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(54) **Title**: DEVICES AND METHODS FOR DETECTION AND QUANTIFICATION OF NITRIC OXIDE IN A BIOLOGICAL MATERIAL

(57) **Abstract**: Disclosed are methods and devices for detecting and quantifying nitric oxide (NO) in a biological material. Exemplary methods include the steps of providing a biological material, contacting the biological material to an electrochemical sensor, performing amperometric measurements using electrodes within the sensor, and detecting an electrical signal indicative of an amount of NO present in the biological material. An exemplary electrochemical sensor includes a substrate, an electrode set disposed on the substrate, and an ionomer coating on the electrode set, an absorbent pad over the electrode set and a gas permeable membrane sealing the pad and the electrode set. In certain embodiments, the sensor provides NO gas capture functionality that allows specific measurement and quantification of nitric oxide.

DEVICES AND METHODS FOR DETECTION AND QUANTIFICATION OF NITRIC OXIDE IN A BIOLOGICAL MATERIAL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of, and incorporates herein by reference in their entireties, U.S. Provisional Patent Application No. 61/470,672, which was filed on April 1, 2011, and U.S. Provisional Patent Application No. 61/570,099, which was filed on December 13, 2011.

BACKGROUND

[0002] Nitric oxide (NO) is a short-lived, radical gas molecule and a key intra- and inter-cellular signaling molecule or biological messenger, playing a role in a variety of physiological and pathological processes. NO, synthesized endogenously from L-arginine, oxygen, and NADPH, by various nitric oxide synthase (NOS) enzymes, activates guanylate cyclase, which elevates cGMP levels, leading to microvascular vasodilation and inhibition of platelet aggregation. In whole blood, NO is rapidly metabolized to nitrite and nitrate by interaction with the heme group of hemoglobin. NO also reacts with thiol (-SH) groups in amino acids and proteins and forms relatively stable nitrosothiols (-S-NO), which circulate in the blood and plasma.

[0003] NO has been implicated in numerous pathologic conditions such as vascular and inflammatory diseases, cancer, sepsis, multiple sclerosis, renal failure, and preeclampsia. For example, sepsis may be considered to be a systemic inflammatory response to infection. Mediators associated with sepsis, such as endotoxin and the pro-inflammatory cytokines IL-1b, IL-2, IL-6, TNF and interferon, may augment iNOS (inducible nitric oxide synthase) levels and thereby increase NO production. NO has therefore been described as a potential biomarker of sepsis. Additionally, NO and NO carriers and metabolites, such as S-nitrosothiols (RSNO), nitrite, and nitrate, may be implicated in the pathogenesis of septic shock.

[0004] Despite NO's central role in many physiological and pathological processes, advances in NO research have been limited by an inability to measure NO levels in blood and

other biological materials. The inability to accurately quantify NO and its primary bioavailable forms have prevented the clinical significance of endogenous NO levels in health and disease from being realized. Accurate NO quantification in biological fluids and gases would allow NO and its carriers and metabolites to be fully characterized and utilized as biomarkers for disease diagnosis and management, and could advance real-time visibility to the onset and progression of disease.

[0005] Accordingly, there is a need for accurate, efficient, and reliable devices and methods for the detection and quantification of NO and its carriers/metabolites in complex biologic materials. A particular need exists for sensors that resist biofouling and are capable of detecting and quantifying NO in complex biological fluids and gases, such as blood and exhaled breath, at low concentrations.

SUMMARY

[0006] In certain embodiments, the devices (e.g., electrochemical sensors) and methods described herein advance detection and quantification of NO levels in biological materials. Measurement of NO provides visibility to the onset of disease (e.g., cardiovascular and inflammatory diseases), before the presentation of other clinical symptoms. These devices and methods are compatible with research and clinical use and provide a unique, practical, and inexpensive approach for detection and quantification of NO in biological materials, such as blood, plasma, and gaseous samples (e.g., exhaled breath). The devices and methods enable more efficient monitoring of NO, and can translate to molecular diagnostic use in multiple unmet clinical needs.

[0007] Advantageously, the electrochemical sensors and associated methods described herein are presently found to demonstrate specificity for the measurement of NO. For example, the oxidation potential associated with NO detection is found to match the known characteristic oxidation potential of NO (i.e., 650-750 mV vs. Ag/AgC), and the detected electrical signals are found to be absent when NO is absent. Signals detected also maybe selectively observed when sensors incorporate a gas permeable barrier over the working electrode. A dose dependent response is presently observed with the use of NO gas, NONOate, RSNO and/or SNO-Albumin. For the decomposition of nitrosothiols, UV irradiation at 300-400 nm is observed to be effective, with 330 nm found to be optimal or near optimal. Additionally, detected signals are found to disappear when UV irradiation is removed, despite the presence of

RSNO. In various embodiments, interferents do not give a significant signal at physiologic levels. Other evidence of specificity has been shown by, for example, decomposing all RSNO and performing measurements, followed by the specific electrochemical removal of NO gas and performing measurements again.

[0008] In one aspect, the invention relates to an electrochemical sensor for the detection and quantification of nitric oxide in a biological material. The electrochemical sensor includes a substrate, an electrode set disposed on the substrate, and an ionomer coating on at least a portion of the electrode set. The electrode set includes a working electrode, a reference electrode, and an auxiliary electrode.

[0009] In certain embodiments, the electrochemical sensor also includes a gas permeable membrane disposed over the electrode set, and an absorbent pad disposed between the gas permeable membrane and the electrode set. The absorbent pad includes an NO acceptor and/or a buffer. In one embodiment, the absorbent pad includes or is soaked with a liquid. In various embodiments, the electrochemical sensor further includes a gasket disposed between the gas permeable membrane and the substrate (e.g., to seal the absorbent pad between the gas permeable membrane and the substrate). The reference electrode and the auxiliary electrode may be the same electrode or different electrodes.

[0010] In some embodiments, the substrate includes rayon, acetate, triacetate, nylon, and/or polyester. The ionomer may include, for example, ethylene/methacrylic acid and/or a fluoropolymer-copolymer. In various embodiments, the reference electrode includes a silver, silver chloride, palladium, and/or platinum. The electrode set may include the reference electrode, the working electrode, and the auxiliary electrode.

[0011] In certain embodiments, the NO acceptor includes oxygen (O_2), a capture reagent, an acid, and/or a neutral pH buffer. In one embodiment, the NO acceptor includes the capture reagent, and the capture reagent includes a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety. The acid may include, for example, sulfuric acid, hydrochloric acid, trichloroacetic acid, and/or other strongly dissociable acids. The neutral pH buffer may include, for example, a phosphate buffer, a tris buffer, a citrate buffer, a tricine buffer, and/or a HEPES buffer. In some embodiments, the gas permeable membrane includes silicon, polytetrafluoroethylene,

polypropylene, polyethylene, and/or polyester. A sealant may be disposed between the gas permeable membrane and an edge of the substrate.

[0012] In another aspect, the invention relates to a method of measuring an amount of NO in a biological material. The method includes the steps of providing the biological material, contacting the biological material to an electrochemical sensor, performing amperometric measurements, and detecting an electrical signal at the working electrode. The biological material includes a stabilizing agent (e.g., an alkylating agent, a chelator, and/or an anticoagulant). The electrochemical sensor includes a gas permeable membrane, an absorbent pad, and an electrode set. The absorbent pad is sealed between the gas permeable membrane and the electrode set. Amperometric measurements are performed using the electrode set. The absorbent pad includes an NO acceptor and/or a buffer. The detected electrical signal is indicative of the amount of NO in the biological material.

[0013] In certain embodiments, the method includes the steps of removing the gas permeable membrane from the electrochemical sensor, sealing the absorbent pad and the electrode set with a cover slip (i.e., a substantially transparent and substantially impermeable cover slip, such as glass), and irradiating the absorbent pad with UV light. The biological material may include an alkylating agent (containing N-ethylmaleimide (NEM)), a chelator (containing ethylenediaminetetraacetic acid (EDTA)), and/or an anticoagulant (e.g., including citrate, EDTA, and/or heparin). In some embodiments, the NO acceptor includes oxygen (O_2), a capture reagent, an acid, and/or a neutral pH buffer. The amperometric measurements may include, for example, chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry. In general, the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

[0014] In another aspect, the invention relates to a method of measuring an amount of NO in a biological fluid. The method includes the steps of providing the biological fluid, incubating an electrochemical sensor in the biological fluid, capturing NO with an NO acceptor, performing amperometric measurements using the electrode set, and detecting an electrical signal at the electrode set. The electrochemical sensor includes a gas permeable membrane disposed over an electrode set. The captured NO is held between the gas permeable membrane and the electrode set. The electrical signal is indicative of the amount of NO in the biological fluid.

[0015] In certain embodiments, the method also includes the step of rinsing the sensor. In some embodiments, the method includes the step of removing the gas permeable membrane from the electrochemical sensor. The method may also include the step of drying an electrode lead of the electrode set. The amperometric measurements may provide a signature current response indicative of nitric oxide oxidation.

[0016] In certain embodiments, the biological fluid includes a stabilizing agent that contains or is an alkylating agent, a chelator, and/or an anticoagulant. For example, the biological fluid may include N-ethylmaleimide (NEM) and/or ethylenediaminetetraacetic acid (EDTA). In one embodiment, the biological fluid includes citrate, EDTA, and/or heparin.

[0017] In some embodiments, the gas permeable membrane includes silicon, polytetrafluoroethylene, polypropylene, polyethylene, and/or polyester. The NO acceptor may include, for example, a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety. For example, the NO acceptor may include the amino acid moiety. The amino acid moiety may be cross-linked to a solid phase (e.g., functionalized magnetic beads or non-magnetic beads). In one embodiment, the amino acid moiety is disposed on an electrode set. The amino acid moiety may be free in solution.

[0018] In certain embodiments, the amperometric measurements include chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry. The biological fluid may include, for example, blood or plasma. In some embodiments, incubating the electrochemical sensor includes tumbling the electrochemical sensor in the biological fluid (e.g., for at least about 10 minutes at a temperature of about 40 °C). In various embodiments, incubating the electrochemical sensor includes recirculating the biological fluid over the gas permeable membrane of the electrochemical sensor (e.g., for at least about one minute at a temperature of about 40 °C).

[0019] In some embodiments, the biological fluid is a gas exhaled from a patient (or animal model). Incubating the electrochemical sensor may include, for example, positioning the gas permeable membrane proximate a mouth of a patient (or animal model). For example, the gas permeable membrane may be positioned within a mask disposed over the mouth of the patient (or an animal model). In one embodiment, the biological fluid includes air, and incubating the electrochemical sensor includes blowing the air across or over the gas permeable

membrane. In various embodiments, incubating the electrochemical sensor includes positioning the electrochemical sensor within tubing and circulating the biological fluid through the tubing.

[0020] In another aspect, the invention relates to a method of measuring an amount of RSNO in a biological material. The method includes the steps of providing the biological material containing a stabilizing agent, applying the biological material to an absorbent pad disposed on an electrode set, irradiating the biological material with UV light (e.g., to convert the RSNO to NO), performing amperometric measurements using the electrode set, and detecting an electrical signal at the electrode set (e.g., at a working electrode). The electrical signal is indicative of the amount of RSNO in the biological material.

[0021] In certain embodiments, the stabilizing agent includes an alkylating agent, a chelator, and/or an anticoagulant. For example, the biological material may include an alkylating agent containing N-ethylmaleimide (NEM), a chelator containing ethylenediaminetetraacetic acid (EDTA), and/or an anticoagulant containing citrate, EDTA, and/or heparin. The amperometric measurements may include, for example, chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry. The amperometric measurements may provide a signature current response indicative of nitric oxide oxidation.

[0022] In another aspect, the invention relates to a method of measuring an amount of RSNO in a biological material. The method includes the steps of providing the biological material, applying anti-albumin immunoaffinity isolation (e.g., magnetic bead immunoaffinity isolation or non-magnetic bead immunoaffinity isolation) to the biological material to produce an immunoaffinity resin, eluting albumin from the immunoaffinity resin to produce an eluate, applying the eluate to an absorbent pad disposed on an electrode set, irradiating the eluate with UV light, performing amperometric measurements using the electrode set, and detecting an electrical signal at the electrode set. The electrical signal, which is detected at the electrode set (e.g., at a working electrode), is indicative of the amount of RSNO in the biological material.

[0023] In certain embodiments, the amperometric measurements include chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry. The amperometric measurements may provide a signature current response indicative of nitric oxide oxidation.

[0024] In another aspect, the invention relates to a method of measuring an amount of RSNO in a biological material. The method includes the steps of providing the biological material, applying anti-albumin immunoaffinity isolation (e.g., magnetic bead immunoaffinity isolation or non-magnetic bead immunoaffinity isolation) to the biological material to produce an immunoaffinity resin, eluting albumin from the immunoaffinity resin to produce an eluate, applying the eluate to a gas permeable membrane disposed over an electrode set, irradiating the eluate with UV light to release NO from the albumin, capturing NO with an NO acceptor, performing amperometric measurements using the electrode set, and detecting an electrical signal at the electrode set (e.g., at a working electrode). The captured NO is held between the gas permeable membrane and the electrode set. The electrical signal is indicative of the amount of RSNO in the biological material.

[0025] In some embodiments, the gas permeable membrane includes silicon, polytetrafluoroethylene, polypropylene, polyethylene, and/or polyester. The NO acceptor may include, for example, a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a thiol, a spin trap, a small molecule radical trap, and/or an amino acid moiety. For example, the NO acceptor may include the amino acid moiety. The amino acid moiety may be cross-linked to a solid phase (e.g., functionalized magnetic or non-magnetic beads). The amino acid moiety, may be disposed on an electrode and/or free in solution. In various embodiments, the amperometric measurements include chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry. The amperometric measurements may provide a signature current response indicative of nitric oxide oxidation.

[0026] In another aspect, the invention relates to a method of manufacturing an electrochemical sensor for the detection and quantification of nitric oxide in a biological material. The method includes the steps of forming (e.g., printing) an electrode set on a substrate, pre-conditioning the electrode set in the presence of an alkaline solution (e.g., KOH) and cyclic voltammetry (e.g., 10 cycle), and coating at least a portion of the electrode set with an ionomer. The electrode set includes a working electrode, a reference electrode, and an auxiliary electrode.

[0027] In certain embodiments, the method also includes the steps of applying a gasket around a working end of the electrode set, positioning an absorbent pad over the working end of the electrode set, and disposing a gas permeable membrane over the working end of the

electrode set. The absorbent pad includes a liquid, an NO acceptor and/or a buffer. The method may also include the step of applying a sealant between the gas permeable membrane and the substrate. The reference electrode and the auxiliary electrode may be the same electrode or different electrodes.

[0028] In some embodiments, the substrate includes rayon, acetate, triacetate, nylon, and/or polyester. The reference electrode may include, for example, silver, silver chloride, palladium, and platinum. The ionomer may include, for example, ethylene/methacrylic acid and a fluoropolymer-copolymer. In one embodiment, the NO acceptor includes oxygen (O_2), a capture reagent, an acid, and/or a neutral pH buffer. The capture reagent may include, for example, a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety. The acid is preferably sulfuric acid, hydrochloric acid, trichloroacetic acid, and/or other strongly dissociable acids. In some embodiments, the neutral pH buffer includes a phosphate buffer, a tris buffer, a citrate buffer, a tricine buffer, and/or a HEPES buffer. The gas permeable membrane may include, for example, silicon, polytetrafluoroethylene, polypropylene, polyethylene, and/or polyester.

[0029] Elements of embodiments described with respect to a given aspect of the invention may be used in various embodiments of another aspect of the invention. For example, it is contemplated that features of dependent claims depending from one independent claim can be used in apparatus and/or methods of any of the other independent claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The objects and features of the invention can be better understood with reference to the drawings described below, and the claims. The drawings are not necessarily to scale, emphasis instead generally being placed upon illustrating the principles of the invention. In the drawings, like numerals are used to indicate like parts throughout the various views.

[0031] While the invention is particularly shown and described herein with reference to specific examples and specific embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention.

[0032] FIG. 1a is a schematic top view of an electrode system printed on a substrate, according to an illustrative embodiment of the invention.

[0033] FIG. 1b is a photograph of a top surface of an electrode system printed on a substrate, according to an illustrative embodiment of the invention.

[0034] FIG. 2 is a schematic top view of an electrode system that includes three electrodes, according to an illustrative embodiment of the invention.

[0035] FIG. 3 is a photograph of a top surface of an electrode system for an electrochemical sensor, according to an illustrative embodiment of the invention.

[0036] FIG. 4a is a schematic top view of an electrode system that includes three electrodes, a gas permeable membrane, an adhesive gasket, and an absorbent pad, according to an illustrative embodiment of the invention.

[0037] FIG. 4b is a photograph of an electrode system that includes three electrodes, a gas permeable membrane, an adhesive gasket, and an absorbent pad, according to an illustrative embodiment of the invention.

[0038] FIG. 5 is a schematic top view of an electrode system that includes three electrodes, an adhesive gasket, a glass cover slip, and an absorbent pad, according to an illustrative embodiment of the invention.

[0039] FIG. 6 is a flowchart of a method for detecting NO in a biological material, according to an illustrative embodiment of the invention.

[0040] FIG. 7 is a plot of current versus voltage obtained from an electrochemical sensor during measurements of NO in a biological material, according to an illustrative embodiment of the invention.

[0041] FIGS. 8, 9, and 10 are flowcharts of methods of detecting NO in a biological material, according to an illustrative embodiment of the invention.

[0042] FIG. 11a is a photograph of an electrode system inserted within tubing, according to an illustrative embodiment of the invention.

[0043] FIGS. 11b through 11e are photographs of a housing for incubating an electrode system in a biological material, according to an illustrative embodiment of the invention.

[0044] FIG. 11f is a photograph of a device, including a pump and the housing of FIGS. 11b through 11e, for incubating an electrode system in a biological material, according to an illustrative embodiment of the invention.

[0045] FIG. 12 is a schematic diagram illustrating the principle of operation for an electrochemical sensor, according to one illustrative embodiment of the invention.

[0046] FIG. 13 is a schematic diagram illustrating the principle of operation for an electrochemical sensor, according to another illustrative embodiment of the invention.

[0047] FIG. 14 is a reaction diagram showing the release of NO from RSNO through biradical photolysis, according to an illustrative embodiment of the invention.

[0048] FIG. 15 is a reaction diagram showing the capture of NO using a metal porphyrin, according to an illustrative embodiment of the invention.

[0049] FIG. 16 is a reaction diagram showing the capture of NO using a thiol trap, according to an illustrative embodiment of the invention.

[0050] FIG. 17 is a reaction diagram showing the capture of NO using a spin trap, according to an illustrative embodiment of the invention.

[0051] FIG. 18 is a reaction diagram showing the capture of NO using a small molecule radical trap, according to an illustrative embodiment of the invention.

[0052] FIG. 19 is a schematic diagram depicting the capture and amplification of NO using ion exchange with NAFION®, according to an illustrative embodiment of the invention.

[0053] FIG. 20 is a reaction diagram showing the capture of NO by its superoxide reactive conversion to peroxynitrite and subsequent stabilized capture to a phenolic moiety that is cross-linked to magnetic or non-magnetic beads or a working electrode surface, according to an illustrative embodiment of the invention.

[0054] FIG. 21 is a flowchart of a method for detecting NO in a biological material, according to an illustrative embodiment of the invention.

[0055] FIGS. 22 and 23 are flowcharts of methods of detecting NO in a biological material, according to an illustrative embodiment of the invention.

[0056] FIGS. 24 through 27 are photographs of an electrochemical sensor system for measuring NO in a biological material, according to an illustrative embodiment of the invention.

[0057] FIG. 28 is a plot of current versus voltage obtained during measurements of NO gas at 100 ppm, according to an illustrative embodiment of the invention.

[0058] FIG. 29 is a plot of current versus voltage obtained during measurements of NO gas at various concentrations, according to an illustrative embodiment of the invention.

[0059] FIG. 30 is a plot of current versus voltage obtained during measurements of various gases that could be interferents in blood, according to an illustrative embodiment of the invention.

[0060] FIG. 31 is a plot of current versus NONOate concentration obtained during measurements of NO gas, according to an illustrative embodiment of the invention.

[0061] FIG. 32 is a plot of current versus voltage obtained during measurements of NO gas from NONOate at various time points, according to an illustrative embodiment of the invention.

[0062] FIG. 33 is a plot of current versus time obtained during measurements of NO gas from NONOate at various time points, according to an illustrative embodiment of the invention.

[0063] FIG. 34 is a plot of current versus voltage obtained during measurements of NO gas in porcine blood, according to an illustrative embodiment of the invention.

[0064] FIG. 35 is a plot of current versus voltage for measurements of NO in circulating bovine blood, according to an illustrative embodiment of the invention.

[0065] FIG. 36 is a plot of current versus voltage for measurements of NO gas exchanged out of circulating bovine blood, according to an illustrative embodiment of the invention.

[0066] FIG. 37 is a plot of current versus voltage for measurements of NO in exhaled breath, according to an illustrative embodiment of the invention.

[0067] FIG. 38 is a plot of current versus voltage for measurements of NO gas in circulating and ventilated bovine blood, according to an illustrative embodiment of the invention.

[0068] FIG. 39 is a plot of current versus voltage for measurements of NO gas in a gas exchanger exhaust, according to an illustrative embodiment of the invention.

[0069] FIG. 40 is a plot of current versus time for NO gas measurements taken every 5 minutes from a gas exchanger exhaust in blood that was ventilated with 10% NO gas, according to an illustrative embodiment of the invention.

[0070] FIG. 41 is a plot of current versus time for NO gas measurements taken every 5 minutes in circulating and ventilated bovine blood, according to an illustrative embodiment of the invention.

[0071] FIGS. 42 and 43 are photographs of an electrode system placed at a bottom of a single well in a 96-well plate, according to an illustrative embodiment of the invention.

[0072] FIG. 44 is a schematic of an experiment performed with an electrode system placed at a bottom of a single well in a 96-well plate, according to an illustrative embodiment of the invention.

[0073] FIG. 45 is a plot of current versus voltage for NO gas measurements taken at various nitrite concentrations, according to an illustrative embodiment of the invention.

[0074] FIGS. 46a, 46b, and 46c are plots of current versus voltage obtained during the measurement of nitric oxide released from S-Nitrosothioglycolic acid, S-Nitrosoglutathione, and S-Nitrosocysteine, respectively, according to an illustrative embodiment of the invention.

[0075] FIGS. 47a, 47b, and 47c are plots of current versus voltage for measurements obtained with known blood interferents (i.e., nitrite, ascorbate and cysteine, respectively), according to an illustrative embodiment of the invention.

[0076] FIG. 48 is a plot of measured electrical current during the release of NO from SNO-Alb in NO-depleted human plasma, in the presence of known interferences, according to an illustrative embodiment of the invention.

[0077] FIG. 49 is a plot of current versus voltage obtained during measurement of NO released from NO-free albumin (de-nitrosylated), according to an illustrative embodiment of the invention.

[0078] FIG. 50 is a plot of current versus voltage obtained during measurements of NO released from SNO-Albumin at various concentrations in NO-depleted human plasma, according to an illustrative embodiment of the invention.

DETAILED DESCRIPTION

[0079] It is contemplated that devices, systems, methods, and processes of the claimed invention encompass variations and adaptations developed using information from the embodiments described herein. Adaptation and/or modification of the devices, systems, methods, and processes described herein may be performed by those of ordinary skill in the relevant art.

[0080] Throughout the description, where devices and systems are described as having, including, or comprising specific components, or where processes and methods are described as having, including, or comprising specific steps, it is contemplated that, additionally, there are devices and systems of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.

[0081] It should be understood that the order of steps or order for performing certain actions is immaterial so long as the invention remains operable. Moreover, two or more steps or actions may be conducted simultaneously.

[0082] The mention herein of any publication, for example, in the Background section, is not an admission that the publication serves as prior art with respect to any of the claims presented herein. The Background section is presented for purposes of clarity and is not meant as a description of prior art with respect to any claim.

[0083] This disclosure is generally directed to devices and methods for detecting and quantifying nitric oxide (NO) in a biological fluid or gas. In certain embodiments, the devices and methods are configured to be used in the research laboratory to help decipher the biochemistry of NO. In some embodiments, the devices and methods are configured to aid in the diagnosis, prognostication, and/or therapy management of diseases in which nitric oxide pathways are implicated. These diseases may include, for example, vascular diseases (e.g., pulmonary hypertension and preeclampsia), inflammatory diseases (e.g., asthma and rheumatoid arthritis), cancer, sepsis, and renal failure. Applications may also include, for example, cardiovascular risk stratification.

