**Title:** NITRIC OXIDE-SPECIFIC ELECTRODE

**Abstract**

An electrode sensor which may be used to specifically and quantitatively measure nitric oxide is provided, as well as a method of preparing and using such an electrode sensor to measure nitric oxide concentration in solution.
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NITRIC OXIDE-SPECIFIC ELECTRODE

FIELD OF THE INVENTION

This invention relates to an electrode sensor that detects nitric oxide. The electrode sensor may be used to specifically and quantitatively measure nitric oxide concentration. In addition, this invention relates to a method of manufacturing the electrode sensor and a method of using the electrode sensor to detect and/or measure nitric oxide in a sample.

BACKGROUND OF THE INVENTION

Nitric oxide (NO) is a key bioregulatory molecule that plays critical roles in the regulation of various biological processes, including the normal physiological control of blood pressure, macrophage-induced cytostasis and cytotoxicity, inhibition of platelet aggregation, and neurotransmission (Moncada et al., Pharmacological Reviews 43(2): 109-142. 1991.). Many tissues in the body endogenously release NO in different amounts (Marletta, Chem. Res. in Toxicology 1(5): 249-257. 1988.; Biochemistry 27: 8706-8711. 1988.) but the actual amounts released are very difficult to quantify. In addition, many diseases, such as endotoxic shock, ischemia reperfusion injury, genetic mutations, which include deamination-related genetic diseases (Wink et al., Science 254: 1001-1003. 1991.) like deamination of cytosine to thymine, cancer, male impotence, and atherosclerosis have been suggested to be caused by defects in the production and/or regulation of NO (Moncada et al.; Masini et al., Agents and Action 33: 53-56. 1991.). Also, drugs, including xenobiotics, can be metabolized to give NO either as the effector molecule or as a harmful metabolite (Feelisch, J. Cardiovasc. Pharmacol. 17: S25-S33. 1991.; Ignarro et al., Biochem. Biophys. Res. Comm. 94: 93-100. 1980.; Servent et al., Biochem. Biophys. Res. Comm. 163: 1210-1216. 1989.; and Haussmann et al., In: Relevance of N-Nitroso Compounds to

The importance of the bioregulation effected by NO is further evidenced by the recent rash of pharmaceutical companies designing drugs around NO. It is hoped that drugs can be developed to control blood pressure, prevent atherosclerosis, treat migraine headaches and impotence, prevent deaths from septic shock, and help protect brain cells threatened by degenerative diseases and strokes.

Accordingly, the ability to specifically and quantitatively measure NO concentrations in solutions, particularly aqueous solutions of biological media, both in vitro and in vivo, and chemical media would be highly advantageous. The ability to measure the concentration of NO by a nondestructive method is an important requirement for further investigation of the mode of action of NO as a key bioregulatory molecule and for the development of therapeutic applications of NO-releasing compounds. Several techniques have been employed to determine the concentration of NO in solution.

One method employs an automated system that analyzes nitrate by reduction with a high-pressure cadmium column to determine amounts of nitrate and/or nitrite in urine, saliva, deproteinized plasma, gastric juice, and milk samples (Green et al., Analytical Biochemistry 126: 131-138. 1982.). The lower limit of detection of the method is said to be 1.0 nmol NO₃⁻ or NO₂⁻/ml. The system reportedly allows quantitative reduction of nitrate and automatically eliminates interference from other compounds normally present in biological fluids. Most samples may be prepared by simple dilution with distilled water, and samples reportedly may be analyzed in an hour. The disadvantage of such a technique in measuring NO is that it does so indirectly, by measuring NO byproducts, which also can be generated from other sources. Accordingly, such a
method is not very accurate in determining NO concentration.

Another method quantitatively analyzes nitrite, an oxidation product of NO, in human plasma to determine NO concentration (Wenkmalm et al., Analyt. Biochem. 187: 359-363. 1990.). Dithionite is used to treat the samples of human plasma to convert nitrite to nitric oxide, with the treated samples being passed over bovine hemoglobin columns. NO is allowed to bind the hemoglobin in columns of bovine hemoglobin covalently bound to agarose. An excess of dithionite is used to ensure that the hemoglobin is reduced to a ferrous, nonoxygenated state. The NO bound to the hemoglobin forms a complex on the column, and the column is then subjected to electron paramagnetic resonance spectrometry, i.e., the column is subjected to a magnetic field and microwave radiation to obtain a characteristic electron paramagnetic resonance spectrum. This method suffers from the same disadvantages as the previously described method. NO concentration is determined indirectly, through the measurement of nitrite. Also, the NO is modified by binding to hemoglobin covalently bound to agarose.

