric diode array	maximal 25 nM 1 nM [102]	Non-specific	0.03–3 μM	5.6	oxidizing agents
ABTS[#] [77, 99]	spectrophotometric	$\varepsilon_{660} = 13000$ M^{-1} $\varepsilon_{750} = 16000$ M^{-1}	non-selective	max. conc. 100 μM	not reported	reducing compounds and radicals
[Fe(II)(SCN)]$^{+}$[##]	spectrophotometric FIA [101]	NO_2^-: 20 ppb NO_3^-: 13 ppb	not reported	NO_2^-: 0.3–3.0 ppm NO_3^-: 1.0–10.0 ppm	<10	not reported
Hemoprotein-fiber-optic sensor*[#] [40, 56]	fiber-optic sensor liquid phase	not reported	selective ccexcept for CO	10–400 μM [56]	0.28	not reported

(continued)

Table 3 (*continued*)

Method	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
Cu(II)(ECR)$_2$ fiber-optic sensor***# [113]	gas phase	8 µM	not reported	0–6 ppm	2.8	not reported

* It is used for NO det., NOS activity, and NOS inhibition measurements; the FIA modification enables the analysis of 40 samples per hour. ** Advantages: large data throughput, speed, precision, parsimony, high information yield, automation; it is used for measurements of NO release from biological systems. *** Applicable to biological systems to determine the rates of simultaneous evolution of NO/O$_2^{\bullet-}$. # Indirect method not used in vivo. ## 30 to 40 samples per hour. *# Earliest fiber-optic sensor.

C. *Fluorimetric probe assays and sensors*

Probe/Sensors	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
DAN* [78]	conventional fluorimetry	9 nM (pH = 11,7)	specific	10 nM–250 nM	not reported	negligible from O$_2^-$, nitrite
DAF** [41, 72, 61, 90]	conventional fluorimetry	5 nM [41]	highly specific	0.01–2.4 µM	poor [61] 0.005	Ca(II), Mg(II), light
TMDABODIPY*** [96]	fluorimetry	2.5 nM	high	0.01–0.4 µM	1.34	not reported
Fluorescein-Au coated fiber-optic sensor# [42]	fluorimetric sensor	20 µM	excellent	0.02–1.0 mM	>1.0	H$_2$O$_2$, O$_2^-$, ONOO–
Cytochrome c-Au coated fiber-optic sensor## [40]	fluorimetric sensor	20 µM	excellent	0.02–1.0 mM	≈1.0	rivoflavin, dopamine
Pyrrolidin-Fe(II) heme complex### [98]	fluorimetric probe	<100 nM	not reported	not reported	good	not reported

* It is used for quantitative measurements of NO generated by vasodilators, and by cultured cells under physiological conditions with minimal interference from nitrite decomposition; it is also used for selectivity measurements. Used in conjuction with HPLC for measurements of NO-metabolites from brain microdialysates [55]. ** Used in conjunction with RP-HPLC for detection of NO production by macrophages stimulated with LPS or other stimulants, and for real-time biological imaging of NO in cells; used in conjunction with a CCD camera or photomultiplier for measuring the fluorescence emission from porcine aortic endothelial cells [61]. *** Under investigation for biological samples. # The sensor was tested to measure NO production by BALB/c mouse macrophages. ## Promising. ### Not applied to biological samples in vivo.

D. *Chemiluminescence probe assays and sensors*

Probe	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
O$_3$* [88]	gas-FI-method	20 pmol [102]	nearly specific	8–186 pM	10 (interday)	
Luminol-H$_2$O$_2$**	solution-FI method	100 fM	non-specific	100 fM–1 nM		nitroso-compounds (minor)
NO-trap/ Luminol-H$_2$O$_2$*** [91]	solution-FI method	2.63×10^5 mVmol^{-1}	nearly specific	2.0×10^{-5}–1.0×10^{-2} mol	1.0	minor from nitrite, nitrate, oxygen, iodide, iodine
Membrane sampler/ Luminol-H$_2$O$_2$# [92]	solution-FI method	0.9×10^{-6} mol	nearly specific	1.8×10^{-6}–2.7×10^{-3} mol	5	minor from BME, DTT, ascorbate, H4BP NADPH

Table 3 (*continued*)