[0084] In certain embodiments, detecting NO or its metabolites (including RSNO, nitrite, and nitrate) in blood is hindered by NO's short half-life in blood, scavenging, and/or

interfering species. An NO sensor with optimized sensitivity and selectivity that can be used in complex biological samples may be required. Nitrites and nitrate levels may be influenced by diet and may have limitations in clinical utility. RSNOs may be blind to these fluctuations, as this NO pool may be created solely by free NO gas, produced by eNOS and iNOS and sequestered by thiols and proteins in blood, producing RSNOs. In some embodiments, free NO is the common entity and is the direct product of NOS activity and therefore the most important molecule to measure. Therefore, in various embodiments, a sensor that can detect free NO is preferred as a clinical biomarker for overall circulating bioavailable NO and its vasodilatory effects in sepsis and other diseases.

[0085] In certain embodiments, an electrochemical sensor is provided for detecting and quantifying NO gas in a biological fluid or gas, such as blood, plasma, culture fluid, and/or exhaled breath. Gaseous samples may come from, for example, a gastrointestinal track, lungs, bronchial tubes, gas inlet or exhaust lines from a blood ventilator, and/or gas exhaust lines from bottled gases. The electrochemical sensor may be used for real-time disease prediction and monitoring, as well as cell physiology and signaling pathway studies. In one embodiment, the electrochemical sensor is an NO sensor that measures NO released from NO-carrying molecules in blood, plasma, and/or other biofluids.

[0086] In various embodiments, the devices and methods described herein require minimal blood volume, thereby facilitating disposable and potentially point-of-care diagnostic use. For example, the blood volume requirement for a sample chamber may be less than about 2 milliliters.

[0087] In some embodiments, the methods and devices described herein are suitable for use in a stand-alone, bench top (or handheld) in vitro diagnostic (IVD) device, and/or for incorporation into an extracorporeal circuit. For example, the device may include a compact disposable cartridge, with low power requirements and a low blood volume requirement. The devices and methods may be used to aid in the diagnosis, prognostication, and therapy management of diseases in which NO pathways are implicated, including: vascular hypertensive diseases including pulmonary hypertension and preeclampsia (eNOS pathway), hypotensive diseases such as sepsis (iNOS pathway), other inflammatory diseases (e.g., asthma), and inflammatory bowel disease (IBD) (iNOS pathway). Additional applications include, for example, cardiovascular risk stratification.

[0088] In certain embodiments, an electrochemical sensor is provided that is selective for NO and specific for all NO species that may exist in a biological system. The sensor is designed for accurate and reproducible quantification of NO, both free or gaseous and bound, in biological and/or environmental fluids and gases, including human blood, culture fluid, and exhaled breath. The sensor enables discrete quantification of each NO species, giving separate measurements of free NO and a variety of bound NO species, including, for example, NO gas and RSNOs (e.g., S-nitroso-albumin, S-nitroso-glutathione, S-nitroso-cysteine, and/or S-nitroso-hemoglobin), nitrite, and nitrate. In one embodiment, the sensitivity and dynamic range of the sensor is sufficient to cover the physiologic range of each analyte of interest.

[0089] In certain embodiments, the sensor isolates and rapidly stabilizes NO, whether as NO gas and/or as NO released from RSNO, allowing accurate and direct quantification. In one embodiment, amperometric measurements accurately and separately quantify (e.g., as separate readouts) free NO and bound NO (e.g., by liberating NO from its bound form) in the sample.

[0090] In some embodiments, the sensor encompasses or includes a two or three electrode set. For example, a two electrode set may include one electrode acting as a working electrode and one electrode acting as a reference electrode and an auxiliary electrode. The three electrode set may include one electrode acting as a working electrode, one electrode acting as a reference electrode, and one acting as an auxiliary electrode.

[0091] The electrochemical sensors described herein may be used to analyze a wide variety of biological materials, including biofluids, tissue, cells, culture media, exhaled breath, and/or gastrointestinal gas. Environmental samples, such as air and water specimens, may also be analyzed. Samples may be collected by a variety of methods, depending on the materials and the NO species of interest.

[0092] In certain embodiments, after collecting a sample of biological material, the NO species (e.g., both free or gaseous and bound) within the sample are stabilized with a stabilizing agent to preserve the state of the NO content and/or convert the NO species to other forms (e.g., an NO proxy). Possible stabilizing agents include, for example, alkylating agents (e.g., N-ethylmaleimide (NEM)), chelators (e.g., ethylenediaminetetraacetic acid (EDTA)), and/or anticoagulants (e.g., citrate, EDTA, and/or heparin). In one embodiment, the stabilizing agent may include one or more analyte capture elements, such as antibodies and/or NO acceptors,

which are free in solution and/or cross-linked to a solid phase and/or incorporated onto an electrode. Samples may be utilized fresh, frozen or stored, using a variety of methods that depend on the samples and the NO species of interest.

[0093] In certain embodiments, a method of detecting and/or measuring NO includes isolating NO gas in a biological sample (e.g., blood, plasma, and/or culture fluid). The method also may include releasing and delivering NO (e.g., from isolated NOx and/or RSNO) as gaseous NO and/or NO converted to a proxy (such as a phenolic moiety, for example, nitrotyrosine) to a sensor electrode. The gaseous NO and/or the proxy may be captured on the sensor electrode, where they may be oxidized and measured for quantification. In some embodiments, measurements are performed real-time (e.g., during incubation of the sensor electrode in the biological sample), for example, in at least 20 second intervals. In various embodiments, a detected electrical signal is indicative of an amount of NO present in the biological sample.

[0094] In certain embodiments, a method of detecting and/or measuring NO includes incubating a sensor or sensor component in a biological material. During incubation, gaseous NO may accumulate within the sensor (e.g., in or around an absorbent pad) beneath a permeable membrane. The NO may then be oxidized on one or more electrodes and quantified in real-time (e.g., at timed intervals) during the incubation period. These real-time measurements allow the NO content of the biological material to be continuously monitored.

[0095] Alternatively or additionally, NO measurements may be performed following the incubation period. For example, the NO that accumulates in the sensor, beneath the gas permeable membrane, may be converted to a proxy (e.g., a stable compound that may be detected with the sensor) or chemically captured within the sensor using an NO acceptor. In various embodiments, the NO acceptor is a material or compound that is capable of accepting NO (e.g., by forming a bond with NO) and subsequently releasing the NO when it is time to perform measurements. For example, using an NO acceptor, NO may accumulate in an absorbent pad positioned between the gas permeable membrane and one or more electrodes within the sensor. The captured NO may then be catalytically released from the NO acceptor (e.g., using UV light). In various embodiments, the released NO is then oxidized by the sensor electrodes and quantified using, for example, amperometric methods. Alternatively or additionally, the NO may be converted to a proxy or a stable new entity, such as a phenolic

moiety (e.g., nitrotyrosine). The proxy may be stored and later utilized for detection and/or quantification (e.g., by oxidizing the proxy on the sensor electrodes). Advantageously, by capturing the NO on an NO acceptor and/or forming the proxy, the quantity of NO that enters the sensor (e.g., through the gas permeable membrane) is stored or preserved in the sensor, thereby minimizing the amount of NO that reacts or decays to form other products that cannot be used as NO indicators.

[0096] Examples of NO acceptors include oxygen (O_2), a thiol (RSH), an amine (RNH), acids (e.g., sulfuric, hydrochloric, trichloroacetic, and/or other strongly dissociable acids), neutral pH buffers (e.g. phosphate buffered saline), N-acetylpenicillamine, a metal porphyrin, a thiocarbamate, a thiol, a spin trap, a small molecule radical trap, an amino acid moiety, and combinations thereof. In one embodiment, NO reacts with O_2 to form nitrite and/or nitrate.

[0097] Nitric oxide levels in blood are described in U.S. Patent No. 7,128,904, titled "Material Containing Metal Ion Ligand Complex Producing Nitric Oxide in Contact with Blood," issued October 31, 2006, and in U.S. Patent Application No. 12/095,656, titled "Polymer Compositions, Coatings and Devices, and Methods of Making and Using the Same," filed March 11, 2009, the disclosures of which are hereby incorporated by reference herein in their entireties.

[0098] Referring to FIGS. 1a, 1b, and 2, in certain embodiments, an electrochemical sensor includes an electrode system 100 for detecting and/or quantifying NO. The electrode system 100 includes an auxiliary electrode 102, a working electrode 104, and a reference electrode 106 disposed on or attached to a substrate 108. The substrate 108 may be or include, for example, a thin sheet of rayon, acetate, triacetate, nylon, polyester, and/or other polymeric material. The set of electrodes 102, 104, 106 includes electrode leads 110 or connectors for electrically connecting the electrodes 102, 104, 106 to other components of the electrochemical sensor (e.g., a potentiostat or device for performing amperometric methods). The set of electrodes 102, 104, 106 includes conductive materials, such as platinum, platinized platinum, palladium, gold, silver, silver chloride, carbon, conductive polymers, and/or composite structures, such as metal-polymer composites (e.g., platinized platinum and PTFE). For example, the reference electrode 106 may include any conductive metal, such as silver, silver

chloride, palladium, and/or platinum. In alternative embodiments, the electrode system 100 does not include the auxiliary electrode 102.

[0099] Referring to FIG. 2, in various embodiments, the electrode system 100 is formed by printing (e.g., screen printing) the set of electrodes 102, 104, 106 onto the substrate 108 using a conductive ink, such as a carbon ink and/or an ink that includes conductive particles. In one embodiment, two or more electrode systems 100 are printed on a single substrate 108. Each electrode system 100 may then be cut or separated from the single substrate 108. The electrodes 102, 104, 106 may be modified with carbon graphite or nanotubes, enzymes, metals, or other mediators.

[0100] In certain embodiments, after forming the set of electrodes 102, 104, 106 on the substrate 108, the electrodes 102, 104, 106 are pre-conditioned. Pre-conditioning may involve, for example, exposing the electrodes 102, 104, 106 to an alkaline solution (e.g., KOH, NaOH, and/or LiOH) and/or applying cyclic voltammetry (e.g., 10 cycle) or chronoamperometry to the electrodes 102, 104, 106 to stabilize a signal from the electrodes 102, 104, 106.

[0101] In one embodiment, the auxiliary electrode 102, the working electrode 104, and/or the reference electrode 106 are coated with an ionomer, such as NAFION®, a polyacrylate, and/or a polysulfonate. In one embodiment, only the working electrode 104 is coated with the ionomer. The ionomer coating may be formed, for example, by applying a solution of between 2% and 20% NAFION®, by weight, to one or more electrodes. The electrode system 100 may be stored prior to use.

[0102] Referring to FIG. 3, in certain embodiments, the electrode system includes a working end 112 and a connection end 114. The working end 112 is for contacting a biological material or portion thereof. The connection end 114 includes the electrode leads 110 that are electrically connected to other components (e.g., a potentiostat) of the electrochemical sensor. Referring to FIG. 4, the electrode system 100 may be trimmed to a required shape. For example, the electrode system 100 may be wider (e.g., to obtain a larger surface area) at the working end 112 than at the connection end 114.

[0103] In the embodiments depicted in FIGS. 4a and 4b, an electrode system 120 includes a gasket 122, an absorbent pad 124, and a gas permeable membrane 126, in addition to the set of electrodes 102, 104, 106. The gasket 122 is disposed around the working end 112 of the electrode system 120 and includes an opening or aperture 128 over the electrodes 102, 104,

106. As depicted, the absorbent pad 124 is positioned within the aperture 128 of the gasket 122, over the working end 112 of the electrodes 102, 104, 106. The gas permeable membrane 126 is placed over the gasket 122 to seal the absorbent pad 124 within the working end 112 of the electrode system 120, between the gas permeable membrane 126 and the electrodes 102, 104, 106. A sealant material 130 (e.g., wax, paraffin, parafilm, and/or silicone) may be placed around an edge of the gas permeable membrane 126, between the gas permeable membrane 126 and the substrate 108. In certain embodiments, the electrode system 120 is intended for single use (i.e., it is disposable).

[0104] The gasket 122 may include or consist of any suitable solid material, such as a polymer (e.g. cellulose acetate, polyesters, and/or polyethylenes). A thickness of the gasket 122 may be from about 0.5 mm to about 5.0 mm. In one embodiment, the gasket 122 is a double sided adhesive gasket, such as double-sided tape.

[0105] In certain embodiments, the absorbent pad 124 includes (e.g., is soaked or saturated with) an NO acceptor. The NO acceptor may be, for example, oxygen (O_2), a capture reagent (e.g., a thiol (RSH) and/or an amine (RNH)), an acid (e.g., sulfuric, hydrochloric, trichloroacetic, and/or other strongly dissociable acids), a neutral pH buffer (e.g. phosphate buffered saline), metal porphyrins, thiocarbamates, thiols, spin traps, small molecule radical traps, amino acid moieties that are free in solution and/or cross-linked to a solid phase (e.g., functionalized magnetic or non-magnetic beads) and/or incorporated onto an electrode. In one embodiment, the absorbent pad 124 includes a base material, such as a foam or any organic fabric such as a cellulose fabric. The absorbent pad 124 may have a thickness of about 0.1 mm or a thickness from about 0.1 mm to about 5 mm. In one embodiment, the absorbent pad has a high surface area to volume ratio to increase an amount of NO acceptor present on or in the absorbent pad.

[0106] In certain embodiments, the gas permeable membrane 126 is a thin sheet or film that permits the passage of gases and resists the passage of liquids and solids. The gas permeable membrane 126 may be a membrane that includes or consists of, for example, silicone, TEFLON® (polytetrafluoroethylene), polypropylene, polyethylene, MYLAR® (e.g., polyester film or plastic sheet), and/or other polymeric materials. A thickness of the gas permeable membrane 126 may be about 0.05 mm, or from about 0.01 mm to about 0.5 mm. In some embodiments, measurements are performed by contacting the gas permeable membrane

126 with a biological material. NO gas from the biological material permeates through the membrane 126 to reach the absorbent pad 124 and/or the electrodes 102, 104, 106.

[0107] Alternatively, the electrode system 120 may not include the gas permeable membrane 126. For example, a method of using the electrode system 120 may include the step of applying a biological material directly to the absorbent pad 124 and/or the electrodes 102, 104, 106. In general, methods for applying the sample to the electrochemical sensor include, for example, droplet application (e.g., with a syringe), sample spotting, passive addition, active addition via fluidics, hydrophobically aided application, and/or fiber tissue aided application.

[0108] In a typical configuration, the electrode leads 110 are connected to a potentiostat (e.g., EmStat, manufactured by PalmSens, of The Netherlands). Together, the electrode system and potentiostat utilize, for example, an amperometric method to measure and quantify NO. The amperometric method may include, for example, chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and/or differential pulse voltammetry.

[0109] Referring to FIG. 5, in certain embodiments, following application of the sample (e.g., incubation of the electrode system 120 in a biological fluid), the gas permeable membrane 126 of the electrode system 120 is replaced with a cover slip 151. For example, the gas permeable membrane 126 may be removed from the electrode system, and the cover slip 151 may be placed freely or adhesively (e.g., using an adhesive on the gasket 122) over the working end 112 of the electrode system 120. The cover slip 151 is preferably impermeable, non-reactive, and transparent. In one embodiment, the cover slip 151 includes or is glass. The cover slip 151 may be used for containment of NO gas and/or head space within the electrode system 120.

[0110] FIG. 6 is a flowchart of a method 600 for measuring NO gas, in accordance with one embodiment of the invention. The method 600 may be used, for example, in conjunction with the electrode system 100, depicted in FIG. 3. The method 600 includes providing (step 601) or collecting a sample of blood, plasma, and/or other biological material(s) (e.g., from a human or other mammal, and/or culture fluid). The sample (e.g., about 1-30 μl worth) is applied (step 602) to the electrode system 100 (e.g., by sample spotting) on an absorbent pad, across the working electrode 104, the reference electrode 106, and the auxiliary electrode 102. A glass cover slip may be applied (step 603) over the electrode set to prevent the escape of NO gas. A potentiostat, which is connected to the electrode leads 110 of the electrode system 100,

is used to apply (step 604) a voltage in a differential pulse manner. For example, the differential pulse may include sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps. Alternatively or additionally, other amperometric methods may be used. From one to about 30 scans may be performed, with or without UV irradiation. Oxidation of NO, which occurs at the surface of the electrode system 100, releases one electron per molecule of NO and provides an electrical signal, which is detected (step 605) using the potentiostat or other measurement device.

[0111] Referring to FIG. 7, in various embodiments, NO is measured or quantified from the detected signal based on a constructed baseline 702 and "peak height" 704 or "area under the curve" calculation. In this case, software was used to construct the baseline 702 at a point in which the signal response was anticipated. The peak height 704 was then determined by the software based on the distance between the baseline and a response or current amplitude, expressed in μA. The signal may provide a relative measure when comparing samples. In one embodiment, the method includes constructing a standard curve and calculating moles of NO measured in the sample. The measured value may be converted to appropriate units (e.g., concentration or flux). In certain embodiments, the applied differential pulsed voltage results in a signature current response indicative of nitric oxide oxidation.

[0112] FIG. 8 is a flowchart of a method 800 for measuring NO gas in accordance with one embodiment of the invention. The method 800 may be used, for example, in conjunction with the electrode system 120 depicted in FIG. 4a. The method 800 includes providing (step 801) or collecting a sample of blood, plasma, and/or other biological material(s) (e.g., from a human or other mammal, and/or culture fluid), and incubating (step 802) the electrode system 120 in the sample (step 802). At step 803, the electrode system 120 is removed from the sample and rinsed (e.g., with water, various pH-dependent buffers, and/or other designed rinses that may afford an advantage toward removing interfering substances). After drying the electrode leads, the gas permeable membrane is removed and a glass cover slip 151 is applied (step 803) over the working end of the electrode system 120, as depicted in FIG. 5. The electrode system 120 is then connected to a potentiostat, and UV irradiation is applied (step 804) for a time period of at least 30 seconds. The potentiostat is used to apply (step 805) a voltage in a differential pulse manner (e.g., sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps). Alternatively or additionally, other amperometric methods may be applied to

perform the measurements. In one embodiment, from one to about 30 scans are performed in the presence and absence (blank) of UV irradiation. Oxidation of NO occurs at the surface of the electrode system 100 to release one electron per molecule of NO and provide a signal, which may be detected (step 806) and analyzed, for example, as described above with respect to FIGS. 6 and 7. In the case where two or more curves provide a measurable response, it may be desirable to increase a signal to noise ratio by adding responses from multiple curves. This could be performed manually or automatically, using software.

[0113] FIG. 9 is a flowchart of a method 900 for measuring NO gas in accordance with one embodiment of the invention. The method 900 may be used, for example, in conjunction with the electrode system 120 depicted in FIG. 4a. The method 900 includes providing (step 901) or collecting a sample of blood, plasma and/or other biological material(s) (e.g., from a human or other mammal, and/or culture fluid). The electrode system 120 is incubated (step 902) in the sample. At step 903, the electrode system 120 is removed from the sample and rinsed (e.g., with water, various pH-dependent buffers, and/or other designed rinses that may afford an advantage toward removing interfering substances). After drying the electrode leads, the gas permeable membrane is removed and a glass cover slip 151 is applied (step 903) over the working end of the electrode system 120, as depicted in FIG. 5. The electrode sensor is connected to a potentiostat. The potentiostat is used to apply (step 904) an amperometric method, such as a differential pulse voltammetry (e.g., sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps). From one to about 30 scans are performed in the presence and absence (blank) of UV irradiation. Oxidation of NO occurs at the surface of the electrode system 100 is detected (step 905) and analyzed, for example, as described above with respect to FIG. 8.

[0114] FIG. 10 is a flowchart of a method 1000 for performing real-time measurements of NO gas in a biological material while the electrode system 120 is incubating or immersed in the biological material, in accordance with one embodiment of the invention. The method 1000 includes providing (step 1001) or collecting the biological material. The electrode system 120 is then incubated (step 1002) in the sample and connected to a potentiostat. During incubation of the electrode system 120, the potentiostat is used to apply (step 1003) an amperometric method, such as differential pulse voltammetry (e.g., sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps). Oxidation of NO on the electrode system 100 is then detected (step 1004). Amperometric scans may be performed at timed intervals (e.g., every 20 seconds) during incubation. Several scans may be performed by repeating steps 1003 and 1004 over the course

of the desired incubation time (step 1005). The results from the scans obtained during incubation are analyzed and may be plotted, as depicted in FIG. 31.

[0115] In one embodiment, the electrochemical sensor is incubated in the biological material or fluid, for example, for at least about 1 minute at a temperature of about 40 °C. The incubation may be performed within tubing or a housing through which the biological material or fluid is circulated. In one embodiment, the incubation occurs in a vessel, such as a culture plate, a flask, and/or a test tube. In various embodiments, the incubation includes tumbling or otherwise agitating the biological fluid around the electrode system. For example, the electrode system 120 may be tumbled in the biological fluid at 40 °C for at least one minute. The electrochemical sensor may be removed after a set incubation time and rinsed. The gas permeable membrane may then be replaced with a glass cover slip, and measurements may be performed. As mentioned, measurements may be performed in real-time (e.g., in at least 20 second time intervals) while the sensor is incubating in the biological fluid. In one embodiment, the biological fluid is a heparinized whole blood sample, plasma, and/or culture fluid (e.g., 1, 10 or 50 ml).

[0116] Referring to FIGS. 11a through 11f, in certain embodiments, the step of incubating the electrode system 120 includes placing the electrode system 120 in a container, such as a tube or housing, and flowing a biological fluid over or around the electrode system 120. For example, in the embodiment depicted in FIG. 11a, the working end 112 of the electrode system 120 is placed within tubing 1102 that is connected to or in-line with a pump (e.g., a peristaltic pump). The tubing 1102 and pump may form a closed circuit filled with the biological fluid, and the biological fluid may be circulated through the tubing 1102 and the pump. In one embodiment, a ratio of gas permeable membrane surface area to volume available for flow past the membrane is maximized with this configuration. The tubing 1102 may be, for example, PEBAX® flat extruded tubing that minimally accommodates the insertion of the electrode system 120. Additional tubing (e.g., 0.25 inch TYGON® tubing) may be attached to ends of the PEBAX tubing. The pump may be used to circulate the biological fluid through the tubing for a desired amount of time (e.g., about one hour). In one embodiment, the continuous circulation occurs for at least about 1 minute at a temperature of about 40 °C. As mentioned, amperometric measurements may be performed during and/or after incubation of the electrode system 120 in the biological fluid.

[0117] In one embodiment, the tubing 1102 is a blood vessel or artery and the pump is a heart. The electrode system may be introduced to the blood vessel or artery using a catheter.

[0118] Referring to FIGS. 11b through 11f, in certain embodiments, the step of incubating the electrode system 120 in a biological fluid (e.g., heparinized whole blood, plasma, and/or culture fluid) includes placing the electrode system 120 within a housing 1112 and pumping the biological fluid through the housing 1112. The housing 1112 is preferably connected to a pump 1113 (e.g., a peristaltic pump) with tubing 1102 to form a closed circuit filled with the biological fluid. In the depicted embodiment, the housing 1112 includes an inlet port 1114 and an outlet port 1116 for connecting the housing 1112 to the pump 1113. A spacer 1118 is used to form a chamber within the housing 1112. The chamber is sealed with a gasket 1120. Securing members 1122 (e.g., screws, nuts, and/or washers) are used to secure the components of the housing 1112 together. During incubation within housing 1112, continuous circulation of the biological fluid may occur, for example, for at least about 1 minute at a temperature of about 40 °C. Measurements of NO may be performed during and/or after the incubation.

[0119] FIG. 12 is a schematic diagram illustrating the principle of operation for an electrochemical sensor 1200, in accordance with certain embodiments of the present invention. As depicted, NO gas (e.g., free gaseous NO, released from heme groups of hemoglobin and/or released from RSNO) enters the electrochemical sensor 1200 through the gas permeable membrane 126. The NO in the presence of oxygen (which also can permeate the membrane) spontaneously oxidizes to nitrite (NO_2), which accumulates at neutral pH on the electrodes of the electrochemical sensor 1200. The electrochemical sensor is then connected to a potentiostat and, in the absence or presence of UV irradiation (i.e., to release NO), an amperometric technique is applied to measure the NO. Oxidation of NO is detected and analyzed, as described herein, and may be viewed on a display 1202.

[0120] FIG. 13 is a schematic diagram illustrating the principle of operation for an electrochemical sensor 1300, in accordance with certain embodiments of the present invention. As depicted, NO gas (e.g., free gaseous NO, released from heme groups of hemoglobin and/or released from RSNO) enters the electrochemical sensor 1300 through the gas permeable membrane 126. The NO in the presence of oxygen (which also can permeate the membrane) spontaneously oxidizes to nitrite (NO_2). In the presence of acid (H^+), the nitrate yields a

nitrosonium ion (NO^+), which condenses with an NO acceptor (e.g., N-acetylpenicillamine or NAP) to form nitrosothiols (RSNO) (e.g., S-nitroso-N-acetylpenicillamine or SNAP). The RSNO then accumulates between the gas permeable membrane 126 and the sensor electrodes. The electrochemical sensor 1300 is then connected to a potentiostat and exposed to UV irradiation, which subsequently releases NO from the RSNO. An amperometric method is used to detect NO, as described herein.