Other methods employed to quantitate NO include chemiluminescence, mass spectrometry (Bazylnski et al., Inorg. Chem. 24: 4285-4288. 1985.), and ultraviolet-visible light spectral changes. In one procedure utilizing chemiluminescence, NO has been quantified by chemiluminescence resulting from the product of NO and ozone (Palmer et al., Nature 327: 524-526. 1987.; Maragos et al., J. Med. Chem. 34: 3242-3247. 1991.). This method also involves modification of NO, in this case by reaction with ozone. In a procedure employing ultraviolet and visible light, spectral changes have been monitored for the conversion of oxyhemoglobin to methemoglobin by NO as an indication of NO concentration (Haussmann et al.). NO is modified in this method by reaction with oxyhemoglobin.
Accordingly, neither one of these methods enables the measurement of NO directly.

Solution methods have been also used to measure NO but seem to lack specificity for NO or reliable quantitation. The use of 3,5-dibromo-4-nitrosobenzene sulphonate (DBNBS) as a spin trap in an electron spin resonance technique to detect NO in a biological system has been reported (Arroyo et al., Biophys. Res. Comm. 170: 1177-1183. 1990.). This method, consequently, involves reaction of NO with modified spin traps. Subsequently, it was demonstrated that the obtained signal may result from simple oxidation of the spin trap, which raises the issue of how specific the spin trap is for NO (Wink et al., Radiat. Phys. Chem. 38: 467-472. 1991.). The use of Fe$^{2+}$ (dithiolate) to trap NO as the nitrosyl also has been used in a spin resonance technique (Mulsch et al., FEBS Letter 294: 252-256. 1991.); however, this technique is not suitable for quantitation due to a lack of biological stability, i.e., the resulting nitrosyl has a half-life of only about 30 seconds in biological systems. Further, it is evident that this method involves the modification of NO by formation of a complex with iron. The iron complex is metabolized, i.e., destroyed, during the process. Also, this method suffers from nitrite interference.

More recently, a modified oxygen electrode has been used to detect NO (Shibuki et al., Neuroscience Res. 9: 69-76. 1990.; Nature 349: 326-328. 1991.). The electrochemical microprobe was developed to detect the release of NO in brain tissue. The output current of the probe was found to correlate linearly with the concentration of NO at the tip. The sensitivity of the probe was reportedly between 3.5 and 106 pA/µM change in NO concentration. However, the validity of this technique has been questioned due to the small current that has been observed (<0.5 pA) and the lack of use of standards at submicromolar concentrations of NO. In addition, the technique measures NO by its oxidation to nitrite, and
those who developed the modified oxygen electrode claim that NO is spontaneously released from sodium nitroprusside and that the release is accurately measured by the electrode. This contradicts what has been shown previously by others, i.e., that sodium nitroprusside does not spontaneously release NO in buffer (Kruszyna et al., Toxicol. Appl. Pharmacol. 91: 429-438. 1987.; Wilcox et al., Chem. Res. Toxicol. 3: 71-76. 1990.), which raises the issue of specificity of this method.

There is a need, therefore, for a method of detecting and measuring NO concentration in a sample in a specific, quantitative, and reproducible manner. The present invention provides an electrode sensor that detects NO in a sample. The electrode sensor may be used to detect and/or measure NO specifically and quantitatively in a variety of solutions, such as aqueous solutions of biological media, both in vitro and in vivo, and chemical media. Accordingly, this invention also provides a method of measuring NO concentration, which utilizes the present inventive electrode sensor, and a method of manufacturing the electrode sensor.

**SUMMARY OF THE INVENTION**

The present invention provides an electrode sensor that detects NO. The electrode sensor may be used to specifically and quantitatively measure NO in a variety of solutions, such as aqueous solutions of biological media, both in vitro and in vivo, and chemical media. This invention also provides a method of measuring NO concentration, which utilizes the present inventive electrode, and a method of manufacturing the electrode.

The present invention further provides a means of monitoring NO production or inhibition effected by drugs and in the design of drugs for the treatment of diseases related to defects in NO regulation and/or production, both in vitro and in vivo. The ability to monitor NO production or inhibition is also useful as a means of detecting and
quantifying defects in NO regulation and/or production, both \textit{in vitro} and \textit{in vivo}, which result from disease, injury, and mutation.

The present invention additionally provides a means of monitoring pollution of which NO is a component.