Probe	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
Microdialysis/ Luminol-H_2O_2[##] [93]	solution-FI method	1 nM	nearly specific	5.0 nM–1.0 μM		minor from small oxidizing or recucing molecules
Luminol-H_2O_2/ Sensor[###] [53]	solution-FI method	1.3 μM	non-specific	5.0–40 μM	5.5	minor from dopamine, uric acid, ascorbic acid, L-cysteine
Luminol-$ONOO^{-@}$ [94]	solution-FI method	4 mV per nM 10 pM (LOD)	nearly specific	8×10^{-11}– 1×10^{-7} M		minor from H_2O_2

* NO recovery depends on the sample matrix. Used for exhaled NO in several cases. The use of the method for biological fluids was applied with the use of head-space gas analysis. ** The method is free from nitrite nitrate and other substances biologically active on endothelium and endothelium derived compounds; it is mainly used for NO determination in perfusates [51]. ***The method is simple and rapid (about five samples per minute). [#] The method was tested with artificial blood samples. [##] The method was used for real-time monitoring of NO release in vivo after injection of drugs, NO activators, cofactors, etc. [###] Response time 8–17 s; disadvantage: consumption of internal solution; sensor life >2 weeks; membrane stability 3 months. [@] In this method the performance is compared to involvement of membrane sampler and no involvement; the mechanism of luminol CL emission and the kinetics of peroxynitrite decomposition are discussed.

E. Electron spin resonance probe assays

Probe	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
Fe(II) Heme-complexes* [84]	classical E.S.R.	1 nmol [102]	not-specific for certain specimen	not reported	10	P_{450}-NO is unstable, mixture of signals; other NO complexes in microsomal preparations of NO-SGC
Fe(II) Non Heme-complexes*[#] [84]	classical E.S.R.	1 nmol [102] high intensity	not specific for certain applications	not reported	not reported	in the case of $Fe(NO)_2L_2$ other paramagnetic species are present; exchange reaction between nitrosylated complexes
DETCs Fe(II) complexes** [84]	monitoring E.S.R.	DETC: 10 nM [103] MGD: 5 nM [106]	spin-specific	not reported		the ESR signal of $Fe(NO)(dithiol)_2$ is complicated by the endogenous $Cu(DETC)_2$ signal
NNO*** [84]	classical E.S.R.	1 nmol	spin-specific	semi-quantitative [107]		ascorbic and glutathione act as reductants to reduce NNO and INO to inactive hydrohylamines
PTIOs[#] [87]	classical E.S.R.	0.1 μM	selective not reactive with other NO-related molecular species	0.1–10 μM [110]		the free forms are susceptible to various reducing compounds that form N-hydroxyPTIOs and PTIs
NOCTs[##] [84]	classical E.S.R	not reported	spin-specific for •NO and •NO_2	detection	not reported	reduction of nitroxide by reductants in cellular systems

Table 3 (continued)

Probe	Mode	Sensitivity LOD	Selectivity	Valid range	%RSD	Interference
DBNBS[###] [86]	classical E.S.R	not reported	non specific	detection	not reported	unsaturated fatty acids, some amino acids cause C-centered radical adducts through nonradical reactions
Nitroxyl probe[***#] [95]	ESR_computed tomography	not reported	non-specific	imaging	not reported	reductants, O_2, ROS