[0121] In certain embodiments, the step of incubating the electrode system 120 in the biological sample includes positioning the gas permeable membrane 126 in front of a patient's mouth to collect exhaled breath. For example, the electrode system 120 may be fastened to an inside surface of a surgical mask. When worn by the patient, the gas permeable membrane 126 may be facing out, directly across from the patient's mouth. The patient (e.g., a person or other mammal) wears the surgical mask (or something similar) with the electrode system 120 and breathes normally for about one hour, more or less. In some embodiments, the electrode system 120 is exposed to other gaseous or environmental samples, such as an air sample, by blowing or forcing the environmental sample over the gas permeable membrane 126 for a desired amount of time. In this case, a flow-thru-cell (e.g., a housing and/or tubing) may be used to efficiently expose the sensor to the environmental sample in which the membrane surface area to volume ratio is maximized. As mentioned, measurements may be performed during (i.e., real-time) and/or after incubation. In certain embodiments, the patient is an animal model (e.g., a laboratory animal).

[0122] In certain embodiments, the methods described herein include the following elements or processes: collecting and stabilizing the NO analyte of interest; isolating the NO analyte of interest; releasing NO from the analyte of interest; capturing NO and/or converting NO to a proxy; and detecting and quantifying NO. Each of these processes is discussed in more detail below.

[0123] Biological samples containing the NO analyte of interest may be collected by a wide variety of methods, depending on the type of biological sample and the NO species of interest. In certain embodiments, the biological sample includes a biological fluid, tissue, cells, culture media, exhaled breath, and/or gastrointestinal gas. Typically the NO species (both free and bound) are stabilized with stabilizing agents to preserve the state of the NO content and prevent decomposition of RSNO and/or conversion of the NO species to other forms. The

stabilizing agent may include, for example, one or more of the following: an alkylating agent (e.g., N-ethylmaleimide (NEM)), a chelator (e.g., ethylenediaminetetraacetic acid (EDTA)), an anticoagulant (e.g., citrate, EDTA, and heparin). In one embodiment, the stabilizing agent includes analyte capture elements, such as antibodies or NO acceptors, which are free in solution and/or cross-linked to a solid phase and/or incorporated onto an electrode. Biological samples may be utilized fresh or can be stored using a variety of methods that depend on the samples and the NO species of interest.

[0124] Isolation of the NO species (either a single analyte and/or multiple analytes) from biological samples may be carried out by a variety of methods, depending on the samples and the NO species of interest. In certain embodiments, the NO species or analyte includes free NO and a variety of bound NO species. For example, the NO analyte may include free gaseous NO, RSNOs (e.g., S-nitroso-albumin, S-nitroso-glutathione, S-nitroso-cysteine, and/or S-nitroso-hemoglobin), nitrite, nitrate, erythrocyte SNOHb, and/or peroxynitrite. In one embodiment, the NO analyte is isolated using capture reagents (e.g., antibodies or NO acceptors) that are free in solution, cross-linked to a solid phase, and/or incorporated onto an electrode. In certain embodiments, NO is isolated using immunoaffinity or affinity. The immunoaffinity or affinity may be, for example, free in solution, cross-linked to a solid phase, and/or incorporated onto an electrode. In one embodiment, NO is isolated using ion exchange (e.g., a solid phase and/or coating on an electrode).

[0125] NO may be liberated or released from the NO species (either a single analyte and/or multiple analytes) by a variety of methods, depending on the samples and the NO species of interest. In certain embodiments, a decomposition catalyst is used to release NO (e.g., release of NO from nitrosothiol). The decomposition catalyst may include, for example, UV irradiation, heat, metal ions (e.g., copper, iron, gold, and silver ions), iodine/iodide, and/or pH changes) (e.g., introduction of an acid or base). Referring to FIG. 14, UV irradiation may release NO from RSNO through biradical photolysis. In one embodiment, the decomposition catalyst is utilized under reaction conditions that are optimized for the specific NO species.

[0126] In certain embodiments, released NO is captured using one or more NO acceptors. The NO acceptors may be, for example, free in solution, cross-linked to a solid phase (e.g., functionalized magnetic or non-magnetic beads), and/or incorporated onto an electrode. Example NO acceptors may include metal porphyrins, thiocarbamates, thiols, spin

traps, small molecule radical traps, and/or amino acid moieties. FIGS. 15, 16, 17, and 18 are reaction diagrams depicting the capture of NO using (i) a metal porphyrin (or thiocarbamate) trap, (ii) a thiol trap, (iii) a spin trap, and (iv) a small molecule radical trap, respectively. FIG. 19 is a schematic diagram depicting the capture of nitrosonium ion (NO^+, the oxidized product of NO) using ion exchange with NAFION®.

[0127] In one embodiment, NO is captured using an NO acceptor that is cross-linked to beads, which may be magnetic or non-magnetic. When tyrosine is used as the NO acceptor, this approach may result in the formation of nitrotyrosine on the beads. This functionalized bead approach may be mediated by peroxynitrite that has been generated in the sample via the production of superoxide by added xanthine and xanthine oxidase. Referring to FIG. 20, with this approach, xanthine/xanthine oxidase may be used to generate superoxide. The superoxide may be reacted with NO to form peroxynitrite, and the peroxynitrite may be reacted with tyrosine to form nitrotyrosine, which may be measured electrochemically.

[0128] In certain embodiments, a method is applied in which it is desirable for capture devices to be associated with a free floating solid support or to be associated with an electrode surface (e.g., the working electrode). This may be accomplished using standard coupling chemistries that immobilize the capture device to either the solid support or the electrode surface. A method may be applied in which a size exclusion or an ionic barrier may be associated with a free floating solid support or associated with the electrode surface, for example, on the working electrode. This may be accomplished by applying a reagent that forms the size exclusion or ionic barrier in a manner that allows for the desired physical characteristic to be functionally expressed. In the case of a free floating solid support, the reagent could be applied in a vaporized or nebulized state while the free floating solid support is tumbled or shaken in a vessel containing the reagent. For an electrode, the reagent could be applied to the surface as a droplet and allowed to dry. In one embodiment, the reagent is applied with spin coating.

[0129] After the NO has been captured, the NO may be detected and measured electrochemically with various methods, depending on the capture technique. In certain embodiments, NO is detected and measured using the electrode system and electrochemical sensor, as described herein. In one embodiment, the electrode system is designed to specifically and selectively detect NO (or NO proxy) via NO (or NO proxy) oxidation on the

electrode, in combination with electrochemical methods that allow NO to be measured and quantified. The electrochemical method may utilize an amperometric method such as, for example, differential pulse voltammetry, anodic square wave voltammetry, and/or chronoamperometry.

[0130] In certain embodiments, the electrochemical sensor is calibrated using a reference standard having a known amount of NO. Example of reference standards include NONOate (i.e., R^1R^2N-(NO^-)-N=O, where R^1 and R^2 are alkyl groups), GSNO, SNO-Alb (Hu), and NO release reaction.

[0131] In certain embodiments, a sensor is provided that includes the working electrode 104, the reference electrode 106, the auxiliary electrode 102, and a barrier layer (e.g., the gas permeable membrane 126). The electrodes 102, 104, 106 may include any conductive material, such as a metal, graphite, or a conductive polymer. In certain embodiments, the working electrode includes platinum, platinized platinum, gold, silver, silver chloride, carbon, and/or composite structures, such as metal-polymer composites (e.g., platinized platinum and PTFE). A composite structure may optimize differentiation of gaseous species (i.e., NO) with respect to interfering oxidative species, in blood or plasma. In certain embodiments, the working electrode has a high surface area to facilitate direct measurement of target analyte (e.g., compared to other methods that use standard additions to determine or back-calculate blood RSNO level). The reference electrode 106 may include platinum, silver, silver chloride, gold, and/or carbon. The reference electrode 106 may be located at the same 'assembly level' where the working electrode 104 is located (e.g., under the same active membrane or gas permeable membrane 126). In certain embodiments, the barrier layer is disposed above the electrodes 102, 104, 106. For example, the barrier layer may be placed as immediately adjacent to the electrodes 102, 104, 106 as possible, while still maintaining an electrolyte between the electrodes 102, 104, 106 and the barrier layer. The barrier layer may include one or more gas permeable membranes such as, for example, silicone, PTFE (TEFLON®), polypropylene, polyethylene, poly(o-phenylenediamine) (P*o*PD), selectively permeable polymers (e.g., polyurethane), MYLAR®, and/or other polymeric materials and/or ionomer layers such as NAFION® with ion channels to inhibit anions from reaching the working electrode.

[0132] The barrier layer may help maximize NO transfer to the electrodes while minimizing the influence of interfering species. For example, the barrier layer may inhibit non-

gaseous species from reaching the electrodes. The barrier layer may also provide size exclusion to prevent large molecules from reaching the electrode. In one embodiment, the barrier layer provides selectivity by charge (such as NAFION®). The electrodes 102, 104, 106 may be positioned or disposed relative to one other within a sample medium.

[0133] In certain embodiments, it may be desirable for an electrode sensor, which includes a reaction chamber (partitioned via a membrane) for stabilizing and/or accumulating NO, to function optimally under pH conditions other than those found in the biological sample being assayed. Referring again to FIG. 4a, for example, nitrosylation of NAP may require the pH of the solution within a reaction chamber (i.e., between the electrodes 102, 104, 106 and the gas permeable membrane 126) to be pH 2 or lower. There may also be other off-line sample preparation methods involving NO capture that require pH conditions to be different from the pH of the biological sample being assayed.

[0134] In certain embodiments, an electrochemical sensor is provided with a reagent kit that is specific to the species of interest. The reagent kit may be provided, for example, in a special collection tube. In one embodiment, the electrode system for the electrochemical sensor is provided in the form of disposable (i.e., single-use) cartridges. A small bench top reader may be included that has a potentiostat, fluidics, and/or a compartment for electrode/sensor cartridges and/or software components.

[0135] In certain embodiments, the barrier layer (e.g., the gas permeable membrane 126) helps to maximize NO transfer to the electrodes while minimizing the influence of interfering species. For example, the barrier layer may inhibit non-gaseous species from reaching the electrodes. The barrier layer may also provide size exclusion to prevent large molecules from reaching the electrode. In one embodiment, the barrier layer provides selectivity by charge.

[0136] In certain embodiments, a catalyst is utilized to liberate NO from a biomolecule, such as RSNO. The catalyst may be disposed on, above, or in the immediate vicinity of the working electrode 104, such that NO generation may be localized to the working electrode 104. For example, the catalyst may be just upstream from the working electrode in a flow circuit, or it may be on an opposite surface of an ultra-low profile reservoir that minimizes reservoir height. The latter configuration may maximize blood/plasma contact with both the catalytic and opposing barrier layer on an opposite side.

[0137] In certain embodiments, an agent is added to prevent transnitrosylation. This stabilizes the NO location or carrier to prevent migration between thiols or proteins during an assay and before catalysis (e.g., with Cu or UV irradiation). The agent used for this purpose may be, for example, *n*-ethylmaleimide (NEM).

[0138] A chelating agent may also be added. The chelating agent may inactivate endogenous metals (e.g., Cu) that may catalyze or scavenge RSNO before it reaches the electrodes. In certain embodiments, the chelating agent is diethylenetriaminepentaacetic acid (DTPA) and/or ethylenediaminetetraacetic acid (EDTA).

[0139] In various embodiments, the methods and devices described herein provide an ability to interrogate an entire RSNO pool, including SNO-Albumin and SNO-Hb in a plasma fraction. In other embodiments, matrix effects are minimized to maximize NO recovery at the electrode. Minimizing matrix effects removes material in the vicinity of the electrode, thereby shortening the path NO needs to travel to reach the electrode within its short half life. Matrix effects may be minimized through minimal sample volume and minimal depth between a far side of an active sensor chamber and the electrode.

[0140] FIG. 21 is a flowchart of a method 2100 for measuring S-nitroso-albumin ((SNO)-albumin), in accordance with one embodiment of the invention. The method 2100 may be used, for example, in conjunction with the electrode system 100 depicted in FIG. 3. The method 2100 includes providing (step 2102) or collecting a sample of blood, plasma and/or other biological material(s) (e.g., from a human or other mammal, and/or culture fluid). The plasma sample (e.g., about 10-30μl worth) is applied (step 2104) to the electrode system 100 by sample spotting on an absorbent pad, across the working electrode 104, the reference electrode 106, and the auxiliary electrode 102. A potentiostat, which is connected to the electrode leads 110 of the electrode system 100, is used to apply (step 2106) an amperometric method , such as differential pulse voltammetry (e.g., sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps). From one to about 30 scans may be performed, with or without UV irradiation (step 2108). The UV irradiation releases NO from SNO-albumin. Oxidation of NO, which occurs at the surface of the electrode system 100, is detected (step 2110) using the potentiostat or other measurement device.

[0141] FIG. 22 is a flowchart of a method 2300 for measuring (SNO)-albumin, in accordance with one embodiment of the invention. The method 2300 may be used, for

example, in conjunction with the electrode system 100 depicted in FIG. 3. The method 2300 includes providing (step 2302) or collecting a biological material (e.g., plasma from a human patient or mammal). SNO-albumin in the sample is isolated (step 2304) and stabilized using anti-albumin magnetic or non-magnetic bead immunoaffinity isolation to produce an immunoaffinity resin or slurry with SNO-albumin bound to it. Albumin is eluted (step 2306) from the immunoaffinity resin, and the eluate is applied (step 2308) to the electrode system 100 by sample spotting on an absorbent pad, across the working electrode 104, the reference electrode 106, and the auxiliary electrode 102. The electrode leads 110 are then dried. The electrode system 100 is connected to a potentiostat, which is used to apply (step 2310) an amperometric method (e.g., differential pulse voltammetry, sweeping from 0.3 volts to 0.85 volts with 0.01 volts steps). From one to about 30 scans may be performed in the presence and absence (blank) of UV irradiation (step 2312). NO is released from SNO-albumin via the UV irradiation. Oxidation of NO occurs at the surface of the electrode system 100 to provide a signal, which may be detected (step 2314) and analyzed, as described herein. In the case where two or more curves provide a measurable response, the signal-to-noise ratio may be increased by adding responses from multiple curves.

[0142] FIG. 23 is a flowchart of a method 2400 for measuring (SNO)-albumin, in accordance with one embodiment of the invention. The method 2400 may be used, for example, in conjunction with the electrode system 120 depicted in FIG. 4a. The method 2400 includes providing (step 2402) or collecting a biological material (e.g., plasma) from a patient. SNO-albumin in the sample is isolated (step 2404) and stabilized using anti-albumin magnetic or non-magnetic bead immunoaffinity isolation to produce an immunoaffinity resin with SNO-albumin bound to it. Albumin is eluted (step 2406) from the immunoaffinity resin, and the eluate is deposited (step 2408) onto the gas permeable membrane 126 of the electrode system 120. UV irradiation is applied (step 2410) to the eluate to release NO from SNO-albumin. At this point, NO can be measured directly. However, in the event where a second release and capture step is desired utilizing a gas permeable membrane, the NO is then captured (step 2412) with an NO acceptor (e.g., N-acetylpenicillamine, which forms S-nitroso-N-acetylpenicillamine (SNAP)). The captured NO is held inside the gas permeable membrane 126, between the gas permeable membrane 126 and the electrodes 102, 104, 106. The electrode system 120 may be rinsed with, for example, water (e.g., deionized water), various pH-dependent buffers, and/or other designed rinses that may afford an advantage toward removing interfering substances.

After rinsing, the electrode leads 110 may then be dried. The electrode sensor is connected to a potentiostat, and an amperometric method (e.g., differential pulse voltammetry) is applied (step 2414), in the presence and absence (blank) of UV irradiation. Oxidation of NO is detected (step 2416) and analyzed, as described above.

[0143] In various embodiments, the devices and methods described herein are used to measure any concentration of NO that is present in a biological material. For example, the devices and methods may be used to measure NO concentrations from about picomolar (pM) to about millimolar (mM). In one embodiment, the devices and methods are used to measure NO concentrations from about nanomolar (nM) to about micromolar (μM).

[0144] In certain embodiments, the devices described herein are suitable for performing measurements over a wide range of time frames. For example, the devices may be used to perform measurements over time periods ranging from a few seconds to several weeks, or more. In one embodiment, the devices remain effective for more than one day, more than one week, more than one month, or more than one year. The sensors may experience minimal or no biofouling, even during extended exposure to biological materials.

EXAMPLES

[0145] The electrochemical sensor and associated methods described herein are presently found to demonstrate specificity for the measurement of NO. For example, in certain embodiments, the oxidation potential associated with NO detection matches known characteristic oxidation potential of NO (i.e., 650-750 mV), although the oxidation potential may vary or shift as assay conditions (e.g., pH) are changed. Further, the detected electrical signals are found to be absent when NO is absent. A dose dependent response has been observed with the use of NO gas, NONOate, RSNO and/or SNO-Albumin. UV irradiation at 300-400 nm has been effective, with 330 nm optimal for the decomposition of nitrosothiols. Additionally, detected signals are found to disappear when UV irradiation is removed, despite the presence of RSNO. In one embodiment, interferents do not give a significant signal at physiologic levels. Other evidence of specificity has been shown by, for example, decomposing all RSNO then measuring, and by burning off NO gas then measuring.

[0146] A series of experiments were performed to evaluate the effectiveness of the methods and electrochemical sensors described herein for detecting and quantifying NO in a biological material. FIGS. 24 through 27 are photographs of an electrochemical sensor system

2400 used for several of these experiments. The electrochemical sensor system 2400 included an electrode system 2402, a UV light source 2404, a computer 2406, and a potentiostat 2408. For some experiments, a peristaltic pump 2410 and a housing 2412 were utilized. The housing 2412 was substantially the same as the housing 1112 described above with respect to FIGS. 11b through 11f. The electrode system 2402 used to obtain the results in FIGS. 28 through 45 was the same as the electrode system 120 depicted in FIG. 4a. The electrode system 2402 used to obtain the results in FIGS. 46a through 50 was the same as the electrode system 100 depicted in FIG. 2.

[0147] FIG. 28 is a plot of current versus voltage obtained during measurements of NO gas at 100 ppm. The NO gas was diluted with scrubbed air in a total volume of 500 ml in a Tedlar bag that contained the electrode system 2402, which was the same as the electrode system 120 shown in FIG. 4a. The bag was placed in a pressure bomb under 120 psi for 10 minutes. Amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. A representative trace is shown for each NO gas concentration.

[0148] FIG. 29 is a plot of current versus voltage obtained during measurements of NO gas at various concentrations, as indicated in the figure. The NO gas was diluted with scrubbed air in a total volume of 500 ml in a Tedlar bag that contained the electrode system 2402. The bag was placed in a pressure bomb under 120 psi for 10 minutes. The electrode system 2402 was then removed from the bag and amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. Representative traces are shown for each NO gas concentration.

[0149] FIG. 30 is a plot of current versus voltage obtained during measurements of various gases that could act as interferents in blood. Various gases at the concentrations indicated in the figure were diluted with scrubbed air in a total volume of 500 ml in a Tedlar bag that contained the electrode system 2402. The bag was placed in a pressure bomb under 120 psi for 10 minutes. The electrochemical sensor 120 was removed from the bag and amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. Representative traces are shown for each gas tested.

[0150] Utilizing NONOate as a source of NO in solution, a dose response study was performed. One milliliter of NONOate in 10 mM phosphate buffered saline (PBS), pH 7.4 at various concentrations, as indicated in the FIG. 31, was continuously re-circulated across the

electrode system 2402 for 90 minutes. The electrochemical sensor was removed from the housing 2412 and rinsed. The gas permeable membrane was removed and a glass cover slip was applied over the electrode set. This resulted in the electrode system 120, as depicted in FIG. 5. Amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. FIG. 31 is a plot of current versus NONOate concentration obtained during measurements of NO gas.

[0151] FIG. 32 is a plot of current versus voltage obtained during measurements of NO gas from NONOate at various time points, as indicated in the figure. One milliliter of 100 μM NONOate in 10 mM PBS, pH 7.4, was continuously re-circulated across the electrode system 2402 for 60 minutes. Amperometric measurements (i.e., differential pulse voltammetry) were taken every 5 minutes. Representative traces are shown for each time point. FIG. 33 contains the same data plotted as current versus time. A representative trace is shown.

[0152] FIG. 34 is a plot of current versus voltage obtained during measurements of NO gas in porcine blood, in accordance with one embodiment of the invention. To perform the measurements, 50 ml of heparinized porcine whole blood was collected. The electrode system 2402 was placed into the blood and incubated with tumbling at 40 °C for 1 hour. The electrode system 2402 was rinsed thoroughly and the gas permeable membrane was removed. The electrode connectors were dried and the electrode system 2402 was connected to a potentiostat. The sensor was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulse voltammetry) were taken.

[0153] FIG. 35 is a plot of current versus voltage for measurements of NO in circulating bovine blood, in accordance with one embodiment of the invention. To incubate the electrode system 2402, the electrode system 2402 was housed in a piece of tubing in-line with an ex-vivo set up of circulating heparinized bovine blood. Incubation was performed with 14 liters of bovine blood, at a flowrate of 5.0 liters/minute, at 37 °C, for 1 hour. The electrode system 2402 was removed from the tubing, the electrode sensor was rinsed thoroughly and the gas permeable membrane was removed. The connectors were dried and the electrode system 2402 was connected to a potentiostat. The sensor was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulse voltammetry) were taken.

[0154] FIG. 36 is a plot of current versus voltage for measurements of NO gas exchanged out of circulating bovine blood. The electrode system 2402 was housed in a piece

of tubing in-line with a gas exchanger system for sampling blood gases in the ex-vivo set up of circulating heparinized bovine blood. Incubation was performed with 15 liters of bovine blood, at a flowrate of 5.0 liters/minute, at 37 °C, for 1 hour. The electrode system 2402 was removed from the tubing and the electrode system 2402 was rinsed thoroughly. The gas permeable membrane was removed, the connectors were dried, and the electrode system 120 was connected to a potentiostat. The electrode system 2402 was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulse voltammetry, resulting in a signature current response indicative of the oxidation of NO) were taken.

[0155] FIG. 37 is a plot of current versus voltage for measurements of NO in exhaled breath, in accordance with an embodiment of the present invention. The electrode system 2402 was fastened to the inside of a surgical mask and worn by a patient so that the gas permeable membrane 126 was facing out and directly across from the patient's mouth. The patient wore the mask with the electrode system 2402 and breathed normally for one hour. The electrode system was rinsed thoroughly and the gas permeable membrane 126 was removed. The connectors were dried and the electrode system 2402 was connected to a potentiostat. The sensor was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulse voltammetry, resulting in a signature current response indicative of the oxidation of NO) were taken.

[0156] Utilizing the system 2400 depicted in FIG. 27, including the peristaltic pump 2410 and the housing 2412, a blood gas exchanger was added in-line to introduce gases to circulating blood. One liter of heparinized bovine blood was added to the system and the blood was stabilized at physiological pO_2, pCO_2, and pH. The electrode system 2402 was placed within tubing used to circulate blood, as depicted in FIG. 11a. A second electrode system 2402 was placed in the housing 2412, as depicted in FIGS. 11b and 11c, in-line with the gas exchanger exhaust. The blood was continuously re-circulated across the electrode systems 2402 at 3.5 liters/min. Referring to FIGS. 38 and 39, blood was ventilated with 10% NO gas via the gas exchanger at various flow rates. After 60 minutes, the electrode systems 2402 were removed from the tubing and the housing 2412 and rinsed. The gas permeable membranes were removed and replaced with glass cover slips. Amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. FIG. 38 is a plot of current versus voltage for measurements of NO gas in circulating and ventilated bovine blood. FIG. 39 is a plot of current versus voltage for measurements of NO gas in the gas exchanger exhaust.

FIGS. 38 and 39 show representative traces for the measurements made for each of the indicated NO gas flow rates.

[0157] Utilizing the system 2400 depicted in FIG. 27, one liter of heparinized bovine blood, with and without NEM added, was pumped into the system 2400 and the blood was stabilized at physiological pO_2, pCO_2, and pH. The electrode system 2402 was placed within tubing used to circulate blood, as depicted in FIG. 11a. A second electrode system 2402 was placed in the housing 2412, as depicted in FIGS. 11b and 11c, in-line with the gas exchanger exhaust. The blood was continuously re-circulated across the electrode system 2402, at 3.5 liters/min. Blood was ventilated with 10% NO gas via the gas exchanger at a flow rate of 0.2 liters/min for 60 minutes. The electrode system 120 was connected to a potentiostat and amperometric measurements (i.e., differential pulse voltammetry) were taken every 5 minutes. FIG. 40 shows representative traces of the NO gas measurements over time from the gas exchanger exhaust. FIG. 41 shows representative traces of the NO gas measurements over time from the blood. The two curves in each of these figures show the influence of NEM on the measurement results.