These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

\textbf{BRIEF DESCRIPTION OF THE DRAWINGS}

Figure 1A is a drawing of the structure of tetramethylpyridylporphyrin (TMPP).

Figure 1B is a drawing of the structure of protoporphyrin IX dimethyl ester (DME).

Figure 2 is a differential pulse voltammogram of current (i, nA) versus potential (V) for 10 \textmu M, 20 \textmu M, and 40 \textmu M NO in 0.1 M phosphate buffer, pH = 7.4, at room temperature, prepared using a glassy carbon electrode (GCE)/Ni(II) TMPP electrode coated with Nafion\textsuperscript{®} (Aldrich Chemical Co., Milwaukee, WI) at an Epa = 0.7 V vs. a standard calomel electrode (SCE).

Figure 3 is a graph of current (i, nA) versus NO concentration (\textmu M) prepared using a GCE/Ni(II) TMPP electrode coated with 4\textmu l Nafion\textsuperscript{®}, which shows the NO response of NO solutions of varying NO concentration in 0.1 M phosphate buffer, pH = 7.4, prepared from a saturated solution of NO at room temperature.

Figure 4 is a graph of current (i, nA) versus DEANO concentration (\textmu M) prepared using a GCE/Ni(II) TMPP electrode coated with 4\textmu l Nafion\textsuperscript{®}, which shows the NO response of DEANO solutions of varying DEANO concentration in 0.1 M phosphate buffer, pH = 7.4, prepared from a 11.2 mM solution of DEANO in 0.01 M NaOH at room temperature.

Figure 5 is a graph of current (i, nA) versus NO concentration (\textmu M) prepared using a GCE/Fe(III) TMPP electrode coated with 4\textmu l Nafion\textsuperscript{®}, which shows the NO
response of NO solutions of varying NO concentration in 0.1 M phosphate buffer, pH = 7.4, prepared from a saturated solution of NO at room temperature.

Figures 6A and 6B are graphs of current (i, µA) versus NO concentration (M) prepared using a 0.0707 cm² GCE/Fe(III) DME electrode and linear sweep voltammetry (scan rate = 20 mV/s), which shows the NO response of NO solutions of varying NO concentration in 0.1 M phosphate buffer, pH = 7.4, prepared from a saturated solution of NO at room temperature.

Figure 7 is a graph of current versus (i, µA) versus potential (V) prepared using a GCE/Fe(III) DME electrode and cyclic voltammetry (scan rate = 20 mV/sec), which shows the current response of NO solutions of varying NO concentration in 0.1 M phosphate buffer, pH 7.2, at room temperature.

Figures 8A and 8B are graphs of current (i, µA) versus potential (V) prepared using a GCE electrode and a GCE/Fe(III) DME electrode, respectively, and cyclic voltammetry (scan rate = 20 mV/sec), which shows the current response of 2 mM NO solution in 0.1 M phosphate buffer, pH 7.2, at room temperature.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides an electrode sensor that detects NO, as well as methods of making and using such an electrode sensor. The electrode may be used to specifically and quantitatively measure NO in a variety of solutions, particularly aqueous solutions of biological media, both in vitro and in vivo, and chemical media.

Specifically, the nitric oxide-specific electrode sensor comprises an electrically conductive substrate, whose amperometric response is substantially unaffected by the presence of nitric oxide, and an adherent and substantially uniform electrochemically active polymeric coating which interacts with NO in such a manner so as to
cause a change in the redox potential of NO and the electrode sensor.

The electrically conductive substrate is preferably electrically conductive carbon, indium tin oxide, iridium oxide, nickel, platinum, silver, or gold. The electrically conductive substrate is preferably electrically conductive carbon, such as basal plane carbon, pyrolytic graphite (BPG), or glassy carbon. The preferred electrically conductive substrate will depend in part on whether oxidation or reduction at the electrode sensor will be taking place during use. For example, a noble metal such as platinum or gold could evolve hydrogen from water reduction which could adversely affect the polymer film(s) on the substrate. The electrically conductive substrate is most preferably glassy carbon.

The electrochemically active polymeric coating may be any suitable polymer which interacts with NO in such a manner so as to cause a change in redox potential of NO and the electrode sensor. The change in the observed current drawn through the electrode sensor at a particular potential can be correlated to the concentration of NO in the sample being evaluated.