* Interpretation of ESR spectra can be complex because of dynamic equilibrium between conformers; low S/N ratio, which improves at low temperatures but exhibits powder pattern signal. Used for detection of the NO mainly: in blood or blood perfusate samples under different treatment conditions of the living organism; in heart tissues; in liver. [*#] Used mainly in biological systems to examine NO influence on the activity of certain enzymes and on the release of iron from ferritin. The NO–Fe–S type signal was observed in cytokine-activated macrophages, pancreatic islets, vascular smooth cells, tumor target cocultured with activated macrophages, and hepatocytes exposed to inflammatory stimuli. [**] Used for continuous monitoring of NO generation from NO donors and enzymatic generation; in vivo NO formation in different organs during ischemia. The Fe(II)(dithiol)$_2$ complexes have higher affinity for NO relative to O_2 and in vivo measurements in real-time are feasible, while Fe(II)(NO)(dithiol)$_2$ complexes are stable in the presence of O_2, and specifically the Fe(II)(NO)(MGD)$_2$ complex can be quantitated. The dithiolate ligands are toxic, especially the lipid soluble and dithiols inhibit the activity of NO-synthase and SOD enzymes. [***] The NNO (reagent) and INO (product) produce different ESR signals; used as probes for investigating the mechanism of vasodilatory action of nitrovasodilators and spin traps; as antagonist in perfused organs. The ESR assay is continuous. The products of the NNO reaction (•NO$_2$, N$_2$O$_3$) can be toxic. [#] Some PTIOs commercially available are not satisfactory for quantitative work due to impurities. The radicals are very stable in aqueous solutions. Liposome protects the PTIOs from the reducing action of reductants. Quantitation is obtained by double integration of experimental signals after normalization with the MnO signal, and very good linear correlation is obtained: The ESR signals of PTIOs and PTIs are somewhat broadened by liposome encapsulation. Used for quantitation of NO release from cells in culture. The S/N can be improved by repeated sweeps in the magnetic field. [##] The rate of formation can be measured; spin concentration can be measured by double integration of the nitroxide and comparison with the spin standard; both •NO and •NO$_2$ can be measured; it is best suited for measurements in lipid phase; imaging in tissues is likely. The compounds are not commercially available; the reduction in cellular systems is a common problem; the trap is generated by photolysis. Used for monitoring NO generation in extracts of cultured liver macrophages (Kupffer cells) and from NO-releasing agents. [###] The obtained spectra are a mixture of more than one signal and need to be simulated by an appropriate computer program for analysis. Caution: several radicals can be trapped; resulting spin adducts are not stable and decompose to radical and non-radical products. Used: for measuring NO formation platelet-related reaction. [***#] Nitroxyl radicals lose their ESR signal by reaction with free radicals through conversion to oxoammonium ion, secondary ammine or hydroxylamine; nitroxyl probes that pass the blood-brain barrier have been synthesized to be used for in vivo measurements in the brain.

developed in our lab. Porphyrine-containing biomolecules, such as hemoglobin, HRP (horseradish peroxidase), cytochrome c, and catalase were tested for their affinity to NO. In this method, the NO is extracted on-line from the biological fluid (such as blood) and brought to the chemiluminescence reaction system for detection. By changing the reaction conditions, the porphyrines are denatured and lose the ability to generate light with the luminol-H_2O_2 system, while the NO is set free. When using this method, the NO half-life time is extended, yielding higher stability, and by using a membrane sampler based on selective trapping of NO from biological fluids, we obtained the highest sensitivity and selectivity [91, 92]. Based on these findings, a new method was developed by employing a microdialysis sampler coated with a perm-selective membrane and chemiluminescence detection. High sensitivity is achieved under optimal conditions and sampling time improvement. This system was used in vivo to monitor the variation of NO in blood and brain tissue of rats and rabbits and proved very successful. The system is sensitive enough to detect variations of NO formation under different pathophysiological conditions. The linear valid range of NO determination is 5 nM–1 μM with a detection limit of 1 nM; real NO concentrations in animals are found to be in the range of 1–5 nM or even less [93]. Further improvement of the above method was reported by using the same FI/CL detection apparatus but with peroxynitrite-luminol CL reagent; reaching a detection limit of 10^{-10} M with dialysis membrane sampler and 10^{-11} M without the sampler [94]. To our knowledge, this is the first system capable of monitoring NO concentrations in real time and in vivo. We anticipate that this system might be useful for NO detection in a number of different fields.

Conclusion

Undoubtedly, NO plays a very important role in the human body with different functions under physiological or pathological conditions. Recent research work produced inconsistent or even contradictory results. Better methods for determination of NO are needed in order to understand its involvement in biological reaction paths and its functions under physiological and pathophysiological conditions.

References

[1] Djamgoz M B A, Sekaran S, Angotzi A R, Haamedi S, Vallerga S, Hirano J, Yamada M (2000) Philos Trans Roy Soc Lond B 355: 1199–1203

[2] Buzard G S, Kasprzak K S (2000) J Environ Pathol Toxicol Oncol 19: 179–199

[3] Ashutosh K (1977) Chest 111: 4–7

[4] Conway P J, Jones N S (2000) Clin Otolaryngol Allied Sci 25: 337–341

[5] Lecomte P V, De Mulder P A, Adriaensen H F, De Hert S G (1999) Acta Anaesthesiol Belgica 50: 105–112

[6] Willenborg D O, Staykova M A, Cowden W B (1999) J Immunol 163: 5278–5286

[7] Mavroudis C (1999) Crit Care Med 27: 877–877

[8] Perner A, Rask-Madsen J (1999) Aliment Pharmac Therapeut 13: 135–144

[9] Zidek Z, Maek K (1998) Intern J Immunopharmacol 20: 319–343

[10] Weinberg J B (1998) Mol Med 4: 557–591

[11] Pieper G M (1998) Hypertension 31: 1047–1060

[12] De Groote K, Van Overmeire B (1998) Tijdschrift voor Geneeskunde 54: 817–823