[0158] Referring to FIGS. 42 and 43, in another experiment, the electrode system 2402 was placed at the bottom of a single well 4302 in a 96-well plate 4304. FIG. 44 is a schematic of the well 4302 and the electrode system 2402 used for the experiment. The electrode system included the working electrode 104, the electrode leads 110, the absorbent pad 124 (with 2 µl of PBS), and the gas permeable membrane 126. As depicted, a bottom portion of the single well 4302 was removed and replaced with the electrode system 2402, so that the gas permeable membrane was exposed to the inside of the well 4302. The well was filled with various amounts of nitrite (as indicated in FIG. 45), in 500mM sodium iodide (NaI) in 25 % acetic acid. The well 4302 was covered with a glass cover slip 151 and inverted. Incubation occurred with stirring, using a stir bar 4306, for ten minutes. The electrode system 2402 was then removed from the plate and rinsed. The gas permeable membrane 126 was replaced with a glass cover slip, and amperometric measurements (i.e., differential pulse voltammetry) were taken with UV irradiation. FIG. 45 shows representative traces of the NO gas measurements for the indicated nitrite concentrations.

[0159] FIGS. 46a, 46b, and 46c are plots of current versus voltage obtained during the measurement of nitric oxide released from S-Nitrosothiogylcolic acid, S-Nitrosoglutathione, and

S-Nitrosocysteine. For these measurements, 30µl of a 500µM RSNO (as indicated) preparation was spotted onto a NAFION® coated electrode system (i.e., the electrode system 100, shown in FIG. 2). Amperometric measurements (i.e., cyclic voltammetry) were taken with and without UV irradiation, as indicated by the two curves in each plot. A representative trace is shown for each RSNO.

[0160] FIGS. 47a, 47b, and 47c are plots of current versus voltage for measurements obtained with known plasma interferents (nitrite, ascorbate, and cysteine, respectively). For these measurements, 30µl of the tested interferents was prepared in NO depleted human plasma at the indicated concentrations. Samples were spotted onto a NAFION® coated electrode system (i.e., the electrode system 100, shown in FIG. 2). Amperometric measurements (i.e., differential pulsed voltammetry) were taken with UV irradiation. Representative traces are shown.

[0161] FIG. 48 is a plot of measured electrical current during the release of NO from SNO-Alb in NO-depleted human plasma, in the presence of known interferences. For these measurements, 30µl of an SNO-Alb preparation was added to NO-depleted human plasma. Samples were spotted onto a NAFION® coated electrode system (i.e., the electrode system 100, shown in FIG. 2). Amperometric measurements (i.e., differential pulsed voltammetry) were taken with UV irradiation. Representative data points are shown.

[0162] FIG. 49 is a plot of current versus voltage obtained during measurements of NO released from De-nitrosylated albumin. To perform the measurements, 30µl of a 100µM de-nitrosylated human serum albumin preparation was spotted onto a NAFION® coated electrode system (i.e., the electrode system 100). Amperometric measurements (i.e., differential pulsed voltammetry) were taken with UV irradiation. Representative traces are shown.

[0163] FIG. 50 is a plot of current versus voltage obtained during measurements of NO released from SNO-Alb in NO-depleted human plasma. For these measurements, 30µl of a SNO-Alb preparation was added to a human plasma (SNO-Alb depleted) sample at various concentrations, as indicated in the figure. Samples were spotted onto a NAFION® coated electrode system (i.e., the electrode system 100). The electrode system was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulsed voltammetry) were taken. A representative trace is shown for each concentration of SNO-Alb.

[0164] To demonstrate reproducibility of the sensor and the methods described herein, a set of measurements were taken of NO released from SNO-Alb in NO-depleted human plasma. To perform the measurements, 30µl of an SNO-Alb preparation was added to an NO-depleted human plasma sample at concentrations of about 100 µM, about 40 µM, about 20 µM, and zero µM. The samples were spotted onto a NAFION® coated electrode system (i.e., the electrode system 100). The electrode system was UV irradiated for all samples except the control while amperometric measurements (i.e., differential pulsed voltammetry) were taken. Fifteen separate measurements were obtained for each of the above concentrations. Table 1 includes the calculated means, standard deviations (SD), and coefficients of variance (CV%) for the set of measurements. In a separate study, the limit of detection for SNO-HSA was determined to be about 10 µM.

Base		Mean	SD	CV%
~100 µM		1.107	0.133	12.005
~40 µM		0.625	0.072	11.469
~20 µM		0.254	0.041	15.942
0 µM		0.000	0.000	0.000
*Note: NO was below detection for 0 µM samples.				

Table 1. Mean, standard deviation, and coefficient of variance.

EQUIVALENTS

[0165] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

INCORPORATION BY REFERENCE

[0166] The entire contents of all patents published, patent applications, websites, and other references cited herein are hereby expressly incorporated herein in their entireties by reference.

[0167] What is claimed is :

1. An electrochemical sensor for the detection and quantification of nitric oxide in a biological material, the electrochemical sensor comprising:

a substrate;

an electrode set disposed on the substrate, the electrode set comprising a working electrode, a reference electrode, and an auxiliary electrode; and

an ionomer coating on at least a portion of the electrode set.

2. The electrochemical sensor of claim 1, further comprising:

a gas permeable membrane disposed over the electrode set; and

an absorbent pad disposed between the gas permeable membrane and the electrode set, the absorbent pad comprising at least one of a nitric oxide acceptor and a buffer.

3. The electrochemical sensor of claim 2, further comprising a gasket disposed between the gas permeable membrane and the substrate to seal the absorbent pad between the gas permeable membrane and the substrate.

4. The electrochemical sensor of any one of the preceding claims, wherein the substrate comprises a material selected from the group consisting of rayon, acetate, triacetate, nylon, and polyester.

5. The electrochemical sensor of any one of the preceding claims, wherein the ionomer comprises at least one of ethylene/methacrylic acid and a fluoropolymer-copolymer.

6. The electrochemical sensor of any one of the preceding claims, wherein the reference electrode comprises a material selected from the group consisting of silver, silver chloride, palladium, and platinum.

7. The electrochemical sensor of any one of the preceding claims, wherein the reference electrode and the auxiliary electrode are the same electrode.

8. The electrochemical sensor of any one of the preceding claims, wherein the reference electrode and the auxiliary electrode are different electrodes.

9. The electrochemical sensor of any one of the preceding claims, wherein the nitric oxide acceptor comprises at least one member selected from the group consisting of oxygen (O_2), a capture reagent, an acid, and a neutral pH buffer.

10. The electrochemical sensor of claim 9, wherein the nitric oxide acceptor comprises oxygen (O_2).

11. The electrochemical sensor of claim 9, wherein the nitric oxide acceptor comprises the capture reagent, the capture reagent comprising at least one member selected from the group consisting of a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety.

12. The electrochemical sensor of claim 9, wherein the nitric oxide acceptor comprises the acid, the acid comprising at least one member selected from the group consisting of sulfuric acid, hydrochloric acid, and trichloroacetic acid.

13. The electrochemical sensor of claim 9, wherein the nitric oxide acceptor comprises the neutral pH buffer, the neutral pH buffer comprising at least one member selected from the group consisting of a phosphate buffer, a tris buffer, a citrate buffer, a tricine buffer, and a HEPES buffer.

14. The electrochemical sensor of any one of the preceding claims, wherein the gas permeable membrane comprises a material selected from the group consisting of silicon, polytetrafluoroethylene, polypropylene, polyethylene, and polyester.

15. The electrochemical sensor of any one of the preceding claims, further comprising a sealant between the gas permeable membrane and an edge of the substrate.

16. A method of measuring an amount of nitric oxide in a biological material, the method comprising:

providing the biological material, wherein the biological material comprises a stabilizing agent;

contacting the biological material to an electrochemical sensor, wherein the electrochemical sensor comprises:

a gas permeable membrane;

an absorbent pad; and

an electrode set, the absorbent pad sealed between the gas permeable membrane and the electrode set, and wherein the absorbent pad comprises at least one of a nitric oxide acceptor and a buffer;

performing amperometric measurements using the electrode set; and

detecting an electrical signal at the electrode set, wherein the electrical signal is indicative of the amount of nitric oxide in the biological material.

17. The method of claim 16, further comprising:

removing the gas permeable membrane from the electrochemical sensor;

sealing the absorbent pad and the electrode set with a cover slip, wherein the cover slip is substantially transparent and substantially impermeable; and

irradiating the absorbent pad with UV light.

18. The method of claim 16 or claim 17, wherein the cover slip comprises glass.

19. The method of any one of claims 16 to 18, wherein the stabilizing agent comprises at least one member selected from the group consisting of an alkylating agent, a chelator, and an anticoagulant.

20. The method of any one of claims 16 to 19, wherein the biological material comprises an alkylating agent comprising N-ethylmaleimide (NEM).

21. The method of any one of claims 16 to 20, wherein the biological material comprises a chelator comprising ethylenediaminetetraacetic acid (EDTA).

22. The method of any one of claims 16 to 21, wherein the biological material comprises an anticoagulant comprising at least one member selected from the group consisting of citrate, EDTA, and heparin.

23. The method of any one of claims 16 to 22, wherein the nitric oxide acceptor comprises at least one member selected from the group consisting of oxygen (O_2), a capture reagent, an acid, and a neutral pH buffer.

24. The method of any one of claims 16 to 23, wherein the amperometric measurements comprise a method selected from the group consisting of chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry.

25. The method of any one of claims 16 to 24, wherein the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

26. A method of measuring an amount of nitric oxide in a biological fluid, the method comprising:

providing the biological fluid;

incubating an electrochemical sensor in the biological fluid, wherein the electrochemical sensor comprises a gas permeable membrane disposed over an electrode set;

capturing nitric oxide with a nitric oxide acceptor, wherein the captured nitric oxide is held between the gas permeable membrane and the electrode set;

performing amperometric measurements using the electrode set; and

detecting an electrical signal at the electrode set, wherein the electrical signal is indicative of the amount of nitric oxide in the biological fluid.

27. The method of claim 26, further comprising rinsing the sensor.

28. The method of claim 26 or claim 27, further comprising removing the gas permeable membrane from the electrochemical sensor.

29. The method of any one of claims 26 to 28, further comprising drying an electrode lead of the electrode set.

30. The method of any one of claims 26 to 29, wherein the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

31. The method of any one of claims 26 to 30, wherein the biological fluid comprises a stabilizing agent comprising at least one member selected from the group consisting of an alkylating agent, a chelator, and an anticoagulant.

32. The method of any one of claims 26 to 31, wherein the biological fluid comprises N-ethylmaleimide (NEM).

33. The method of any one of claims 26 to 32, wherein the biological fluid comprises ethylenediaminetetraacetic acid (EDTA).

34. The method of any one of claims 26 to 33, wherein the biological fluid comprises at least one member selected from the group consisting of citrate, EDTA, and heparin.

35. The method of any one of claims 26 to 34, wherein the gas permeable membrane comprises a material selected from the group consisting of silicon, polytetrafluoroethylene, polypropylene, polyethylene, and polyester.

36. The method of any one of claims 26 to 35, wherein the nitric oxide acceptor comprises at least one member selected from the group consisting of a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety.

37. The method of claim 36, wherein the nitric oxide acceptor comprises the amino acid moiety.

38. The method of claim 37, wherein the amino acid moiety is cross-linked to a solid phase.

39. The method of claim 38, wherein the solid phase comprises functionalized beads.

40. The method of claim 37, wherein the amino acid moiety is disposed on an electrode.

41. The method of claim 37, wherein the amino acid moiety is free in solution.

42. The method of any one of claims 26 to 41, wherein the amperometric measurements comprise a method selected from the group consisting of chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry.

43. The method of any one of claims 26 to 42, wherein the biological fluid comprises blood or plasma.

44. The method of any one of claims 26 to 43, wherein incubating the electrochemical sensor comprises tumbling the electrochemical sensor in the biological fluid.

45. The method of claim 44, wherein the tumbling occurs for at least about 10 minutes at a temperature of about 40 °C.

46. The method of any one of claims 26 to 45, wherein incubating the electrochemical sensor comprises recirculating the biological fluid over the gas permeable membrane of the electrochemical sensor.

47. The method of claim 46, wherein the recirculating occurs for at least about one minute at a temperature of about 40 °C.

48. The method of any one of claims 26 to 47, wherein the biological fluid is a gas exhaled from a patient.

49. The method of any one of claims 26 to 48, wherein incubating the electrochemical sensor comprises positioning the gas permeable membrane proximate a mouth of a patient.

50. The method of any one of claims 26 to 49, wherein incubating the electrochemical sensor comprises positioning the gas permeable membrane within a mask disposed over the mouth of the patient.

51. The method of any one of claims 26 to 50, wherein the biological fluid comprises air, and wherein incubating the electrochemical sensor comprises blowing the air across the gas permeable membrane.

52. The method of any one of claims 26 to 51, wherein incubating the electrochemical sensor comprises positioning the electrochemical sensor within tubing and circulating the biological fluid through the tubing.

53. A method of measuring an amount of RSNO in a biological material, the method comprising:

providing the biological material comprising a stabilizing agent;

applying the biological material to an absorbent pad disposed on an electrode set;

irradiating the biological material with UV light;

performing amperometric measurements using the electrode set; and

detecting an electrical signal at the electrode set, wherein the electrical signal is indicative of the amount of RSNO in the biological material.

54. The method of claim 53, wherein the stabilizing agent comprises at least one member selected from the group consisting of an alkylating agent, a chelator, and an anticoagulant

55. The method of claim 53 or claim 54, wherein the biological material comprises an alkylating agent comprising N-ethylmaleimide (NEM).

56. The method of any one of claims 53 to 55, wherein the biological material comprises a chelator comprising ethylenediaminetetraacetic acid (EDTA).

57. The method of any one of claims 53 to 56, wherein the biological material comprises an anticoagulant comprising at least one member selected from the group consisting of citrate, EDTA, and heparin.

58. The method of any one of claims 53 to 57, wherein the amperometric measurements comprise a method selected from the group consisting of chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry.

59. The method of any one of claims 53 to 58, wherein the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

60. A method of measuring an amount of RSNO in a biological material, the method comprising:

providing the biological material;

applying anti-albumin immunoaffinity isolation to the biological material to produce an immunoaffinity resin;

eluting albumin from the immunoaffinity resin to produce an eluate;

applying the eluate to an absorbent pad disposed on an electrode set;

irradiating the eluate with UV light;

performing amperometric measurements using the electrode set; and

detecting an electrical signal at the electrode set, wherein the electrical signal is indicative of the amount of RSNO in the biological material.

61. The method of claim 60, wherein the anti-albumin immunoaffinity isolation comprises at least one of magnetic bead immunoaffinity isolation and non-magnetic bead immunoaffinity isolation.

62. The method of claim 60 or claim 61, wherein the amperometric measurements comprise a method selected from the group consisting of chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry.

63. The method of any one of claims 60 to 62, wherein the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

64. A method of measuring an amount of RSNO in a biological material, the method comprising:

providing the biological material;

applying anti-albumin immunoaffinity isolation to the biological material to produce an immunoaffinity resin;

eluting albumin from the immunoaffinity resin to produce an eluate;

applying the eluate to a gas permeable membrane disposed over an electrode set;

irradiating the eluate with UV light to release nitric oxide from the albumin;

capturing nitric oxide with a nitric oxide acceptor, wherein the captured nitric oxide is held between the gas permeable membrane and the electrode set;

performing amperometric measurements using the electrode set; and

detecting an electrical signal at the electrode set, wherein the electrical signal is indicative of the amount of RSNO in the biological material.

65. The method of claim 64, wherein the anti-albumin immunoaffinity isolation comprises at least one of magnetic bead immunoaffinity isolation and non-magnetic bead immunoaffinity isolation.

66. The method of claim 64 or claim 65, wherein the gas permeable membrane comprises a material selected from the group consisting of silicon, polytetrafluoroethylene, polypropylene, polyethylene, and polyester.

67. The method of any one of claims 64 to 66, wherein the nitric oxide acceptor comprises at least one member selected from the group consisting of a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety.

68. The method of claim 67, wherein the nitric oxide acceptor comprises the amino acid moiety.

69. The method of claim 68, wherein the amino acid moiety is cross-linked to a solid phase.

70. The method of claim 69, wherein the solid phase comprises functionalized beads .

71. The method of claim 68, wherein the amino acid moiety is disposed on an electrode.

72. The method of claim 68, wherein the amino acid moiety is free in solution.

73. The method of any one of claims 64 to 72, wherein the amperometric measurements comprise a method selected from the group consisting of chronoamperometry, potentiometry, cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry.

74. The method of any one of claims 64 to 73, wherein the amperometric measurements provide a signature current response indicative of nitric oxide oxidation.

75. A method of manufacturing an electrochemical sensor for the detection and quantification of nitric oxide in a biological material, the method comprising:

forming an electrode set on a substrate, the electrode set comprising a working electrode, a reference electrode, and an auxiliary electrode;

pre-conditioning the electrode set in the presence of an alkaline solution and cyclic voltammetry; and

coating at least a portion of the electrode set with an ionomer.

76. The method of claim 75, further comprising:

applying a gasket around a working end of the electrode set;

positioning an absorbent pad over the working end of the electrode set, wherein the absorbent pad comprises a nitric oxide acceptor; and

disposing a gas permeable membrane over the working end of the electrode set.

77. The method of claim 76, further comprising applying a sealant between the gas permeable membrane and the substrate.

78. The method of any one of claims 75 to 77, wherein the substrate comprises a material selected from the group consisting of rayon, acetate, triacetate, nylon, and polyester.

79. The method of any one of claims 75 to 78, wherein the reference electrode and the auxiliary electrode are the same electrode.

80. The method of any one of claims 75 to 78, wherein the reference electrode and the auxiliary electrode are different electrodes.

81. The method of any one of claims 75 to 80, wherein the reference electrode comprises a material selected from the group consisting of silver, silver chloride, palladium, and platinum.

82. The method of any one of claims 75 to 81, wherein the alkaline solution comprises KOH.

83. The method of any one of claims 75 to 82, wherein the cyclic voltammetry is 10 cycle.

84. The method of any one of claims 75 to 83, wherein the ionomer comprises at least one of ethylene/methacrylic acid and a fluoropolymer-copolymer.

85. The method of any one of claims 76 to 84, wherein the nitric oxide acceptor comprises at least one member selected from the group consisting of oxygen (O_2), a capture reagent, an acid, and a neutral pH buffer.

86. The method of claim 85, wherein the nitric oxide acceptor comprises oxygen (O_2).

87. The method of claim 85, wherein the nitric oxide acceptor comprises the capture reagent, the capture reagent comprising at least one of a thiol (RSH), an amine (RNH), a metal porphyrin, a thiocarbamate, a spin trap, a small molecule radical trap, and an amino acid moiety.

88. The method of claim 85, wherein the nitric oxide acceptor comprises the acid, the acid comprising at least one member selected from the group consisting of sulfuric acid, hydrochloric acid, and trichloroacetic acid.

89. The method of claim 85, wherein the nitric oxide acceptor comprises the neutral pH buffer, the neutral pH buffer comprising at least one member selected from the group consisting of a phosphate buffer, a tris buffer, a citrate buffer, a tricine buffer, and a HEPES buffer.

90. The method of any one of claims 76 to 89, wherein the gas permeable membrane comprises a material selected from the group consisting of silicon, polytetrafluoroethylene, polypropylene, polyethylene, and polyester.