The electrochemically active polymeric coating will typically be comprised of a metallized or doped polymer, such as metallized polymeric porphyrins, metallized polyphthalocyanines, polyvinylmetallocenes, metallized polyacetylenes, metallized polypyrrolines, and polymeric substituted glyoximes. The metallized or doped polymer may contain any suitable metal which will interact with NO, such as transition or amphoteric metals and preferably nickel, cobalt, or iron.

The electrochemically active polymeric coating is preferably a metallized polymeric porphyrin or a metallized polyphthalocyanine, most preferably a metallized polymeric porphyrin. The metallized polymeric porphyrin compounds should not form metal-oxo bridges (M=O-M) with the substrate. Suitable porphyrin compounds include pyrroles,
pyridines, and ether porphyrins. The electrochemically active polymeric coating is most preferably comprised of the metallized polymeric porphyrin compounds of tetramethyl pyridine pyrrole and dimethyl ester porphyrin, especially tetramethyl pyridine pyrrole (TMPP) and dimethyl ester porphyrin (DME) metallized with nickel, cobalt, and iron. These most preferred metallized porphyrin compounds of TMPP and DME are respectively depicted in Figures 1A and 1B, wherein M is any suitable metal ion, such as Ni$^{2+}$, Co$^{2+}$, or Fe$^{3+}$, X is a suitable anion to render the compound neutral, such as ClO$_4^-$ in the case of TMPP and Cl$^-$ in the case of DME, and n is an integer sufficient to render the compound neutral, such as 4 in the case of Ni$^{2+}$ and Co$^{2+}$ TMPP, 5 in the case of Fe$^{3+}$ TMPP, and 1 in the case of Fe$^{3+}$ DME (not shown). The particular TMPP and DME compounds utilized in the examples herein are depicted in Figures 1A and 1B.

The electrochemically active polymeric coating should be adherent to and substantially uniform over the substrate. While the polymeric coating may be of any suitable thickness, it is preferably between about 0.01 $\mu$m and about 50 $\mu$m in thickness.

It will be appreciated by one who is skilled in the art that electrochemically active polymeric coatings will differ in affinity for various substrates. The electrochemically active polymeric coating as used in the present inventive electrode sensor preferably has a high affinity for the particular substrate being used.

The electrode sensor may additionally comprise a gas-permeable membrane coating that is permeable to NO but excludes interfering ions and compounds, particularly nitrite. The gas-permeable membrane coating may be of any suitable material, preferably a perfluorinated compound such as Nafion® (Aldrich Chemical Co., Milwaukee, WI). The layer of gas-permeable membrane coating deposited onto the surface of the electrode sensor may be of any suitable thickness, preferably about 0.5-50 $\mu$m. The resulting membrane-coated NO electrode sensor is more selective to NO
than an uncoated electrode due to the membrane exclusion of interfering species such as nitrite.

One who is skilled in the art will appreciate that the sensor of the present invention may vary in size, according to the particular application at hand. Therefore, the present inventive sensors may range from macroelectrode sensors to microelectrode sensors and nanoelectrode sensors, utilizing, for example, the edges of carbon films and suitable metal films, as well as carbon fibers. Such submicron size electrodes are more suitable for intracellular and in vivo use.

The present inventive nitric oxide-specific electrode sensors may be prepared in any suitable manner. An adherent and substantially uniform coating of an electrochemically active polymer as previously described is formed on a surface of an electrically conductive substrate as also previously described, by any suitable means, preferably by electrolytic polymerization.

The precursor (e.g., monomer, dimer, or oligomer) used to form the electrochemically active polymeric coating can be electrolytically polymerized onto a surface of the electrically conductive substrate by immersing the substrate in an appropriate electrolyte solution containing the precursor in combination with a supporting electrolyte. The electrolyte solution will typically additionally contain a suitable solvent. Examples of solvents that may be used in the electrolyte solution include acetonitrile, methanol, dimethyl formamide, dimethyl sulfoxide, propylene carbonate, and the like. The supporting electrolyte preferably is a perchlorate, sulfuric acid, phosphoric acid, boric acid, tetrafluoro-potassium phosphate, quaternary ammonium salt, or similar compound.

The coating of the gas-permeable membrane as previously described, e.g., Nafion®, may be applied onto the sensor by any suitable means. For example, a solution of the membrane material, e.g., Nafion®, may be used to coat the electrode, and the electrode then may be allowed
to dry so as to produce a uniform film. The sensitivity of the gas-permeable membrane-coated electrode sensor is further increased by soaking the electrode sensor in a sodium hydroxide solution for at least about 24 hours and preferably a few days.