[13] Weight S C, Nicholson M L (1998) Eur J Vas Endovas Surg 16: 98–103

[14] Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V (1998) J Invest Dermatol 110: 1–7

[15] Goldman A, Brown L R, Schoenfeld W G, Spencer M N, Chackerian C Jr, Giver L P, Dothe H, Rinsland C P, Coudert L H, Dana V, Mandin J-Y (1998) J Quantit Spectr Rad Trans 60: 825–838

[16] Aarna I, Suuberg E M (1997) Fuel 76: 475–491

[17] Petr V, Gustav E (1997) Collect Czech Chem Commun 62: 1355–1384

[18] Krafft P, Metnitz P G H, Fridrich P, Krenn C G, Hammerle A F, Steltzer H (1997) Clin Intens Care 8: 27–32

[19] Burnett A L (1995) Urology 45: 1071–1083

[20] Barton M (2000) Cardiovasc Res 46: 20–23

[21] Zhang R, Hirsch O, Mohsen M, Samuni A (1994) Arch Biochem Biophys 312/2: 385–391

[22] Athena W X, Ziegler D M (1991) Anal Biochem 198: 143–148

[23] Bechman J S, Beckman T W, Chen J, Marshall P A, Freeman B A (1990) Proc Natl Acad Sci USA 87: 1620–1624

[24] Tsubaki M, Yu N T (1982) Biochem 21/6: 1140–1144

[25] Benko B, Yu N T (1983) Proc Natl Acad Sci USA 80: 7042–7046

[26] Bredt D S, Snyder S H (1989) Proc Natl Acad Sci USA 86: 9030–9033

[27] Furchgott R F, Zawadzki J V (1980) Nature 288/27: 373–376

[28] Lopez B M, Noval J A, Bonet L G G, Estrada C (2000) Brain Res 869: 244–250

[29] Bonner F T (1996) Meth Enzymol 268: 50–57

[30] Wink D A, Grisham M B, Mitchell J B, Ford P C (1996) Meth Enzymol 268: 12–31

[31] Nappi A J, Vass E (1997) Biochim Biophys Acta 1336: 295–301

[32] Gryglewski R J, Palmer R M J, Moncada S (1986) Nature 320: 454–456

[33] Palmer R M J, Ashton D S, Moncada S (1988) Nature 333: 664–666

[34] Knowles R G, Palacios M, Palmer R M, Moncada S (1989) Proc Natl Acad Sci USA 86: 5159–5162

[35] Ohshima H, Oguchi S, Adachi H, Iida S, Suzuki H, Sugimura T, Esumi H (1992) Biochem Biophys Res Commun 183/1: 238–244

[36] Evmiridis N P, Yao D (2000) Anal Chim Acta 410: 167–175

[37] Rivot J P, Barraud J, Montecot C, Jost B, Besson J M (1997) Brain Res 773: 66–75

[38] Meyer J, Richter N, Hecker M (1997) Anal Biochem 247: 11–16

[39] Mesaros S, Grunfeld S, Mesarosova A, Bustin D, Malinski T (1997) Anal Chim Acta 339: 265–270

[40] Barker S L R, Kopelman R, Meyer T E, Cusanovich M A (1998) Anal Chem 70: 971–976

[41] Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T (1998) Anal Chem 70: 2446–2453

[42] Barker S L R, Kopelman R (1998) Anal Chem 70: 4902–4906

[43] Giraldez R R, Zweier J L (1998) Anal Biochem 261: 29–35

[44] Sonoda M, Kobayashi J, Takezawa M, Miyazaki T, Nakajima T, Shimomura H, Koike K, Satomi A, Ogino H, Omoto R, Komoda T (1997) Anal Biochem 247: 417–427

[45] Kosaka H, Tanaka S, Yoshii T, Kumura E, Seiyama A, Shiga T (1994) Biochem Biophys Res Commun 204: 1055–1060