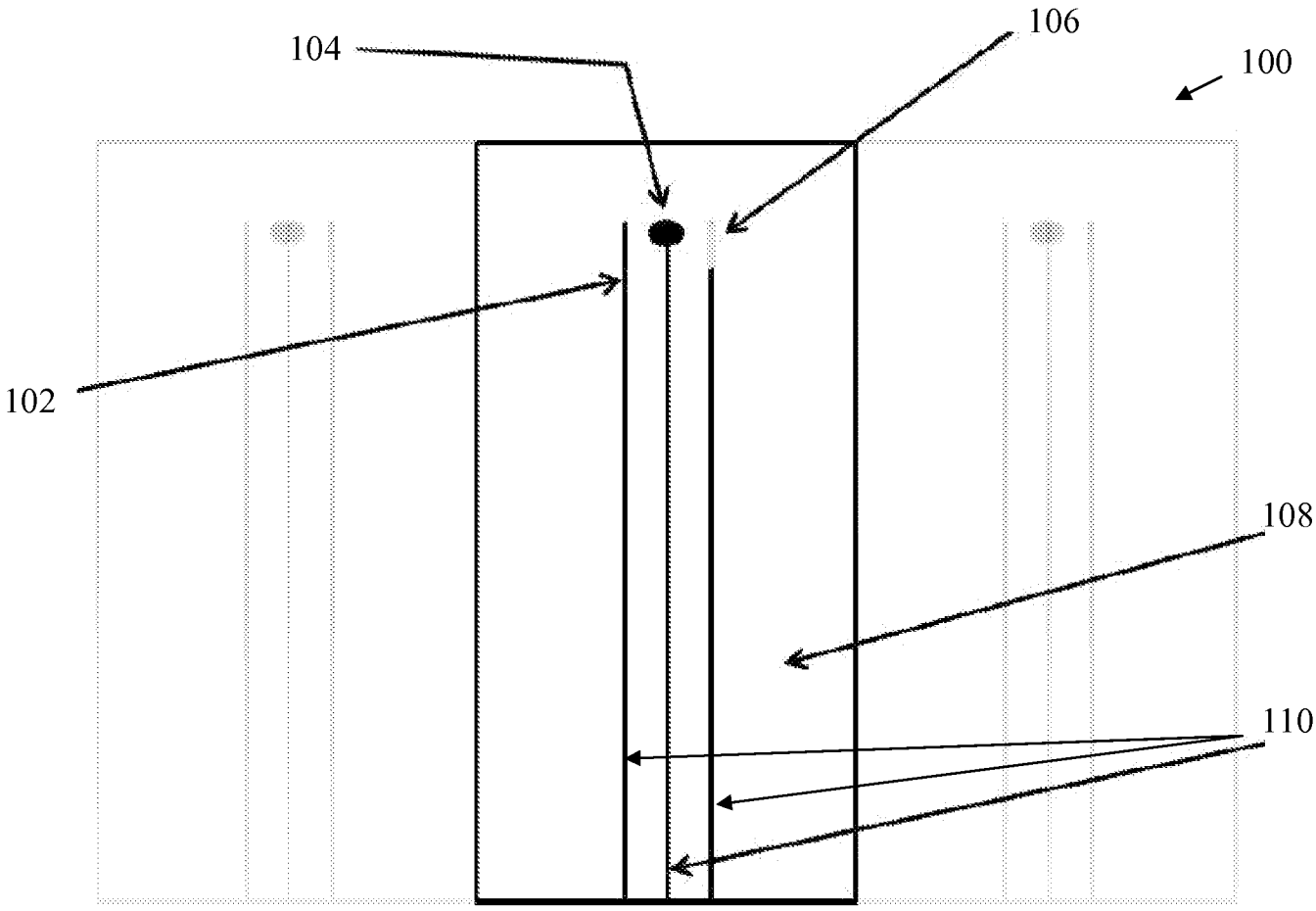

FIG. 1a

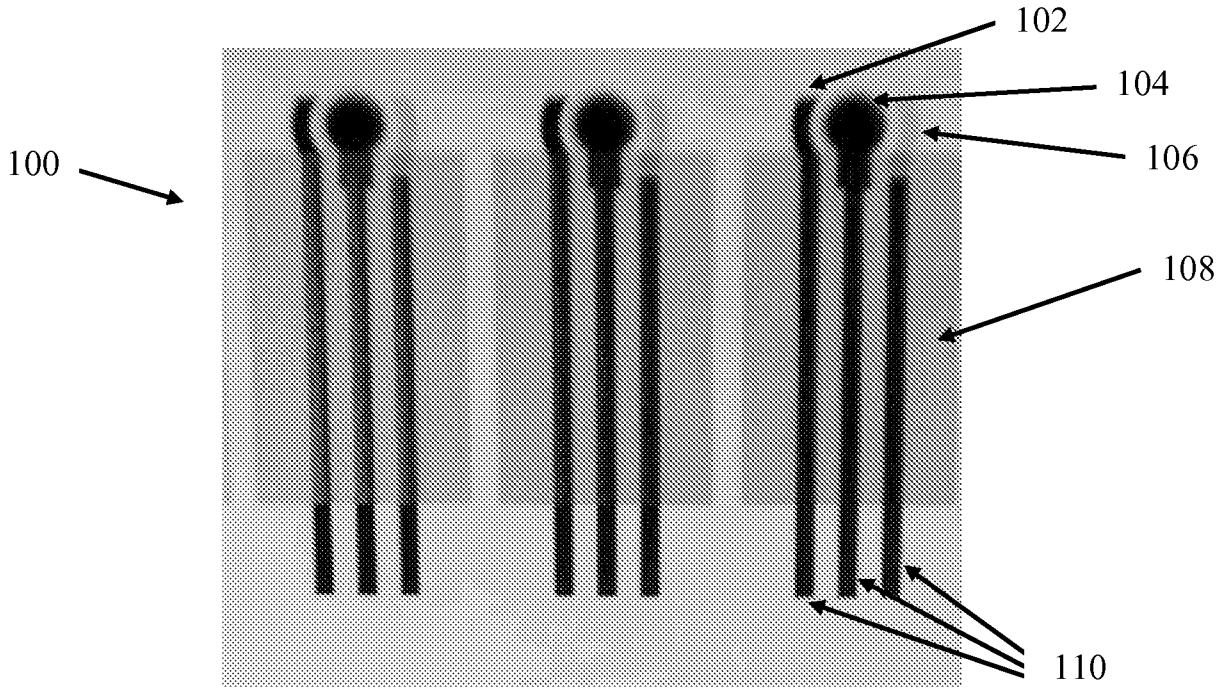

FIG. 1b

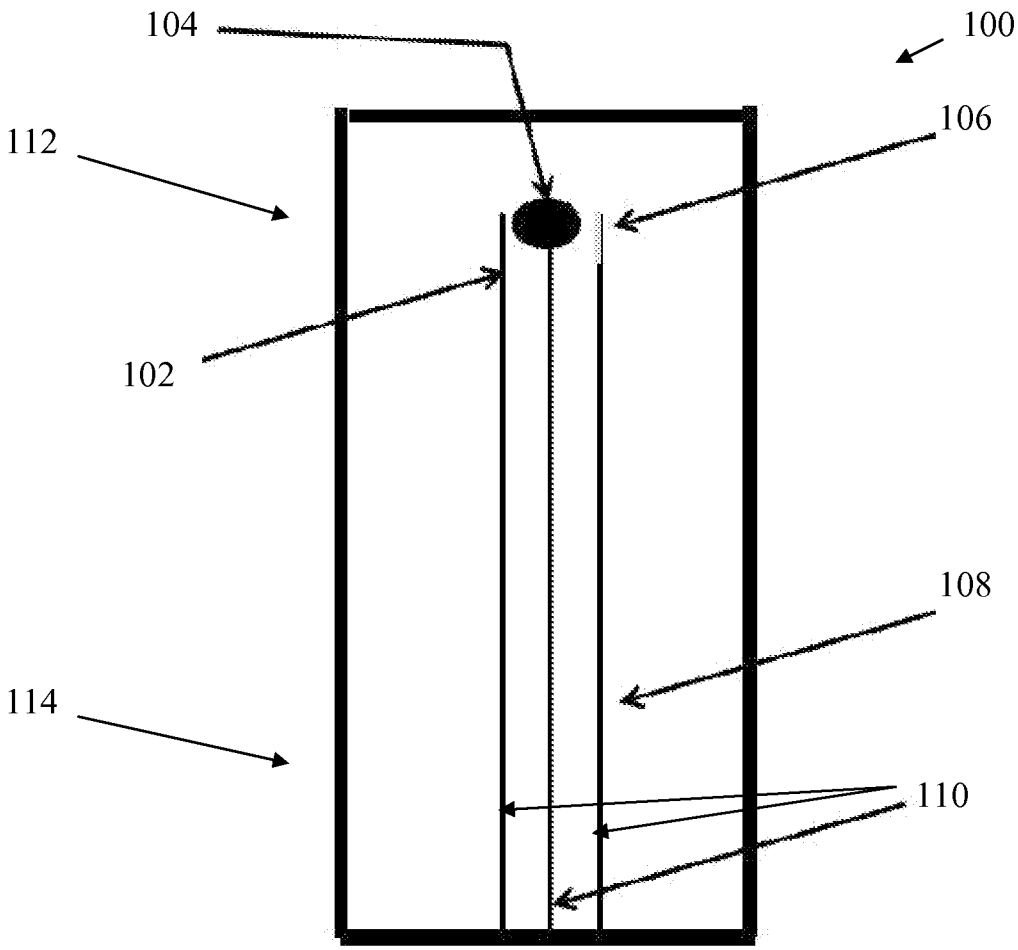

FIG. 2

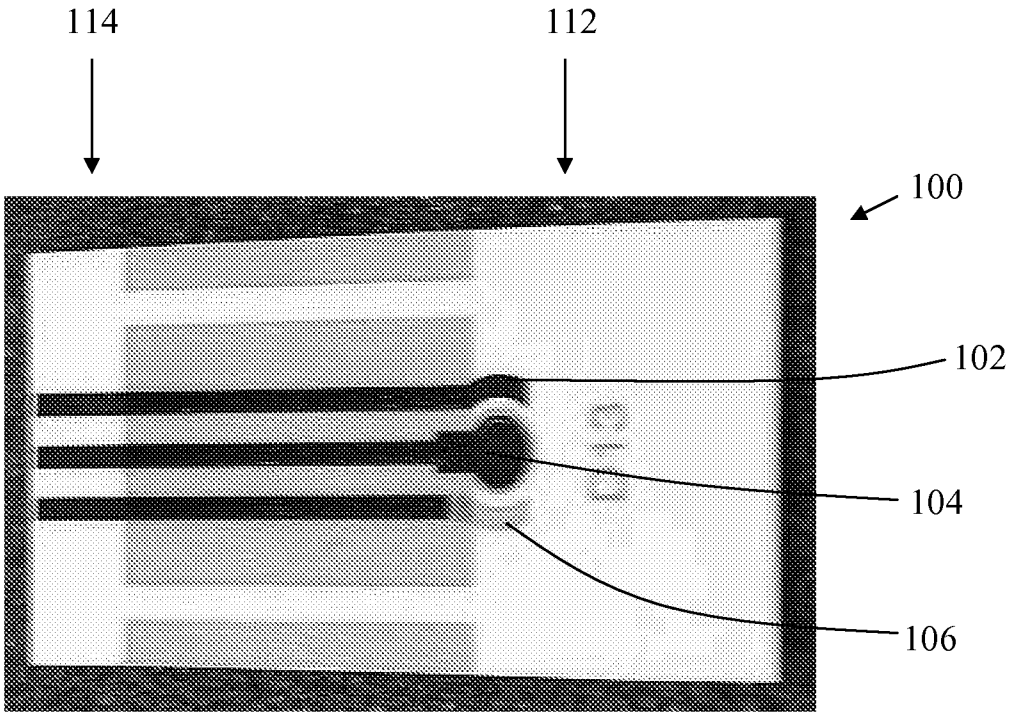

FIG. 3

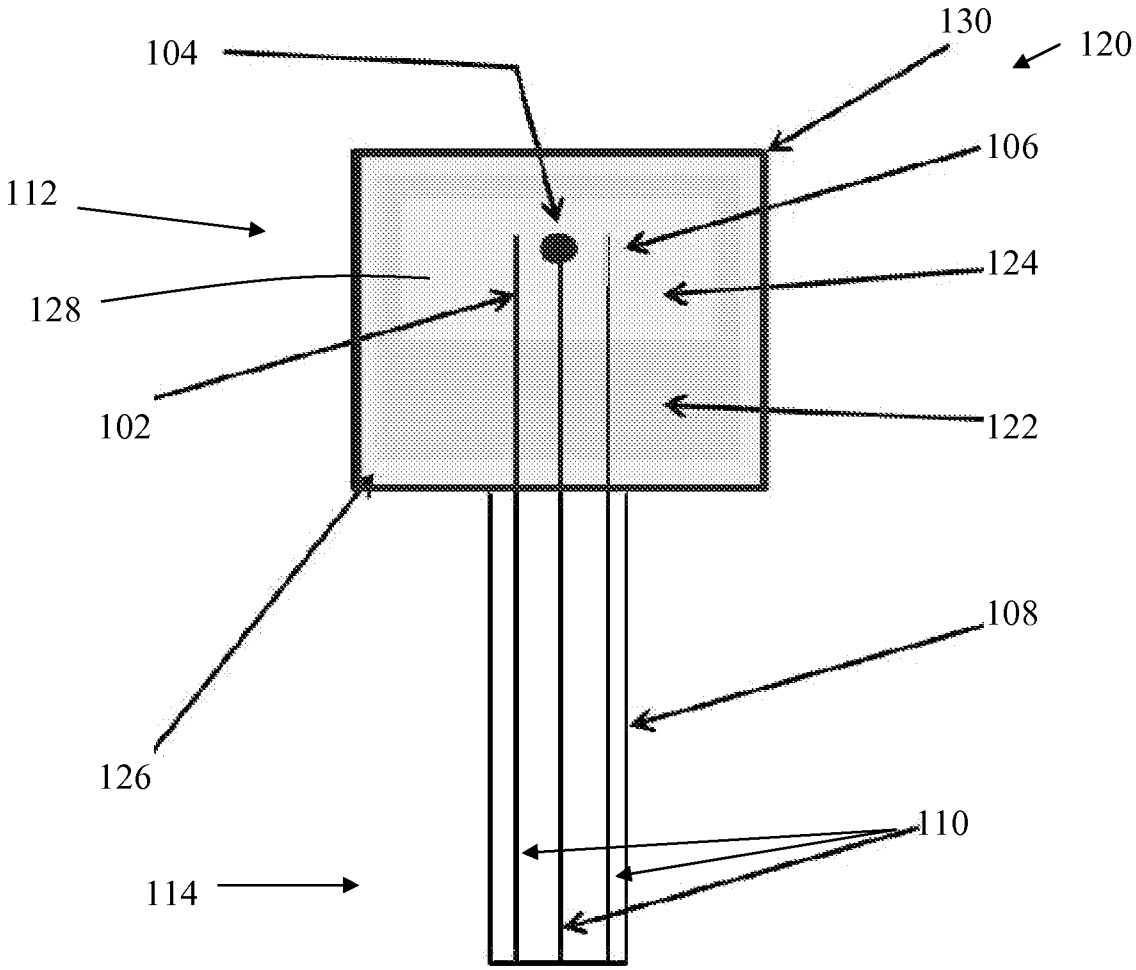

FIG. 4a

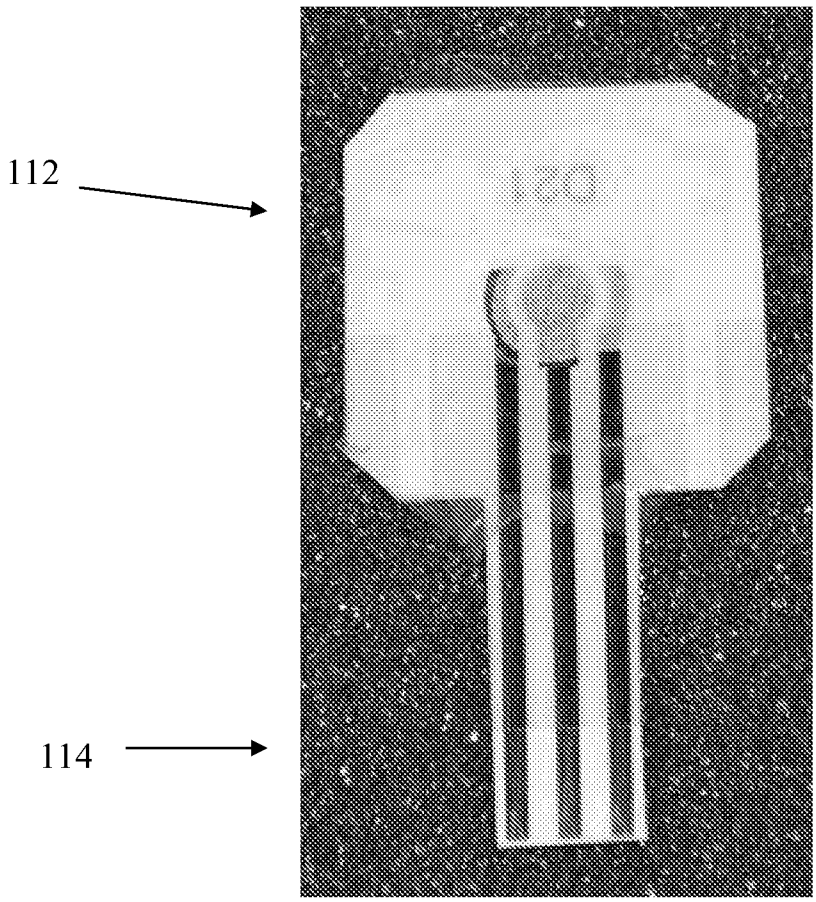

FIG. 4b

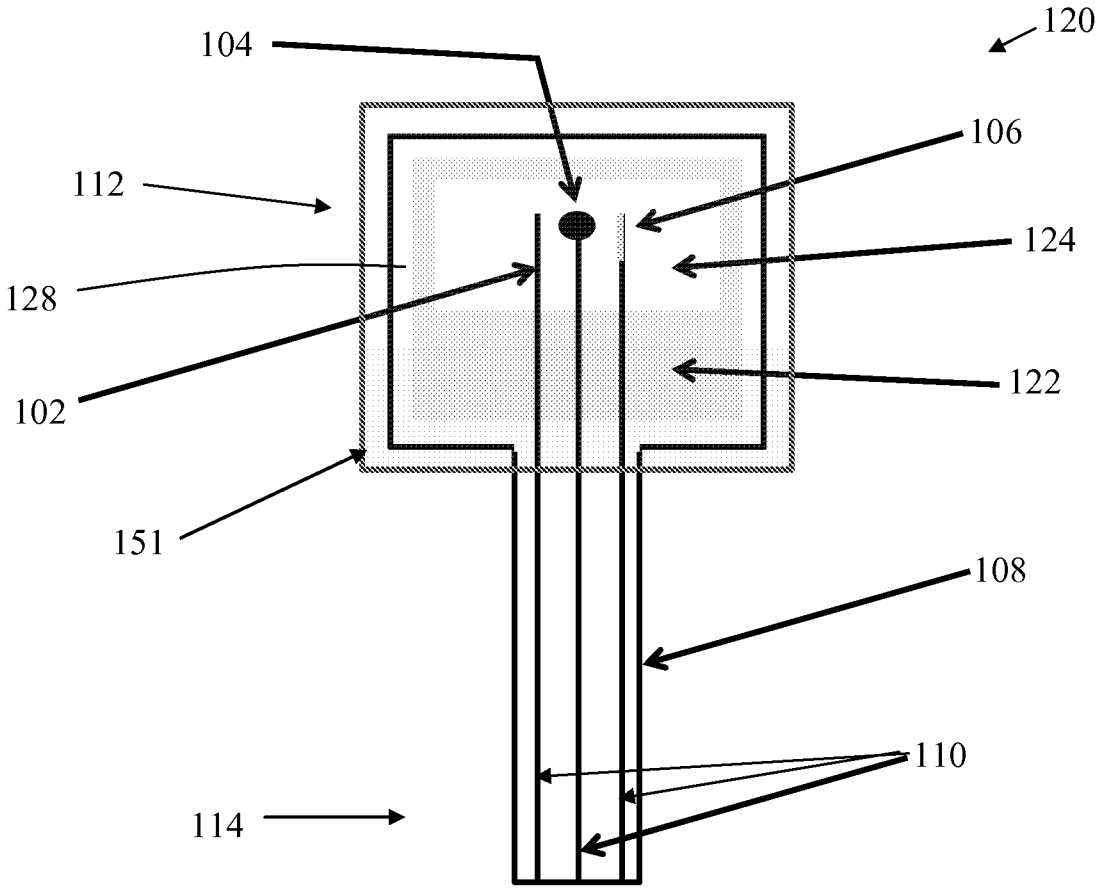

FIG. 5

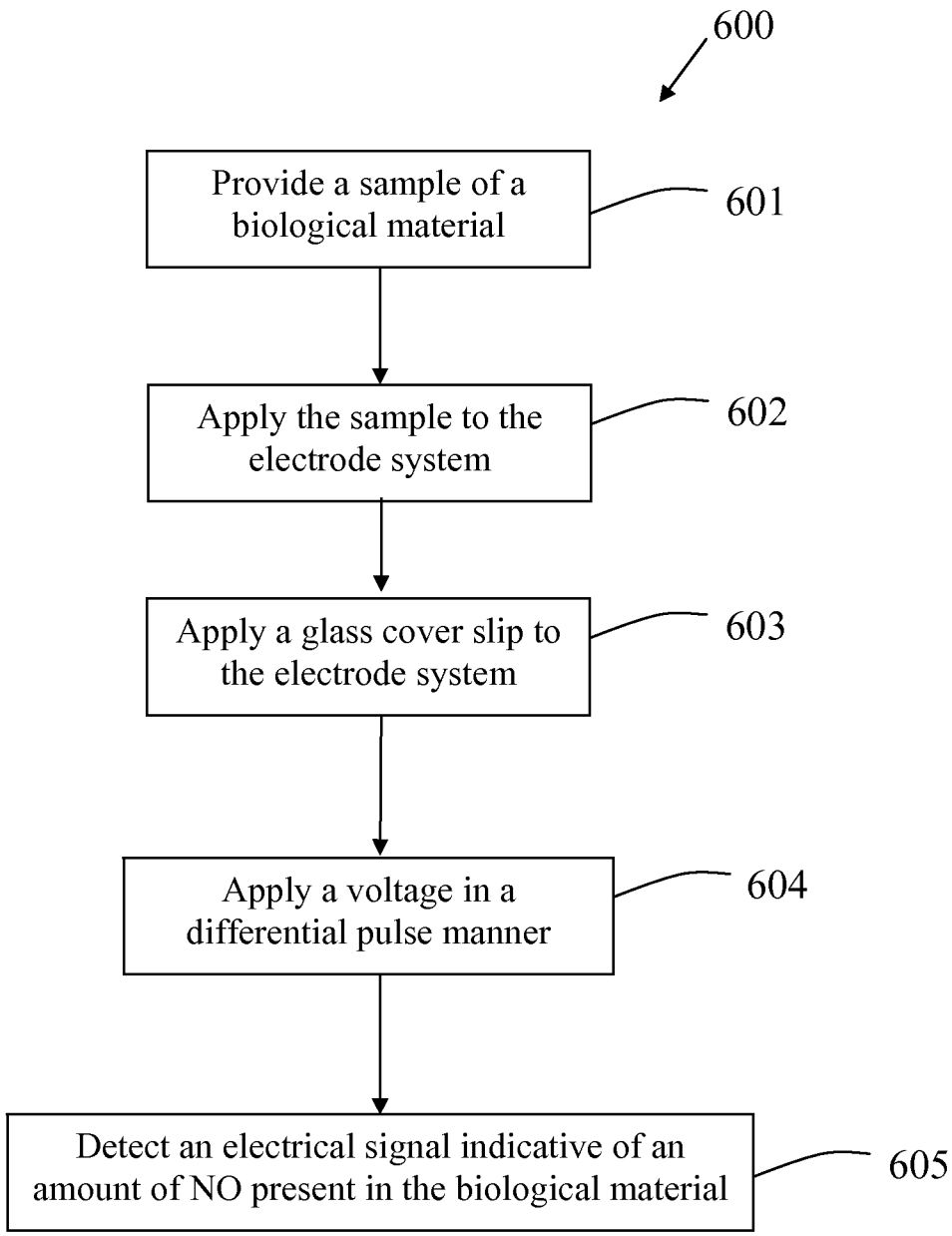
600
Provide a sample of a biological material
601
Apply the sample to the electrode system
602
Apply a glass cover slip to the electrode system
603
Apply a voltage in a differential pulse manner
604
Detect an electrical signal indicative of an amount of NO present in the biological material
605

FIG. 6

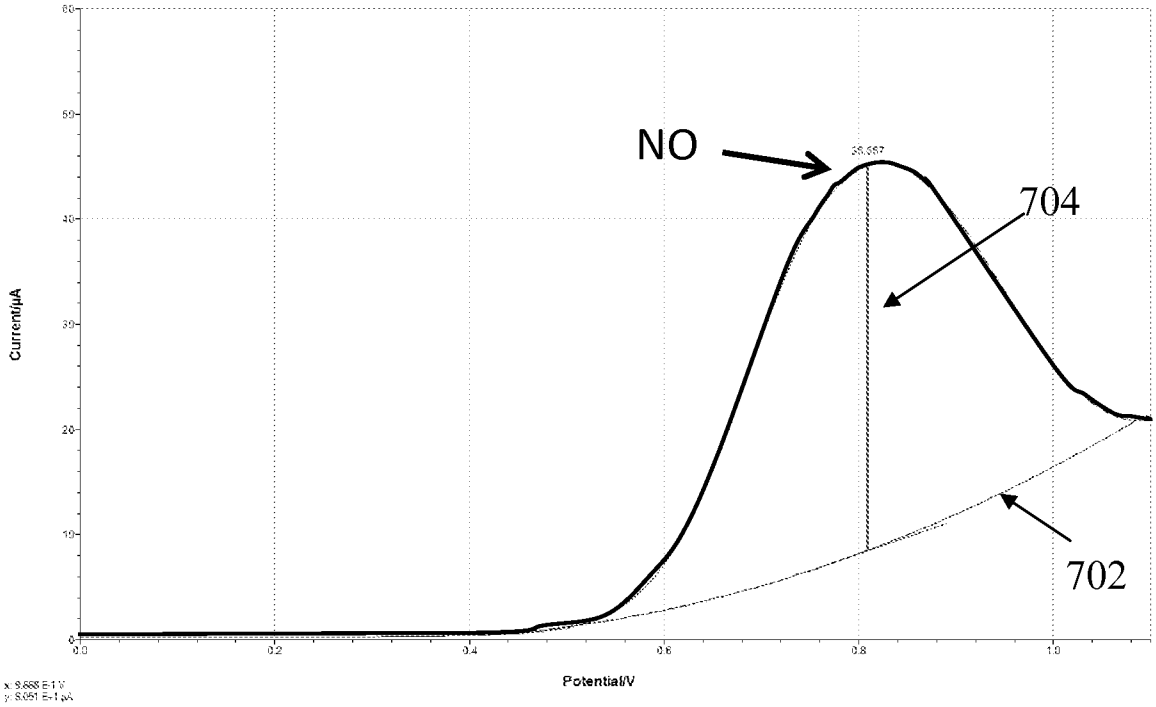

FIG. 7

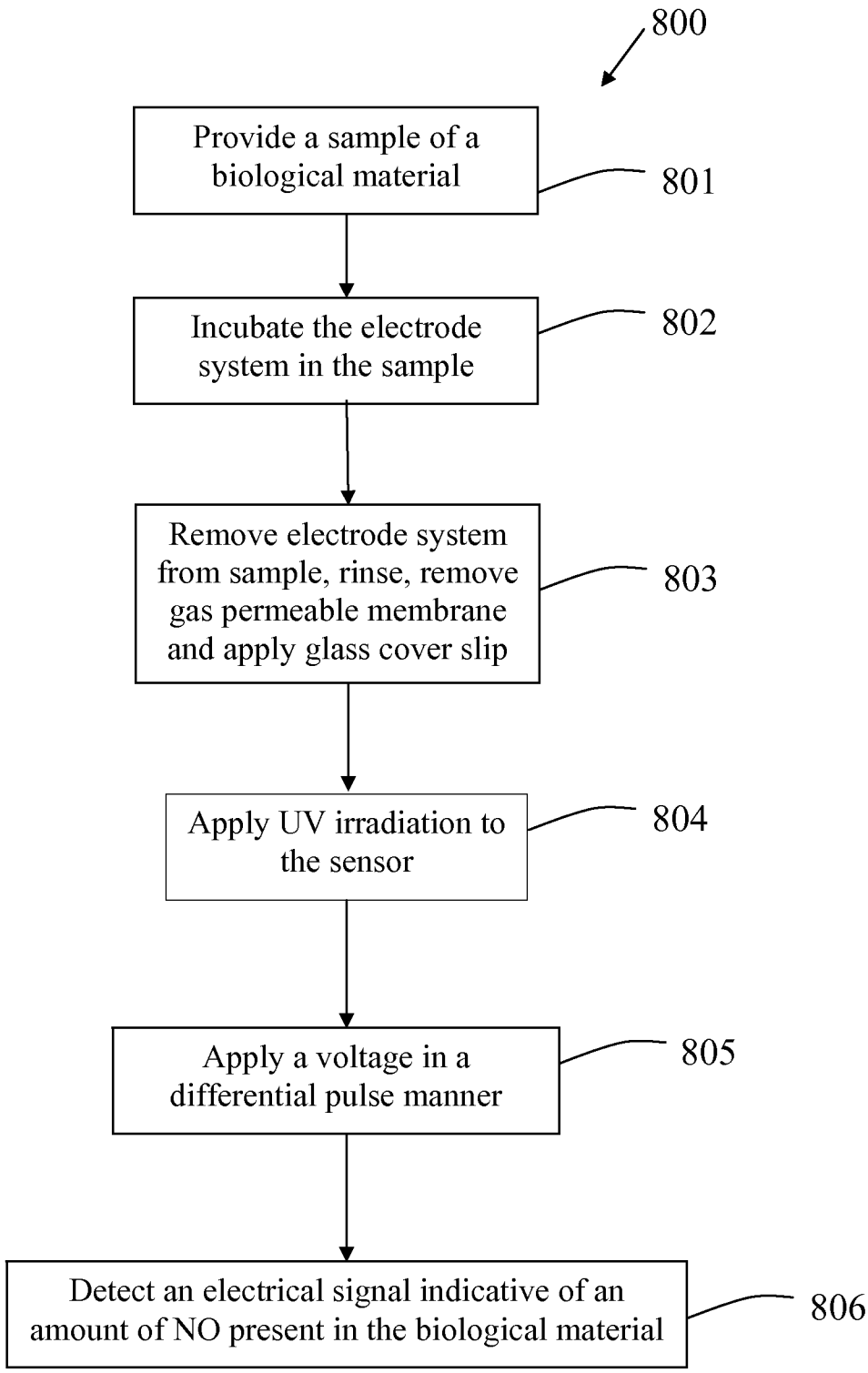
800
Provide a sample of a biological material
801
Incubate the electrode system in the sample
802
Remove electrode system from sample, rinse, remove gas permeable membrane and apply glass cover slip
803
Apply UV irradiation to the sensor
804
Apply a voltage in a differential pulse manner
805
Detect an electrical signal indicative of an amount of NO present in the biological material
806