The present invention also provides a method of detecting and/or measuring NO concentration in a sample by utilizing the present inventive electrode sensor. NO will directly interact with or bind the polymeric coating on the substrate, thereby changing the redox potential of NO and the electrode sensor so as to change the current drawn through the electrode sensor when employed as a working electrode at a particular potential in a manner related to the concentration of NO in the sample being evaluated. For example, metalloporphyrins, which contain metals such as iron, manganese, nickel, and cobalt, are capable of binding NO and are believed to form metal nitrosyls which provide a different oxidation or reduction potential than NO or the electrode sensor alone. The NO concentration in a sample may be determined by comparing the observed current drawn through the electrode sensor as a working electrode at a fixed potential with the currents observed at the same potential using samples of known NO concentration.

The method of detecting the presence or absence of NO in a sample, therefore, comprises connecting the nitric oxide-specific electrode sensor of the present invention to a potentiostat, such as Model 273 of Princeton Applied Research, calibrating the potentiostat and electrode sensor for a sample known to be devoid of NO, and detecting the presence or absence of nitric oxide in an unknown sample by comparing the measured current to the current for the sample known to be devoid of NO. A change in the observed current indicates the presence of NO in the unknown sample.

Similarly, the method of measuring the concentration of NO in a sample comprises connecting the nitric oxide-specific electrode sensor of the present invention to a potentiostat, calibrating the potentiostat and electrode
sensor for samples of known NO concentration, and measuring NO concentration in an unknown sample by comparing the measured current to the current for the samples of known NO concentration.

The present inventive method of detecting and/or measuring NO may be carried out on any suitable sample, although it is preferably carried out in an aqueous sample. The sample may be of a biological or chemical medium, and the evaluation may take place either in vitro or in vivo.

The potential applied to the electrode will depend upon the type of polymeric compound used to coat the substrate. It is preferred that the applied potential have a greater absolute value than the peak potential of the oxidation or reduction reaction in a cyclic voltammogram, e.g., a -0.45 V applied potential for a -0.40 V peak potential, or a +0.55 V applied potential for a +0.50 V peak potential, all relative to a reference electrode potential.

The detection and/or measurement of NO in a sample may be alternatively accomplished by contacting the electrode sensor of the present invention with the sample being tested for some determined period of time sufficient to allow interaction of NO with the electrochemically active polymeric coating and then removing the electrode sensor from the sample, connecting the electrode sensor to a potentiostat previously calibrated for known concentrations of NO, and comparing the observed current with the current for the electrode sensor having been exposed to similar samples of known NO concentration for the same period of time.

The present inventive method of measuring NO concentration is useful, for example, monitoring NO production or inhibition effected by drugs, in the design of drugs for the treatment of diseases related to defects in NO regulation and/or production, as well as a means of detecting and quantifying defects in NO regulation and/or
production, which result from disease, injury, and mutation, all in vitro or in vivo.

The present invention additionally provides a means of monitoring pollution of which NO is a component.

The following examples serve to further illustrate the present invention and are not intended to limit the scope of the invention.

**Example 1**

This example illustrates the preparation of several TMPP-coated NO electrode sensors.

A glassy carbon electrode (GCE, diameter = 2 mm) was coated with the conductive polymeric porphyrin Ni²⁺ tetramethylpyridylporphyrin (TMPP, prepared for use in this example although commercially available from Midcentury Chemicals, Posen, IL) (see Figure 1A) by cyclic voltammetry employing a platinum rod as an auxiliary electrode and a standard calomel electrode (SCE) as a reference electrode in a 5 ml solution of 0.1 M NaOH and the monomeric porphyrin and cycling between 0 and +1.0 V (versus SCE) for 15 cycles at a 50 mV/sec scan rate. Glassy carbon electrodes were similarly prepared using Co²⁺ and Fe³⁺ TMPP, except using a controlled potential, rather than the cyclic voltammetry, of about +0.75 V (versus SCE) maintained for about 4 minutes.

**Example 2**

This example illustrates the treatment of a Ni²⁺ TMPP-coated NO electrode sensor to render it insensitive to interference from nitrite during measurements of NO concentration in samples.

Possible sources of interference in measuring NO with the electrode sensor include nitrite, nitrate, and nitrous oxide. Nitrate ions and nitrous oxide do not generate a response in the examined potential range, i.e., +0.4 V to +0.9 V. A response to nitrite can be obtained in the
examined potential range, although the response to NO is 100 times more sensitive than the response to nitrite.