[46] Drapier J-C, Pellat C, Henry Y (1991) J Biol Chem 266: 10162–10167

[47] Green L C, Wagner D A, Glogowski J (1982) Anal Biochem 126: 131–138

[48] Pai T G, Payne W J, Legall J (1987) Anal Biochem 166: 150–157

[49] Mordvintcev P, Mulsch A, Busse R, Vanin A (1991) Anal Biochem 199: 142–146

[50] Malinski T, Taha Z (1992) Nature 358: 676–678

[51] Kikuchi K, Nagano T, Hayakawa H, Hirata Y, Hirobe M (1993) Anal Chem 65: 1794–1799

[52] Maskus M, Pariente F, Wu Q, Toffanin A, Shapleigh J P, Abruna H D (1996) Anal Chem 68: 3128–3134

[53] Zhou X, Arnold M A (1996) Anal Chem 68: 1748–1754

[54] Kelm M, Dahmann R, Wink D, Feelisch M (1997) J Biol Chem 272/15: 9922–9932

[55] Woitzik J, Abromeit N, Schaefer F (2001) Anal Biochem 289: 10–17

[56] Blyth D, Ayllot J W, Moir J W B, Richardson D J, Russel D A (1999) Analyst 124: 129–134

[57] Liu Z-G, Liu X-C, Yim A P C, He G-W (2001) Ann Thorac Surg 71: 133–137

[58] Kitamura Y, Uzawa T, Oka K, Komai Y, Ogawa H, Takizawa N, Kobayashi H, Tanishita K (2000) Anal Chem 72: 2957–2962

[59] McMurtry M S, Kim D H, Dinh-Xuan T, Archer S L (2000) Analysis 28: 455–465

[60] Xian Y, Zhang W, Xue J, Ying X, Jin L (2000) Anal Chim Acta 415: 127–133

[61] Berkels R, Dachs C, Roesen R, Klaus W (2000) Cell Calcium 27: 281–286

[62] Xian Y, Zhang W, Xue J, Ying X, Jin L (2000) Analyst 125: 1435–1439

[63] Yamada K, Nabeshima T (1998) Microsc Res Techniq 43: 68–74

[64] Lopez B L, Davis-Moon L, Ballas S K, Ma X-L (2000) Am J Hematol 64: 15–19

[65] Combet S, Balligand J-L, Lameire N, Goffin E, Devuyst O (2000) Kidney Int 57: 332–338

[66] Piacentini G L, Suzuki Y, Bodini A (2000) BioDrugs 13: 279–288

[67] Tsukahara H, Hiraoka M, Kobata R, Hata I, Ohshima Y, Jiang M Z, Noiri E, Mayumi M (2000) Redox Report 5: 23–28

[68] Silkoff P E (2000) Clin Chest Med 21: 345–360

[69] Bruckheimer E, Rimar S, Dubois A B, Douglas J S (2000) J Clin Monitor Comput 16: 21–23

[70] Palm J P, Graf P, Lundberg J O N, Alving K (2000) Eur Respir J 16: 236–241

[71] Tuncel M, Dogrukol-Ak D, Erkasap N (2001) J Chromatogr B 751: 257–263

[72] Itoh Y, Ma F H, Hoshi H, Oka M, Noda K, Ukai Y, Kojima H, Nagano T, Toda N (2000) Anal Biochem 287: 203–209

[73] Pinelli E, Gebhard D, Mommaas A M, Hoeij M, Langermans J A M, Ruitenberg E J, Rutten V P M (2000) Veterin Parasitol 92: 181–189

[74] Leshem Y Y, Pinchasov Y J (2000) Exp Botany 51: 1471–1473

[75] Malinski T, Mesaros S, Tomboulian P (1996) Meth Enzymol 268: 58–69

[76] Christodoulou D, Kudo S, Cook J A, Krishna M C, Miles A, Grisham M B, Murugesan R, Ford P C, Wink D A (1996) Meth Enzymol 268: 69–83

[77] Nims R W, Cook J C, Krishna M C, Christodoulou D, Poore C M, Miles A M, Grisham M B, Wink D A (1996) Meth Enzymol 268: 93–105

[78] Miles A M, Wink D A, Cook J C, Grisham M B (1996) Meth Enzymol 268: 105–120

[79] Wink D A, Grisham M B, Miles A M, Nims R W, Krishna M C, Pacelli R, Teague D, Poore C M B, Cook J A, Ford P C (1996) Meth Enzymol 268: 120–130

[80] Wishnok J S, Glogowski J A, Tannenbaum S R (1996) Meth Enzymol 268: 130–141

[81] Granger D L, Taintor R R, Boockvar K S, Hibbs J B Jr (1996) Meth Enzymol 268: 142–151