FIG. 8

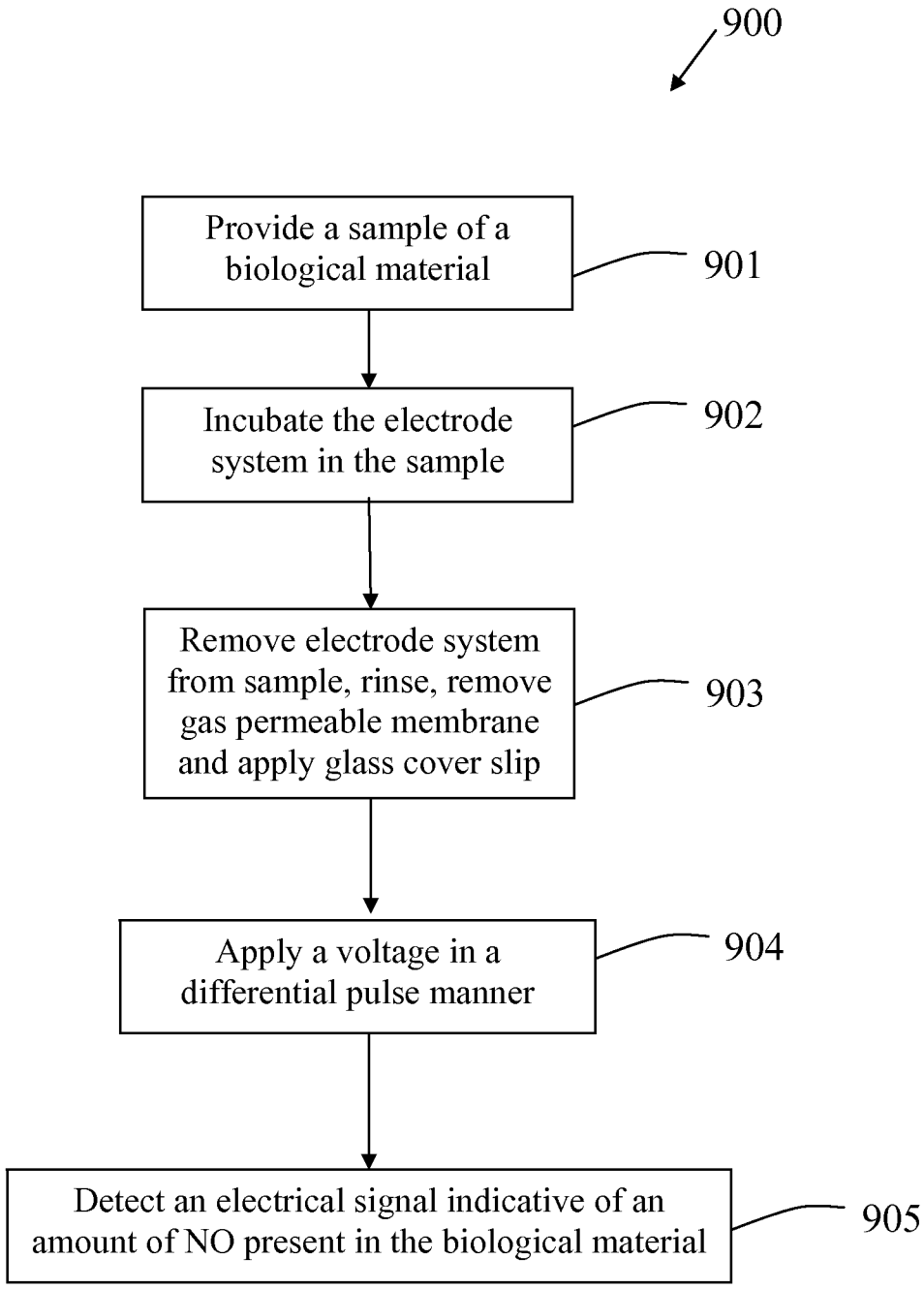
900
Provide a sample of a biological material
901
Incubate the electrode system in the sample
902
Remove electrode system from sample, rinse, remove gas permeable membrane and apply glass cover slip
903
Apply a voltage in a differential pulse manner
904
Detect an electrical signal indicative of an amount of NO present in the biological material
905

FIG. 9

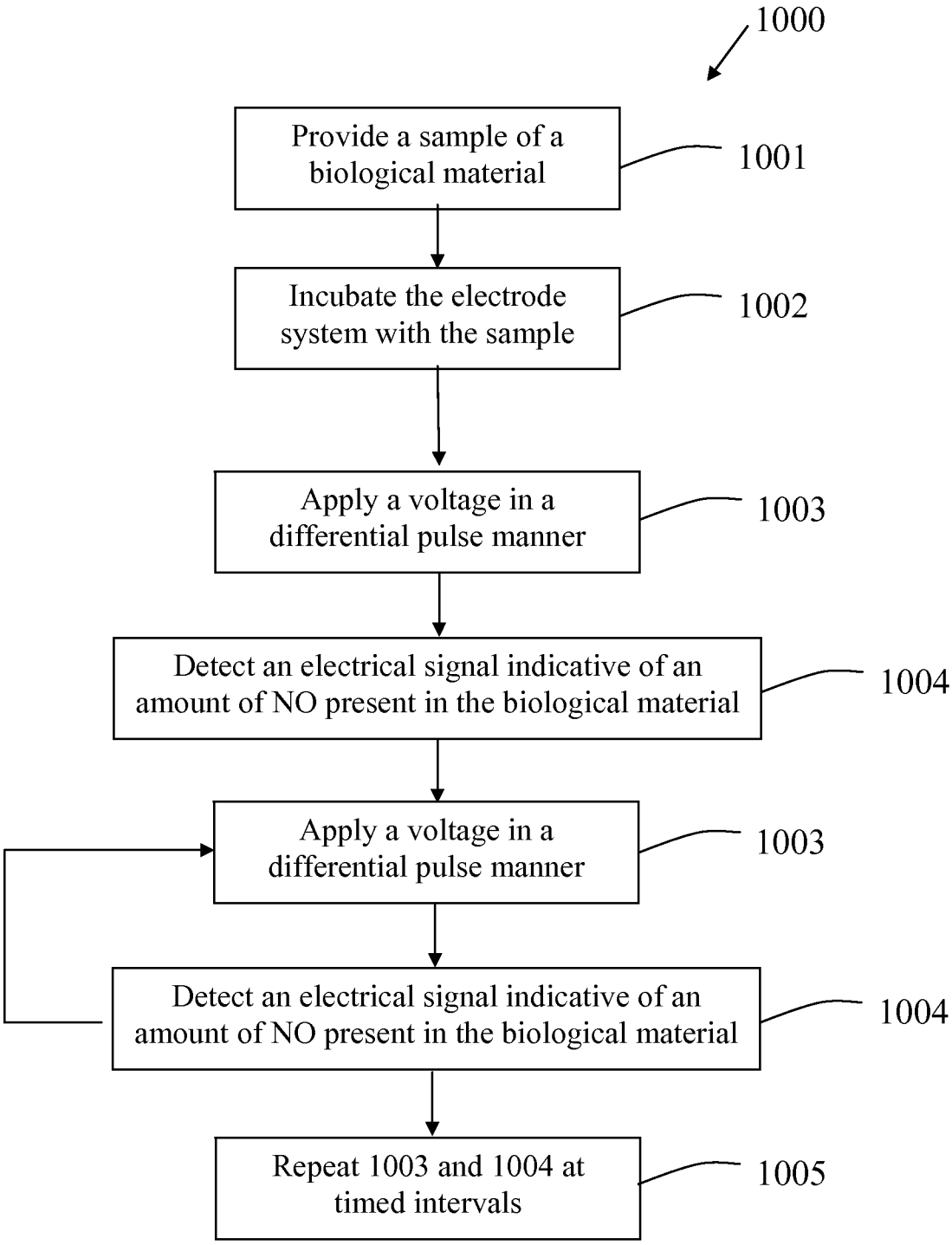
1000
Provide a sample of a biological material
1001
Incubate the electrode system with the sample
1002
Apply a voltage in a differential pulse manner
1003
Detect an electrical signal indicative of an amount of NO present in the biological material
1004
Apply a voltage in a differential pulse manner
1003
Detect an electrical signal indicative of an amount of NO present in the biological material
1004
Repeat 1003 and 1004 at timed intervals
1005