In order to eliminate the interference from nitrite and other ions, the Ni²⁺ TMPP-coated NO electrode sensor of Example 1 was further coated with Nafion®. The Nafion® coating was effected by exposing the electrode sensors to 4 μl of a commercially available Nafion® solution comprising 5 wt. % Nafion® in a mixture of lower aliphatic alcohols and water (9:1) (Aldrich Chemical Co.) and then allowing the Nafion® coating to dry so as to generate a uniform film on the electrode sensor. This Nafion® electrode sensor was then soaked in a 0.01 M NaOH solution for 24 hours.

Example 3

This example illustrates the preparation of a DME-coated NO electrode sensor.

Protoporphyrin IX dimethyl ester (DME, Porphyrin Products, Logan, UT) was metalated with iron in dimethyl formamide at 100°C by a standard procedure (Mikami et al., J. Biochem. (Tokyo) 105: 47. 1989.). A glassy carbon electrode (diameter = 2 mm) was coated with the conductive polymeric porphyrin iron (III) protoporphyrin IX dimethyl ester (see Figure 1B) by immersing the electrode in an argon-blanketed dichloromethane solution (1.0 mM) of the metalloporphyrin monomer and cycling the electrode potential repeatedly between 0.0 and +1.3 V vs. SCE. After electropolymerization, the coated electrode was rinsed thoroughly with pure solvent.

Example 4

This example illustrates the use of TMPP-coated electrode sensors to measure NO concentration in aqueous solutions.

A stock solution of saturated NO was prepared anaerobically in 0.1 M phosphate buffer at pH 7.4. The stock solution was then used to prepare aqueous solutions
of NO at desired concentrations. All solutions were freshly degassed and stored under nitrogen prior to use.

An electrochemical cell was prepared with a platinum rod counter electrode, a SCE reference electrode, and the TMPP-coated glassy carbon working electrodes prepared in accordance with Examples 1 and 2. A 0.1 M phosphate buffer at pH 7.4 was used as the supporting electrolyte during measurement of NO concentration. All measurements were performed in 5 ml of the phosphate buffer.

A baseline scan was taken using differential pulse voltammetry (range = +0.4 - +0.9 V vs. SCE). Linear sweep voltammetry (range = 0 - +0.9 V vs. SCE) could have been similarly used to determine a baseline.

Aliquots of the NO stock solution were introduced into the electrochemical cell by means of a gas-tight syringe. The final dilution of the NO stock solution in the phosphate buffer was taken as the final NO concentration.

The response obtained by differential pulse voltammetry using the Ni^{2+} TMPP electrode of Example 2 with 10, 20, and 40 µM NO solutions is shown in Figure 2 (anodic peak (oxidation) potential (Epa) = 0.7 V vs. SCE). A linear response of current versus NO concentration was observed for the same electrode for concentrations of NO of 20, 40, 60, and 80 µM as shown in Figure 3 (Epa = 0.7 V vs. SCE). A linear response of current versus NO concentration was similarly observed for the Fe^{3+} TMPP electrode of Example 1 for concentrations of NO up to 140 µM as shown in Figure 5 (Epa = 0.7 V vs. SCE).

Measurements were also taken for the NO-releasing compound \{Et₂N-N(N=O)-O\}Na, known as DEANO (Maragos et al., J. Med. Chem. 34: 3242-3247. 1991.), and a linear response of current versus DEANO concentration was observed for the Ni^{2+} TMPP electrode of Example 2 for DEANO concentrations up to 200 µM as shown in Figure 4 (Epa = 0.7 V vs. SCE).
Example 5

This example illustrates the use of a DME-coated electrode sensor to measure NO concentration in aqueous solutions.

A stock solution of saturated NO was prepared anaerobically in 0.1 M phosphate buffer at pH 7.4. The stock solution was then used to prepare aqueous solutions of NO at desired concentrations. All solutions were freshly degassed and stored under nitrogen prior to use.

An electrochemical cell was prepared with a platinum rod counter electrode, a SCE reference electrode, and the DME-coated glassy carbon working electrode prepared in accordance with Example 3. A 0.1 M phosphate buffer at pH 7.4 was used as the supporting electrolyte during measurement of NO concentration. All measurements were performed in 5 ml of the phosphate buffer.

A baseline scan was taken using differential pulse voltammetry (range = +0.4 - +0.9 V vs. SCE). Linear sweep voltammetry (range = 0 - +0.9 V vs. SCE) could have been similarly used to determine a baseline.

Aliquots of the NO stock solution were introduced into the electrochemical cell by means of a gas-tight syringe. The final dilution of the NO stock solution in the phosphate buffer was taken as the final NO concentration.