[82] Yokoi I, Habu H, Kabuto H, Mori A (1996) Meth Enzymol 268: 152–159

[83] Gross S S (1996) Meth Enzymol 268: 159–168

[84] Kalyanaraman B (1996) Meth Enzymol 268: 168–188

[85] Osipov A N, Gorbunov N V, Day B W, Elsayed N M, Kagan V E (1996) Meth Enzymol 268: 193–203

[86] Ichimori K, Arroyo C M, Nakazawa H (1996) Meth Enzymol 268: 203–211

[87] Akaike T, Maeda H (1996) Meth Enzymol 268: 211–222

[88] Brien J F, Mclaughlin B E, Nakatsu K, Marks G S (1996) Meth Enzymol 268: 83–93

[89] Campanella L, Tomassetti L P M (2000) Sens Actuators B 68: 351–359

[90] Broillet M C, Randin O, Chatton J Y (2001) FEBS Lett 491: 227–232

[91] Yao D, Vlessidis A G, Evmiridis N P (2001) Anal Chim Acta 435: 273–280

[92] Yao D, Prodromidis M I, Vlessidis A G, Karayannis M I, Evmiridis N P (2001) Anal Chim Acta 450: 63–72

[93] Yao D, Vlessidis A G, Evmiridis N P, Evangelou A, Karkabounas S, Tsampalas S (2002) Anal Chim Acta 458: 281–289

[94] Kang Dai, Vlessidis A G, Evmiridis N P (2003) Talanta 59: 55–65

[95] (a) Utsumi H, Yamada K (2003) Arch Biochem Biophys 416: 1–8; (b) Koshiishi I, Sato R, Utsumi H (2003) Development of novel nitroxyl spin probe for evaluation of $O_2^{\bullet-}$, NO, and ONOO-generations by ESR/spin probe method, submitted

[96] Zhang X, Wang H, Li J-S, Zhang H-S (2003) Anal Chim Acta 481: 101–108

[97] Shioya T, Swager T M (2002) Chem Commun 13: 1364–1365

[98] Soh N, Imato T, Kawamura K, Maeda M, Katayama Y (2002) Chem Commun 22: 2650–2651

[99] Nims R W, Darbyshire J F, Saaverda J E, Christodoulou D, Hanbauer I, Cox G W, Grisham M B, Laval F, Cook J A, Krishna M C, Wink D A (1995) Methods 7: 48–54

[100] Guevara I, Iwanejko J, Dembinska-Kiec A, Pankiewicz J, Wanat A, Anna P, Golabek I, Bartus S, Malczewska-Malec M, Szczudlik A (1998) Anal Chim Acta 274: 177–188

[101] Andrade R, Viana C O, Guadagnin S G, Reyes F G R, Rath S (2003) Food Chem 80: 597–602

[102] Archer S (1993) FASEB J 7: 349–360

[103] Tsuchiya K, Takasugi M, Minakuchi K, Fukuzawa K (1996) Free Radic Biol Med 21: 733–737

[104] Fujii H, Berliner L J (1999) Mag Resonance Med 42: 599–602

[105] Komarov A, Mattson D, Jones M M, Singh P K, Lai C-S (1993) Biochem Biophys Res Commun 195: 1191–1198

[106] Reinke L A, Moore D R, Kotake Y (1996) Anal Biochem 243: 8–14

[107] Kotake Y, Moore D R, Sang H, Reinke L A (1999) Nitric Oxide 3: 114–122

[108] Lecour S, Maupoil V, Zeller M, Laubriet A, Briot F, Rochette L (2001) J Cardiovasc Pharmac 37: 55–63

[109] Korth H-G, Sustmann R, Lommes P, Paul T, Ernst A, de Groot H, Hughes L, Ingold K U (1994) J Am Chem Soc 116: 2767–2777

[110] Yoshimura Y, Maeyama H, Nakazawa H (2001) Anal Sci [Suppl] 17: i519–i522

[111] Khan N, Swartz H (2002) Mol Cell Biochem 234/235: 341–357

[112] Bedioui F, Villeneuve N (2003) Electroanalysis 15: 5–18

[113] Dacres H, Narayanaswamy N (2003) Sens Actuators B 90: 222–229

[114] Menon N K, Patricza J, Binder T, Bing R J (1991) J Mol Cell Cardiol 23: 389–393

[115] Luchsinger B P, Rich E N, Gow A G, Williams E M, Stamler J S (2003) Proc Natl Acad Sci USA 100: 461–466