FIG. 10

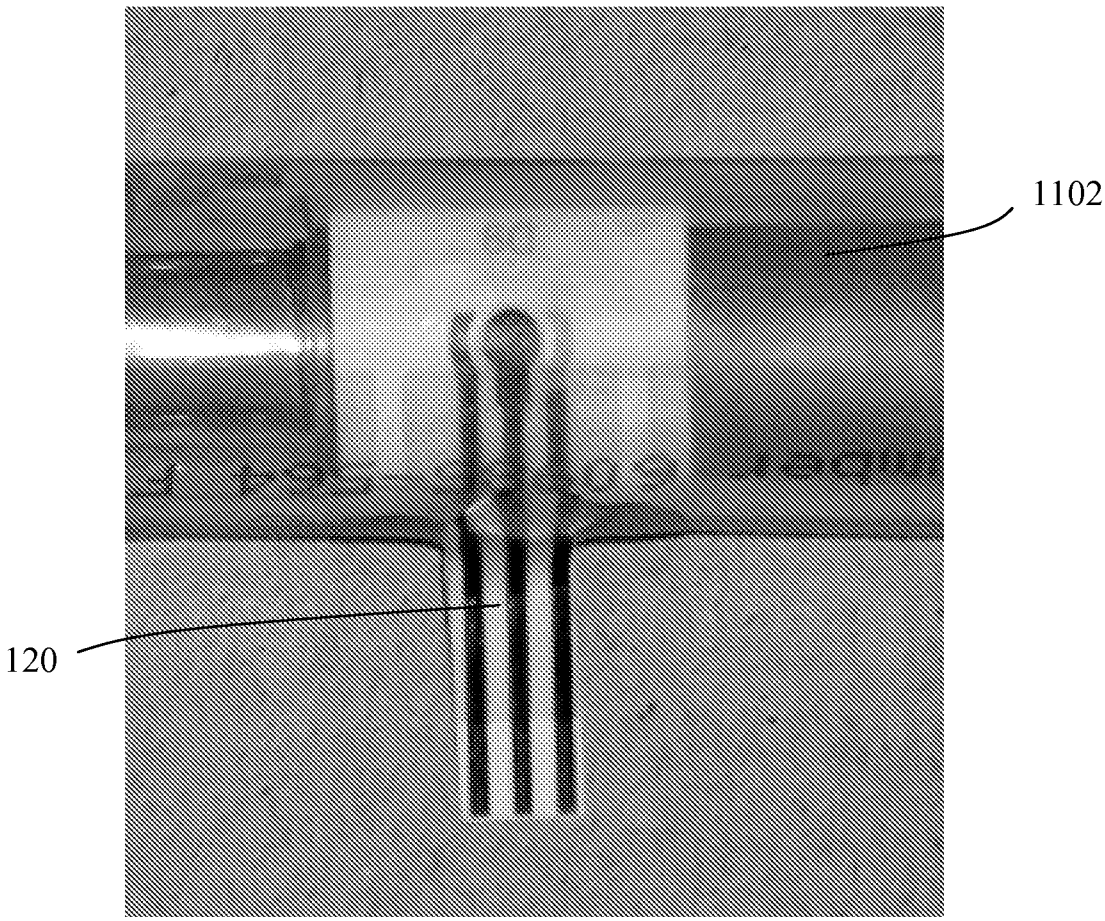

FIG. 11a

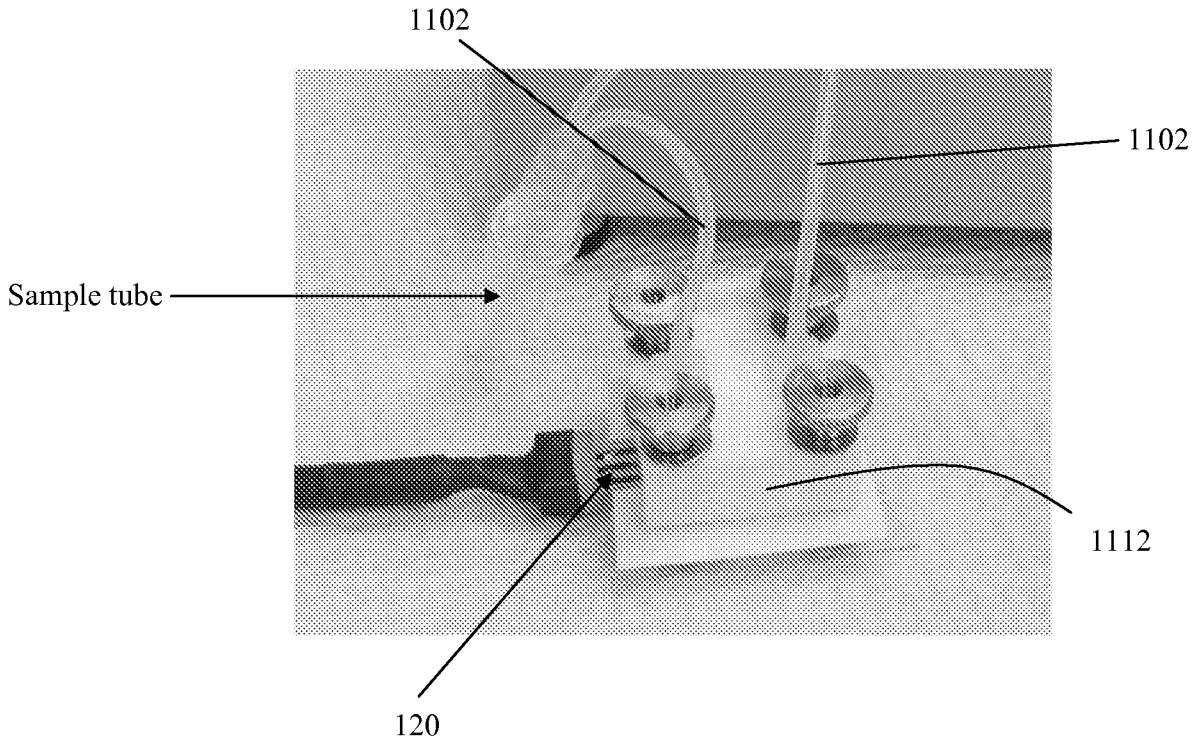

FIG. 11b

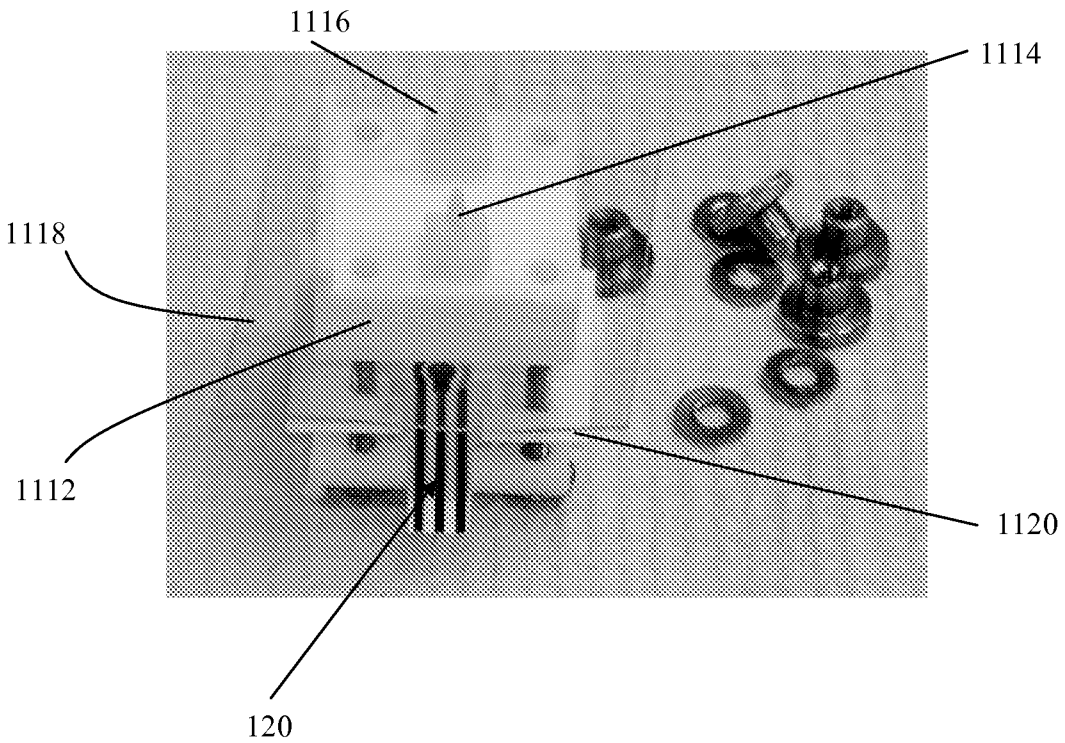

FIG. 11c

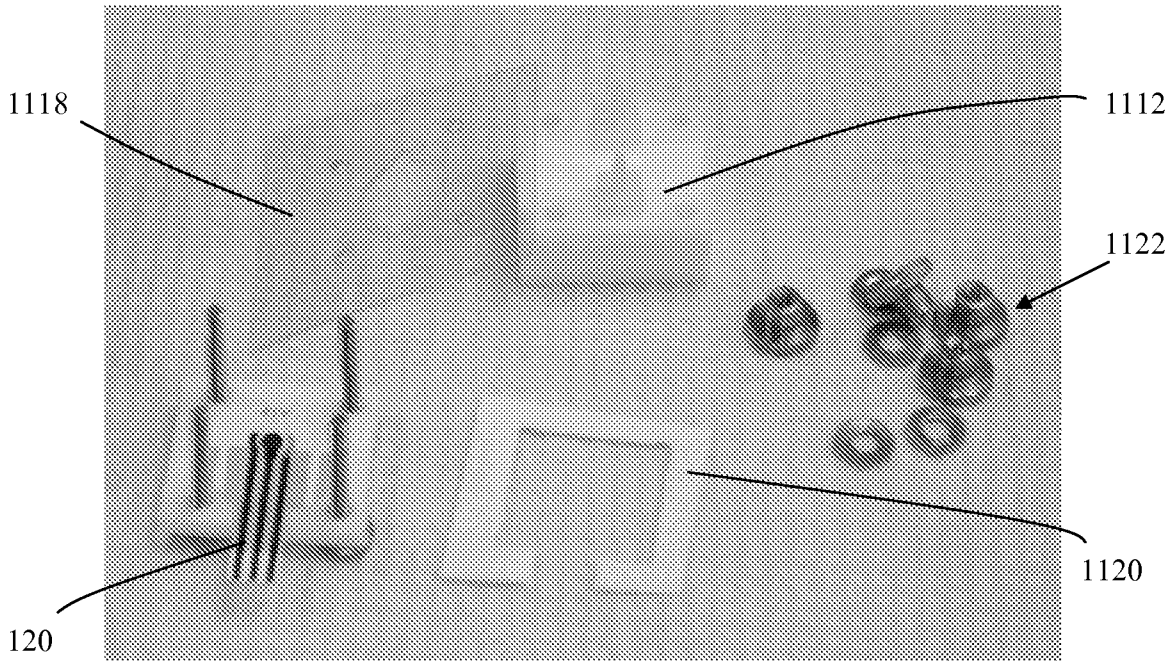

FIG. 11d

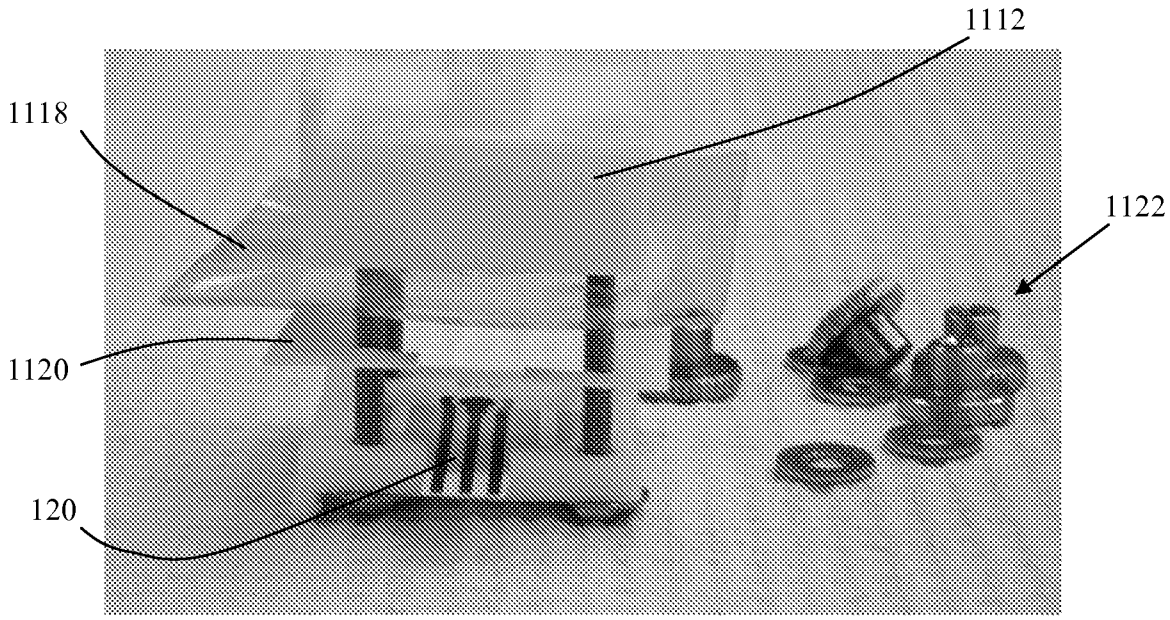

FIG. 11e

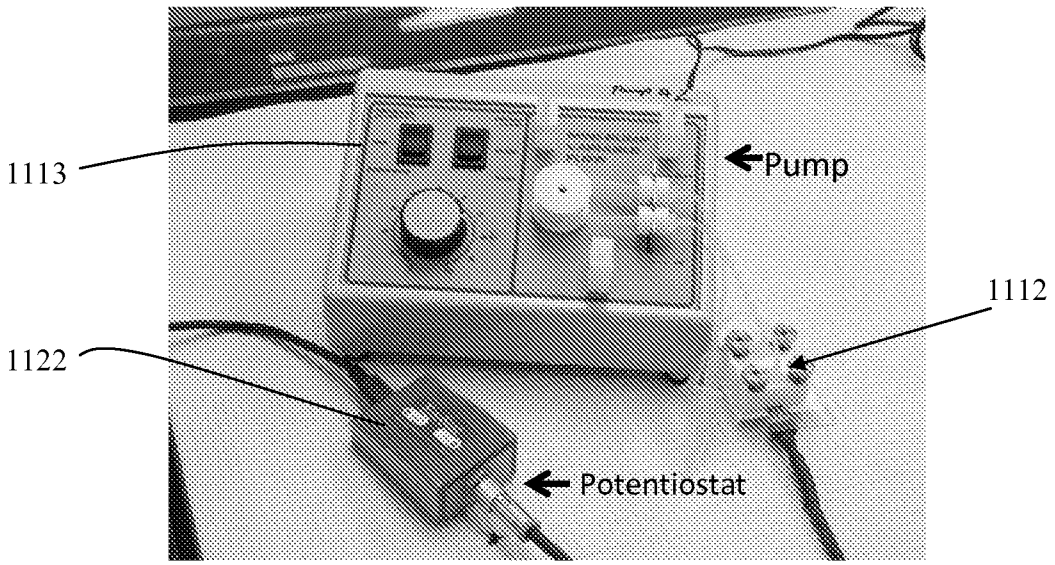

FIG. 11f

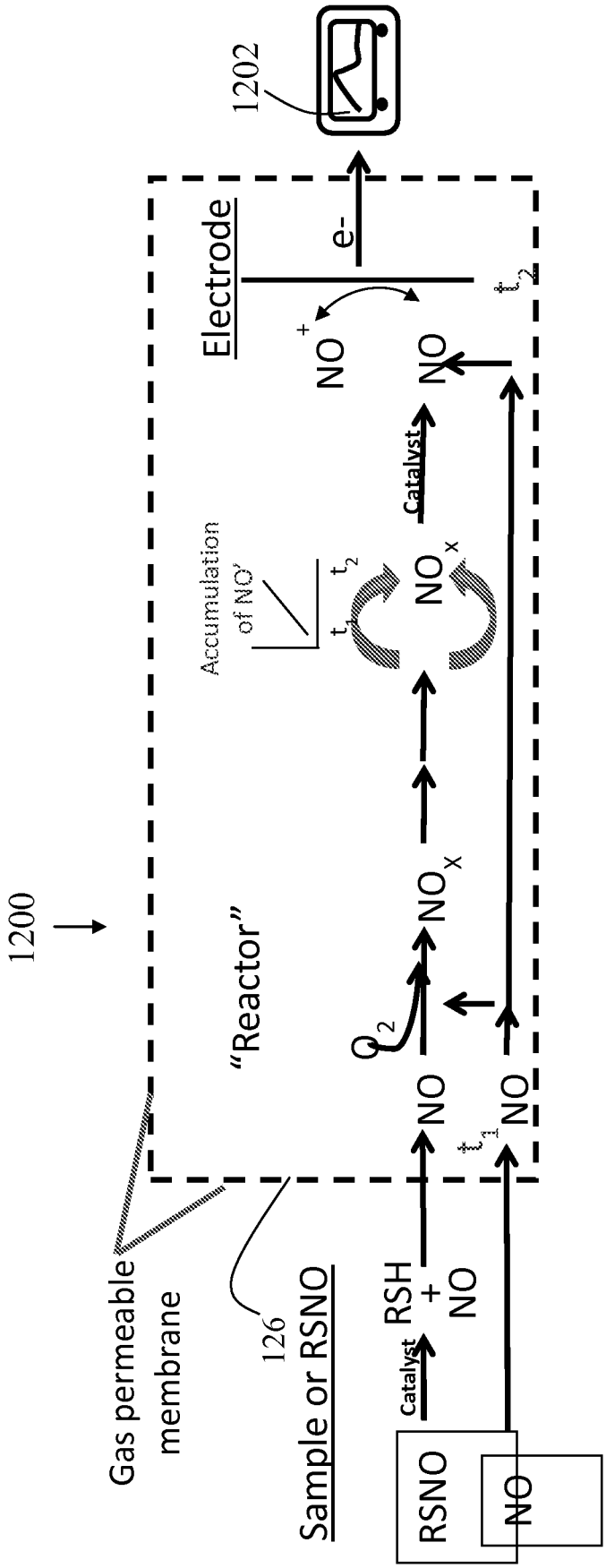
1200
1202
Electrode
e-
t_2
NO^+
NO
Catalyst
Accumulation of NO'
t_1
t_2
NO_x
"Reactor"
O_2
NO_x
t_1
NO
NO
Gas permeable membrane
126
Sample or RSNO
Catalyst
RSH + NO
RSNO
NO

FIG. 12

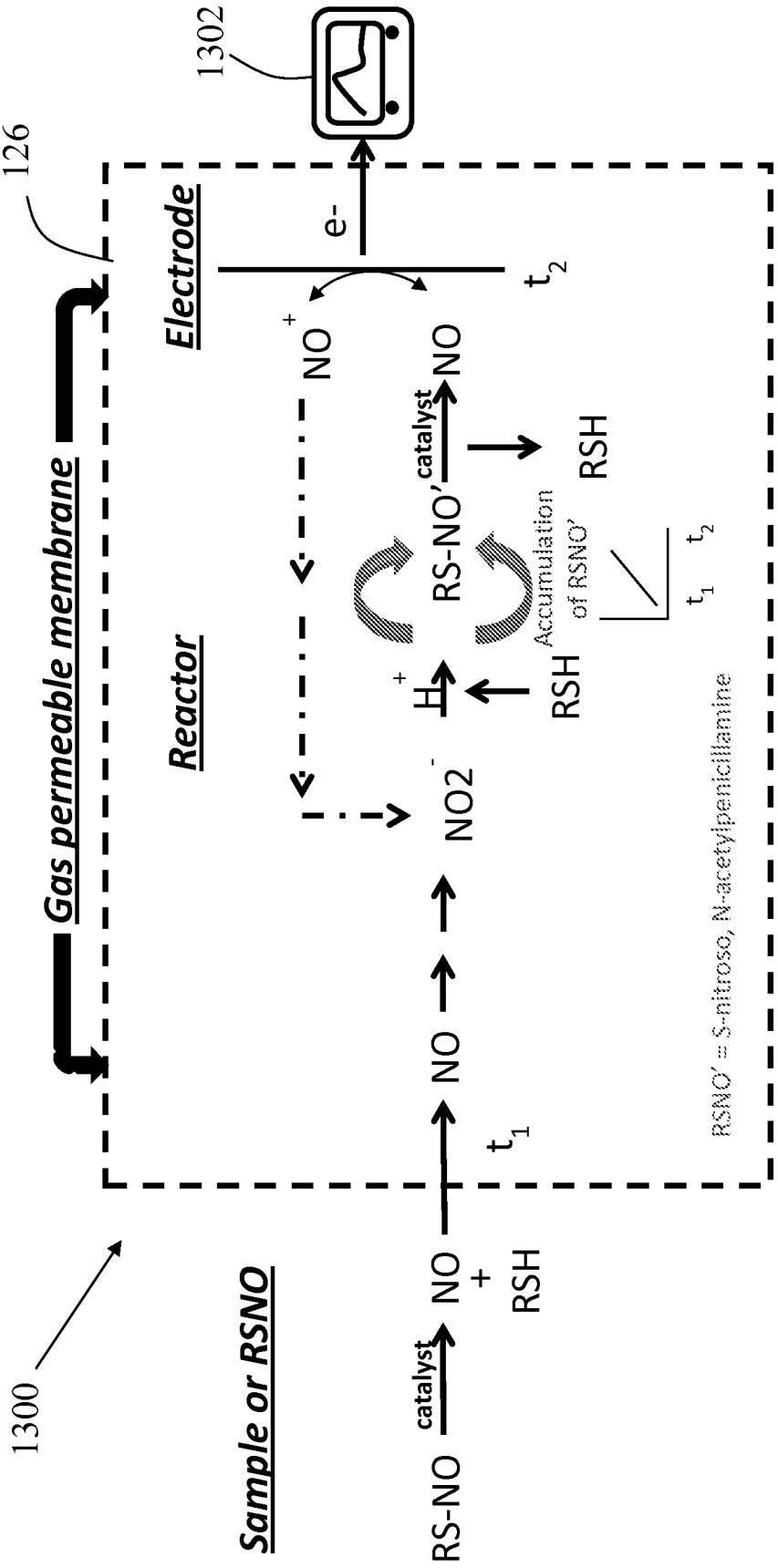
1300
126
1302
Sample or RSNO
Gas permeable membrane
Reactor
Electrode
RS-NO
catalyst
NO
+
RSH
NO
NO2-
H+
RS-NO'
catalyst
NO
RSH
Accumulation of RSNO'
RSH
t1
t2
t1
t2
e-
NO+
RSNO' = S-nitroso, N-acetylpenicillamine

FIG. 13

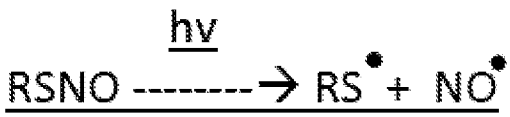

FIG. 14

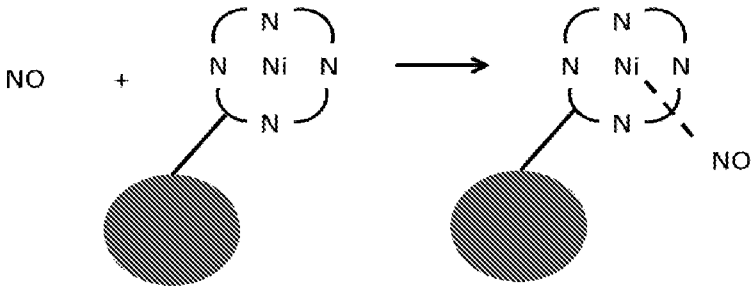

FIG. 15

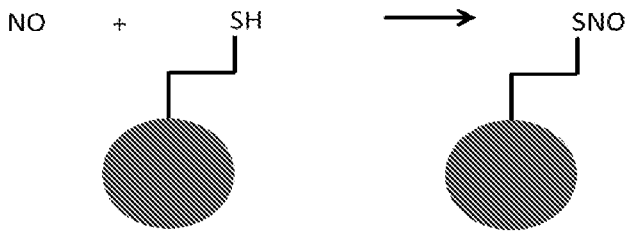

FIG. 16

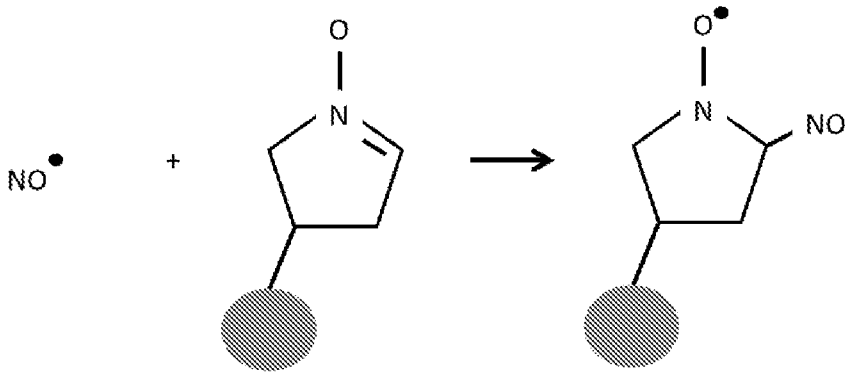

FIG. 17

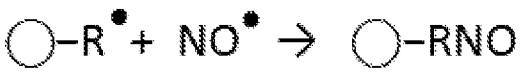

FIG. 18

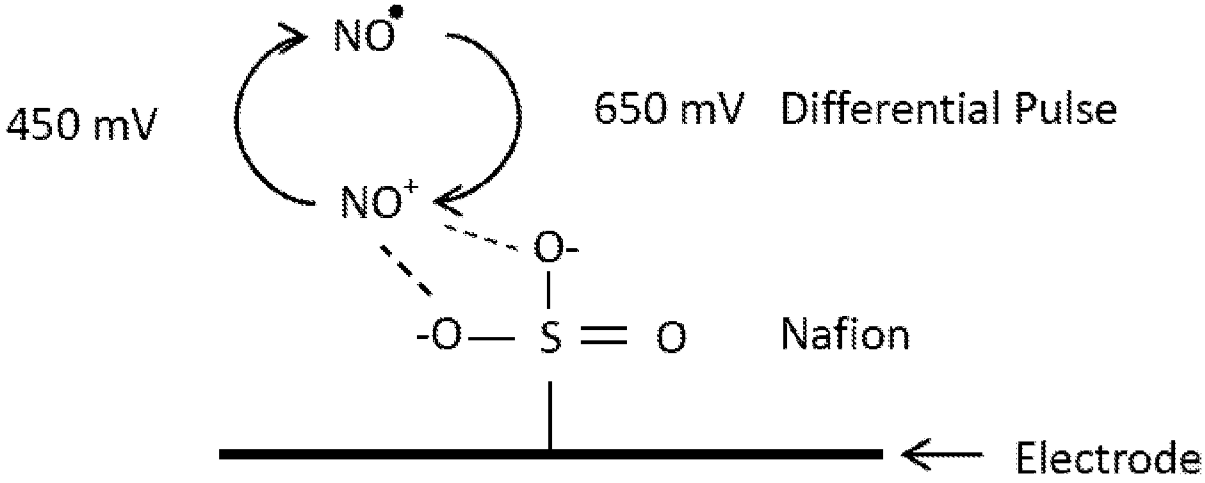
NO•
450 mV
650 mV Differential Pulse
NO+
O-
-O— S = O
Nafion
Electrode

FIG. 19

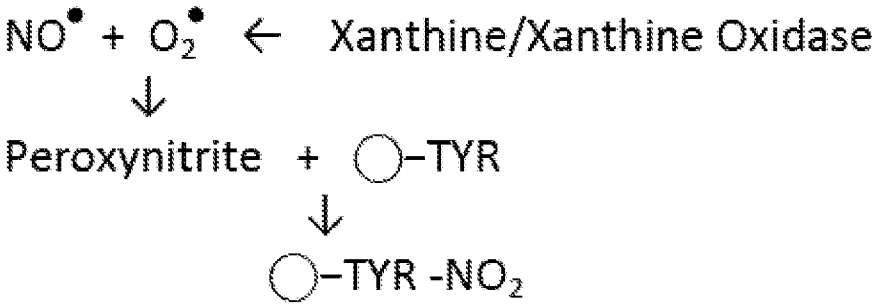
NO• + O2•
Xanthine/Xanthine Oxidase
Peroxynitrite + ◯–TYR
◯–TYR -NO2

FIG. 20

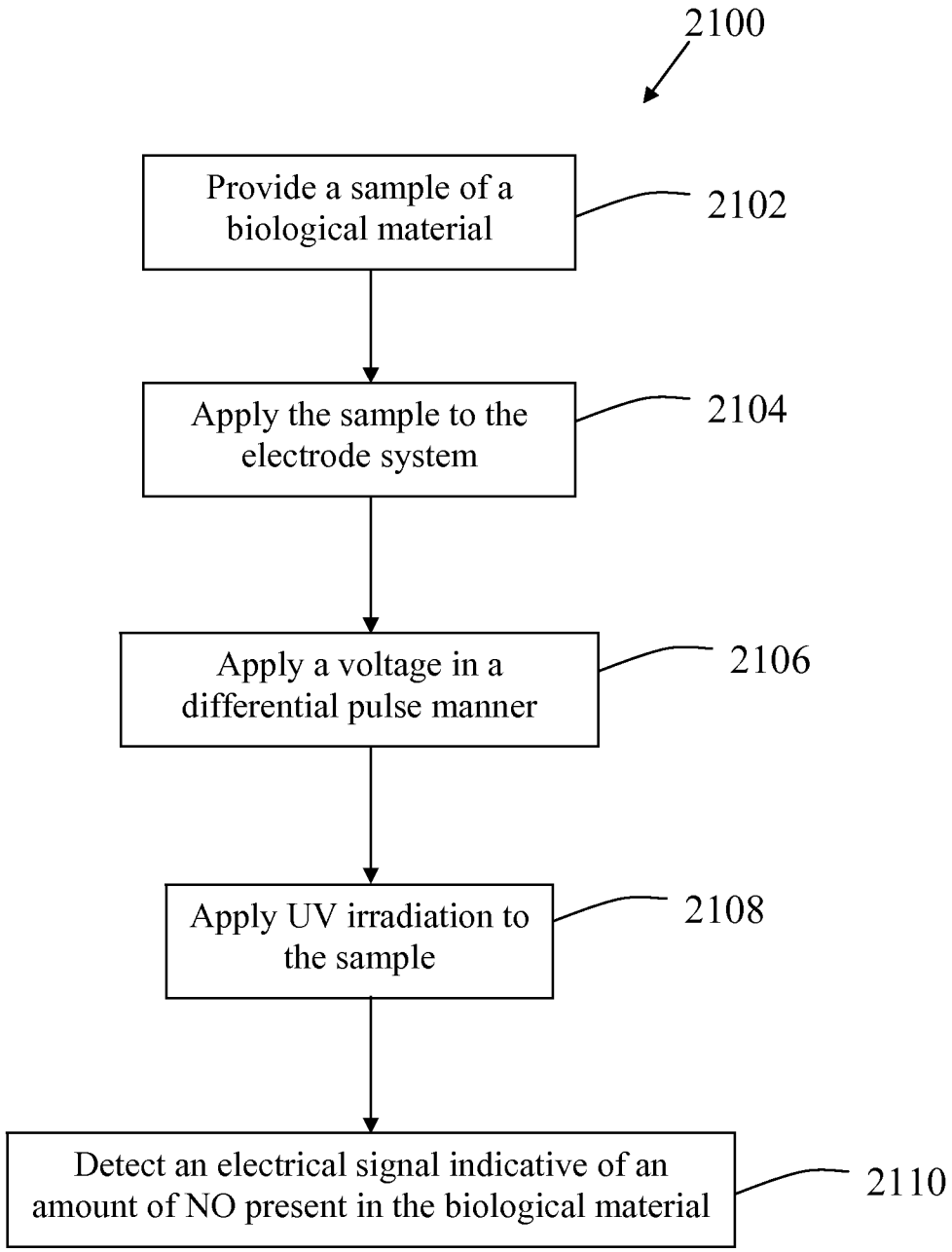
2100
Provide a sample of a biological material
2102
Apply the sample to the electrode system
2104
Apply a voltage in a differential pulse manner
2106
Apply UV irradiation to the sample
2108
Detect an electrical signal indicative of an amount of NO present in the biological material
2110

FIG. 21

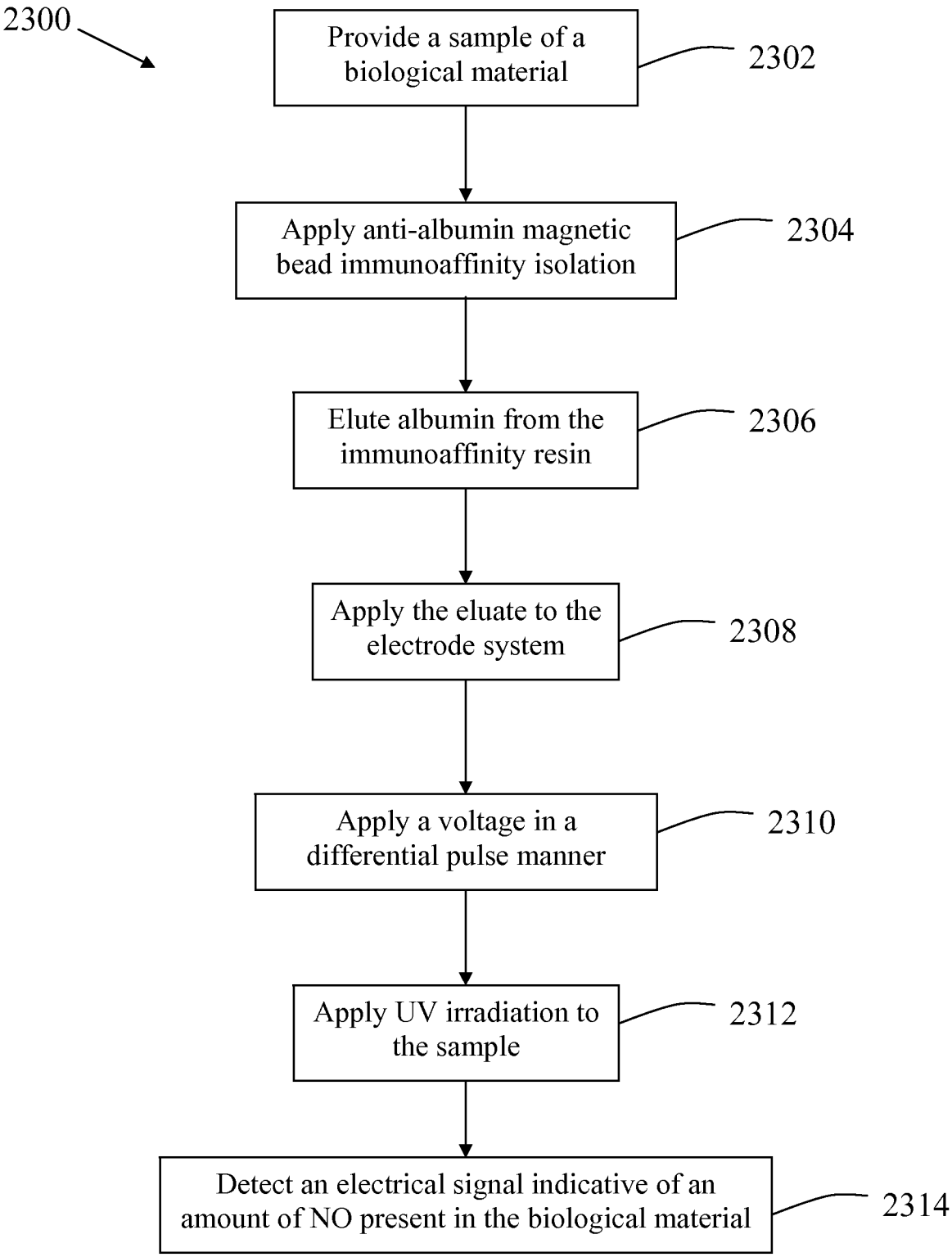
2300
Provide a sample of a biological material
2302
Apply anti-albumin magnetic bead immunoaffinity isolation
2304
Elute albumin from the immunoaffinity resin
2306
Apply the eluate to the electrode system
2308
Apply a voltage in a differential pulse manner
2310
Apply UV irradiation to the sample
2312
Detect an electrical signal indicative of an amount of NO present in the biological material
2314

FIG. 22

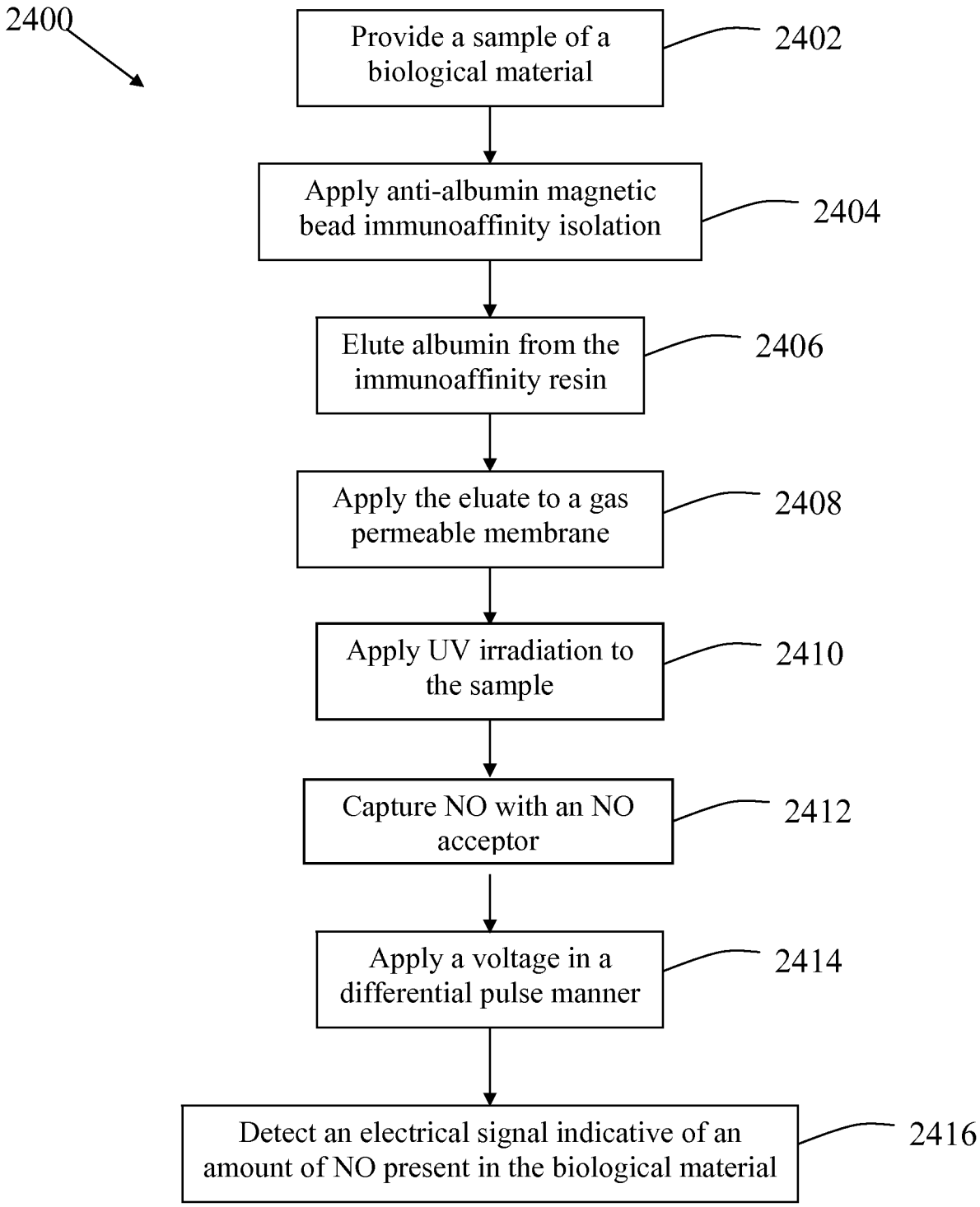
2400
Provide a sample of a biological material
2402
Apply anti-albumin magnetic bead immunoaffinity isolation
2404
Elute albumin from the immunoaffinity resin
2406
Apply the eluate to a gas permeable membrane
2408
Apply UV irradiation to the sample
2410
Capture NO with an NO acceptor
2412
Apply a voltage in a differential pulse manner
2414
Detect an electrical signal indicative of an amount of NO present in the biological material
2416