A linear response of current versus NO concentration was observed for the Fe\textsuperscript{3+} DME electrode of Example 3 for concentrations of NO of 0.2 to 1.0 M NO as shown in Figures 6A and 6B. (Epa = 0.7 V vs. SCE).

Example 6

This example further illustrates the use of an Fe\textsuperscript{3+} DME-coated NO electrode sensor to measure NO concentration in an aqueous solution.

A stock solution of saturated NO was prepared anaerobically in 0.1 M phosphate buffer at pH 7.2. The stock solution was then used to prepare aqueous solutions
of NO at desired concentrations. All solutions were freshly degassed and stored under nitrogen prior to use.

An electrochemical cell was prepared with a platinum rod counter electrode, a SCE reference electrode, and a Fe(III) DME-coated glassy carbon working electrode, prepared in accordance with Example 3. A 0.1 M phosphate buffer at pH 7.2 was used as the supporting electrolyte during measurement of NO concentration. All measurements were performed in 5 ml of the phosphate buffer.

A baseline scan was taken using cyclic voltammetry (20 mV/sec, cathodic peak (reduction) potential (Epa) = -1.0 V vs. SCE). Differential pulse voltammetry could have similarly been used to establish a baseline.

Aliquots of the NO stock solution were introduced into the electrochemical cell by means of a gas-tight syringe. The final dilution of the NO stock solution in the phosphate buffer was taken as the final NO concentration.

The response obtained by cyclic voltammetry using the Fe$^{3+}$ DME electrode of Example 3 with 0, 0.4, 0.7, and 0.9 μM NO solutions is shown in Figure 7 (cathodic peak (reduction) potential (Epa) = -1.0 V vs. SCE). The responses obtained by cyclic voltammetry using the a GCE electrode without any electrochemically active polymeric coating and the Fe$^{3+}$ DME electrode of Example 3 with 2 mM NO solutions were then compared as shown in Figures 8A and 8B, respectively. In contrast to the Fe$^{3+}$ DME electrode sensor, the glassy carbon electrode without the electrochemically active polymeric coating was relatively unresponsive to NO concentration.

All of the publications identified herein are hereby incorporated by reference in their entireties.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred electrode sensor and methods may be used and that it is intended that the invention may be practiced
otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.
WHAT IS CLAIMED IS:

1. A nitric oxide-specific electrode sensor, which comprises:
   an electrically conductive substrate whose amperometric response is substantially unaffected by the presence of nitric oxide and
   an adherent and substantially uniform electrochemically active polymeric coating formed on a surface of said electrically conductive substrate, said electrochemically active polymeric coating capable of interacting with NO in such a manner so as to cause a change in the redox potential of NO and the electrode sensor.

2. The electrode sensor of claim 1, wherein said electrochemically active polymeric coating is formed on the surface of said electrically conductive substrate by electrolytic polymerization.

3. The electrode sensor of claim 2, wherein said electrically conductive substrate is selected from the group consisting of electrically conductive carbon, indium tin oxide, iridium oxide, nickel, platinum, silver, and gold.

4. The electrode sensor of claim 3, wherein said electrically conductive substrate is carbon.

5. The electrode sensor of claim 4, wherein said electrochemically active polymeric coating is a metallized or doped polymer.

6. The electrode sensor of claim 5, wherein said electrochemically active polymer coating is selected from the group consisting of metallized polymeric porphyrins, metallized polyphthalocyanines, polyvinylmetallicocenes,
metallized polyacetylenes, metallized polypyrrolines, and polymeric substitituted glyoximes.

7. The electrode sensor of claim 6, wherein said electrochemically active polymeric coating is selected from the group consisting of metallized polymeric porphyrins and metallized polyphthalocyanines.

8. The electrode sensor of claim 7, wherein said electrochemically active polymeric coating is a metallized polymeric porphyrin which does not form metal-oxo bridges with said substrate.

9. The electrode sensor of claim 8, wherein said electrochemically active polymeric coating is selected from the group consisting of metallized polymeric pyroles, pyridines, and ether porphyrins.

10. The electrode sensor of claim 9, wherein said electrochemically active polymeric coating is selected from the group consisting of metallized polymeric tetramethyl pyridine pyrrole and dimethyl ester porphyrins.

11. The electrode sensor of claim 10, wherein said electrochemically active polymeric coating is selected from the group consisting of polymeric tetramethyl pyridine pyrroles and dimethyl ester porphyrins metallized with Ni$^{2+}$, Co$^{2+}$, or Fe$^{3+}$.

12. The electrode sensor of claim 11, wherein said electrochemically active polymeric coating is Ni$^{2+}$ or Fe$^{3+}$ polymeric tetramethyl pyridine pyrrole.

13. The electrode sensor of claim 11, wherein said electrochemically active polymeric coating is Fe$^{3+}$ polymeric dimethyl ester porphyrin.
14. The electrode sensor of claim 2, which additionally comprises an gas-permeable membrane coating that is permeable to nitric oxide and is not permeable to nitrite.

15. The electrode sensor of claim 11, which additionally comprises an gas-permeable membrane coating that is permeable to nitric oxide and is not permeable to nitrite.

16. A method of manufacturing a nitric oxide-specific electrode sensor, which comprises contacting an electrically conductive substrate whose amperometric response is substantially unaffected by the presence of nitric oxide with a compound which forms an electrochemically active polymeric coating capable of interacting with NO in such a manner so as to cause a change in the redox potential of NO and the electrode sensor.

17. The method of claim 16, wherein said electrochemically active polymeric coating is formed on the surface of said electrically conductive substrate by electrolytic polymerization.

18. The method of claim 17, wherein said electrically conductive substrate is selected from the group consisting of electrically conductive carbon, indium tin oxide, iridium oxide, nickel, platinum, silver, and gold.

19. The method of claim 18, wherein said electrochemically active polymeric coating is a metallized or doped polymer.
20. The method of claim 19, wherein said electrochemically active polymeric coating is selected from the group consisting of metallized polymeric porphyrins and metallized polyphthalocyanines.

21. The method of claim 20, wherein said electrically conductive substrate is carbon and said electrochemically active polymeric coating is selected from the group consisting of polymeric tetramethyl pyridine pyroles and dimethyl ester porphyrins metallized with Ni\(^{2+}\), Co\(^{2+}\), or Fe\(^{3+}\).

22. The method of claim 21, wherein said electrochemically active polymeric coating is Ni\(^{2+}\) or Fe\(^{3+}\) polymeric tetramethyl pyridine pyrrole.

23. The method of claim 21, wherein said electrochemically active polymeric coating is Fe\(^{3+}\) polymeric dimethyl ester porphyrin.

24. The method of claim 21, which additionally comprises applying a coating of an gas-permeable membrane that is permeable to nitric oxide and excludes nitrite.

25. A method of detecting nitric oxide in solution, which method comprises connecting an electrode sensor of claim 1 to a potentiostat, calibrating the potentiostat and electrode sensor for known concentrations of nitric oxide in solution, contacting said electrode sensor with an unknown sample, and detecting the presence or absence of nitric oxide in said unknown sample by comparing the measured current to a current for a sample of known nitric oxide concentration at a particular potential.

26. The method of claim 25, wherein said sample of known nitric oxide concentration has no nitric oxide.
27. The method of claim 25, wherein said the concentration of nitric oxide in said unknown sample is determined by comparing the measured current to currents observed for samples of of more than one known nitric oxide concentration.

28. The method of claim 27, wherein said electrode sensor is an electrode sensor of claim 11.

29. The method of claim 27, wherein said electrode sensor is an electrode sensor of claim 14.

30. The method of claim 27, wherein said electrode sensor is an electrode sensor of claim 15.
FIG. 1a

\[
\text{TMPP} = \text{CH}_3N^+ \quad N \quad N \quad N \quad N \quad N \quad N \quad N
\]

\[
M = \text{Ni}^{+2}, \text{Co}^{+2}, \text{Fe}^{+3}
\]

\[
X = \text{ClO}_4^-
\]

\[
n = 4, 5
\]
FIG. 1b

\[ \text{DME} = \]

\[ M = \text{Fe}^{+3} \]

\[ X = \text{Cl}^- \]
FIG. 3

\[ y = -17.000 + 14.110x \quad R^2 = 0.997 \]

FIG. 4

\[ y = 19.749 + 9.631x \quad R^2 = 0.994 \]
FIG. 5

\[ y = 80.000x \quad r = 1.000 \]
**FIG. 6a**

\[ y = 12.938 + 38.551x \quad r = 0.987 \]

**FIG. 6b**

\[ y = 4.3916 + 44.159x \quad r = 0.994 \]
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 GOIN33/00 GOIN27/30

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 GOIN

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
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<td>1,16, 2-7, 17-20, 25</td>
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Patent family members are listed in annex.

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1 Date of the actual completion of the international search

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