FIG. 23

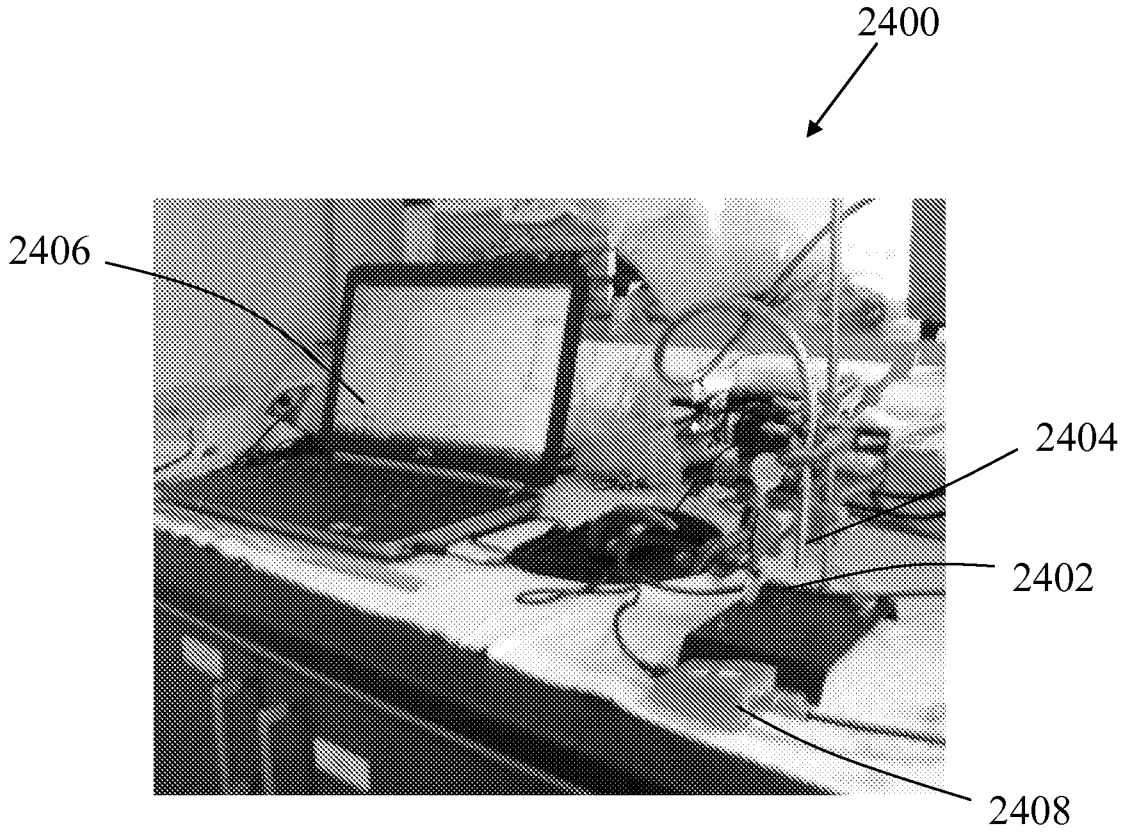

FIG. 24

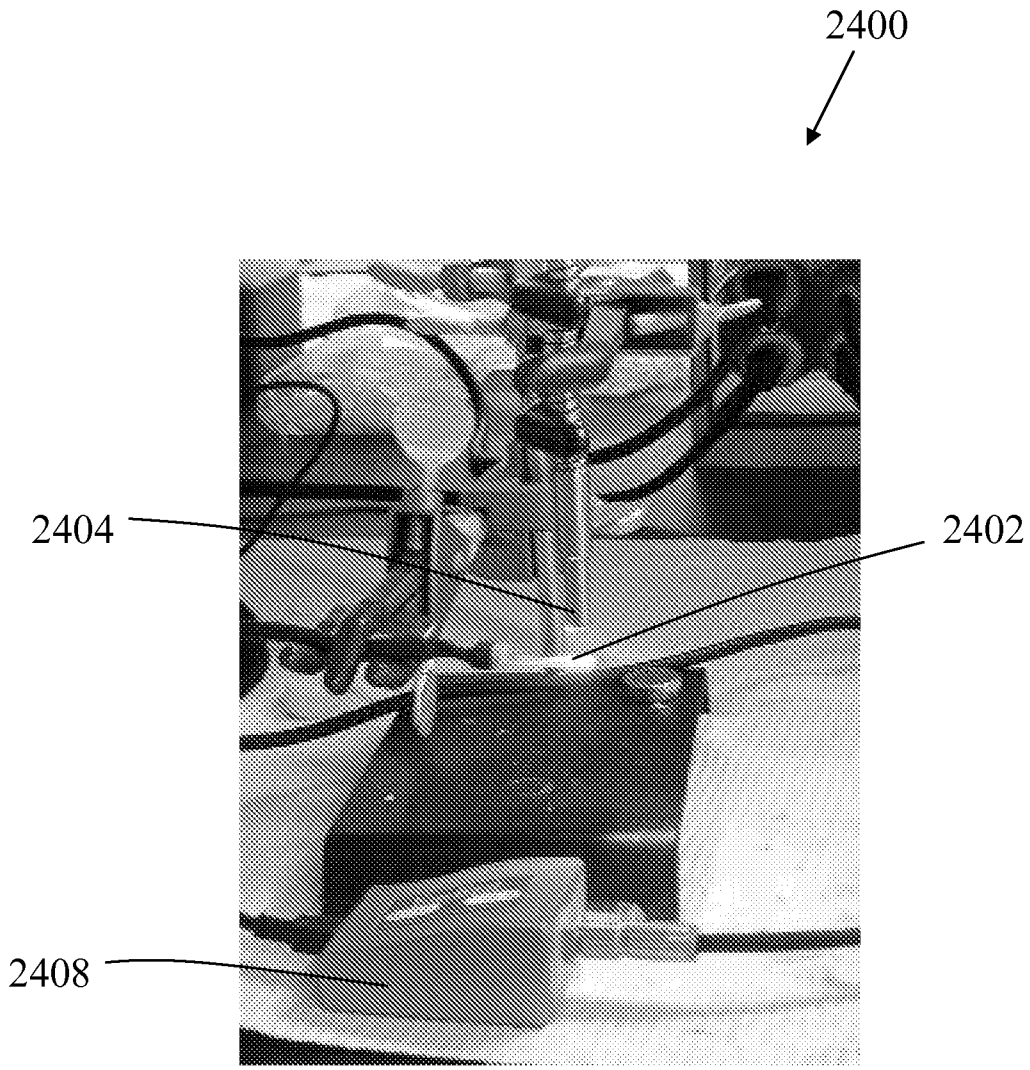

FIG. 25

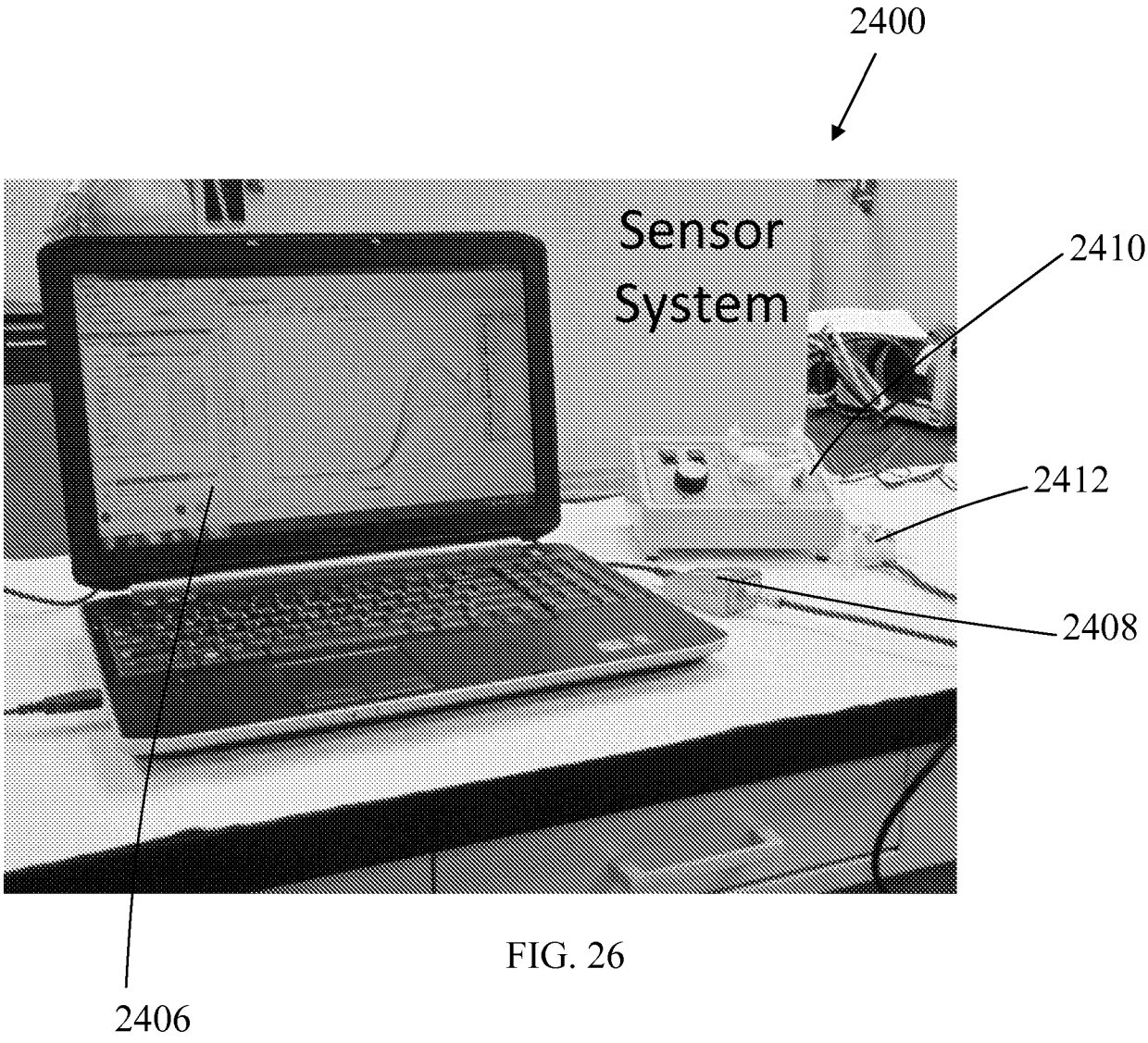

FIG. 26

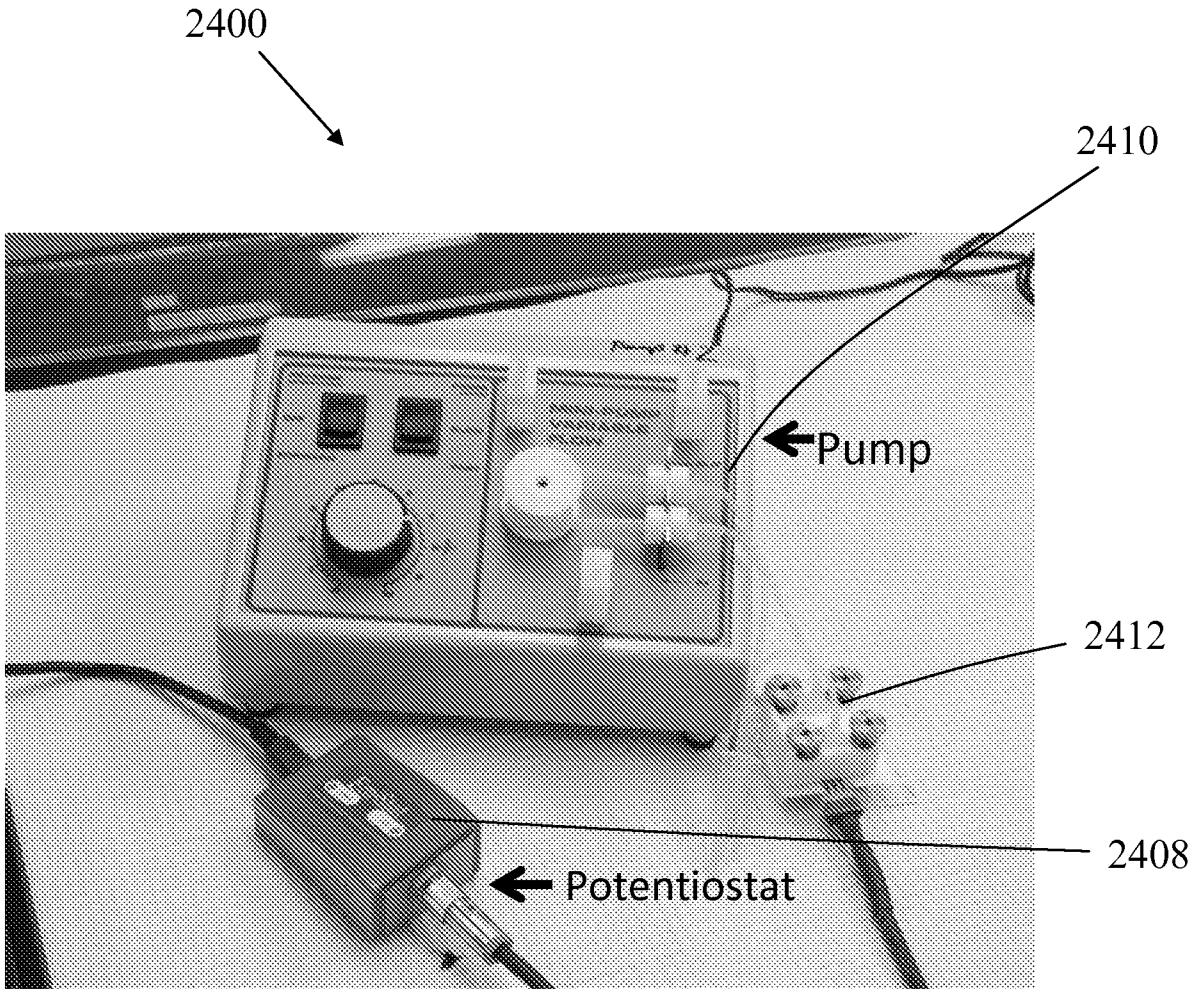

FIG. 27

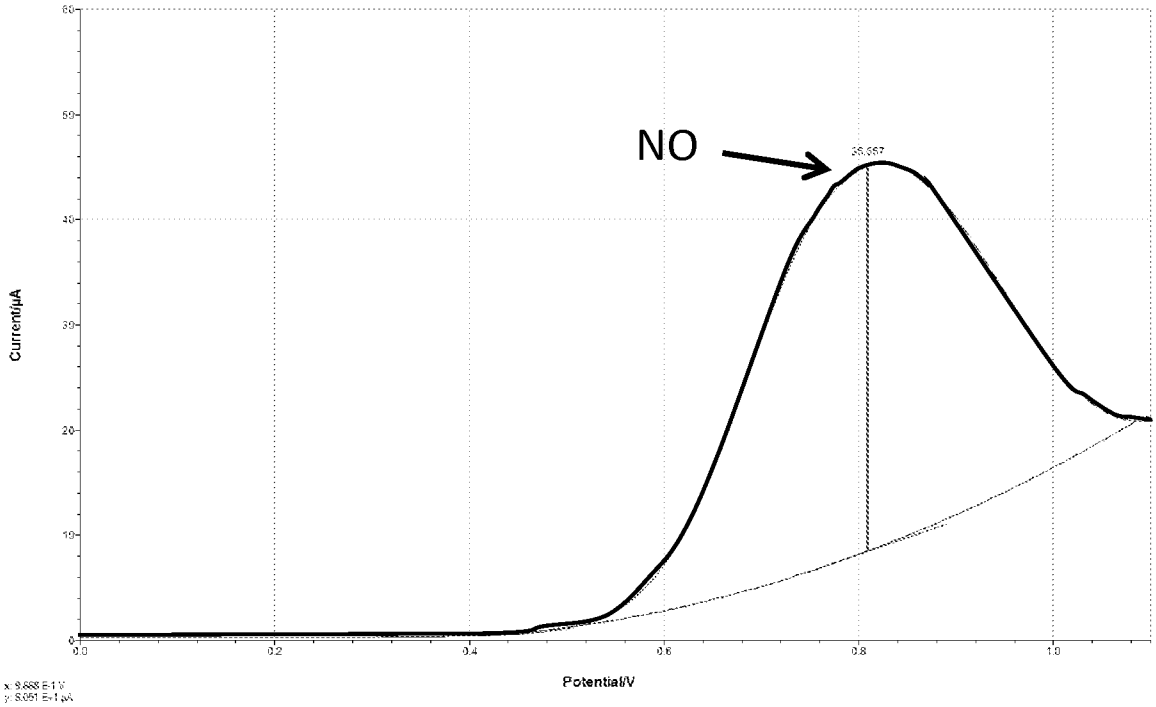
NO
Current/μA
Potential/V

FIG. 28

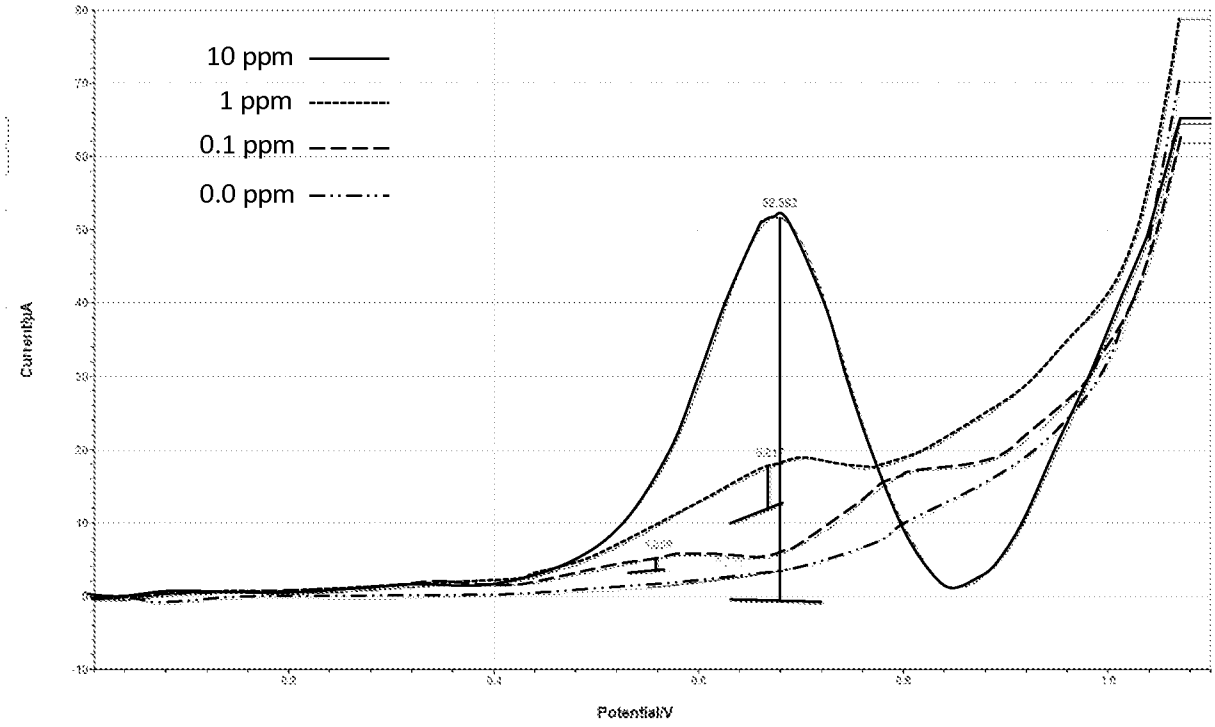
10 ppm
1 ppm
0.1 ppm
0.0 ppm
Current/μA
Potential/V

FIG. 29

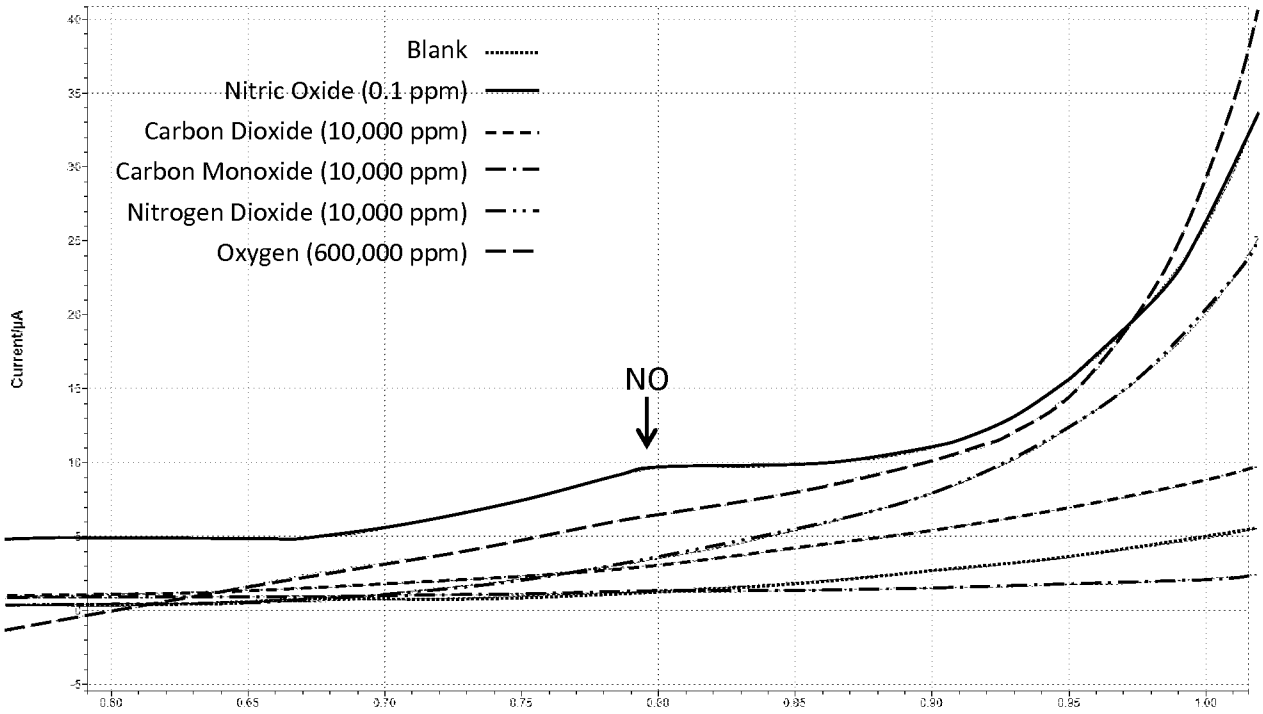
Blank
Nitric Oxide (0.1 ppm)
Carbon Dioxide (10,000 ppm)
Carbon Monoxide (10,000 ppm)
Nitrogen Dioxide (10,000 ppm)
Oxygen (600,000 ppm)
NO
Current/µA

FIG. 30

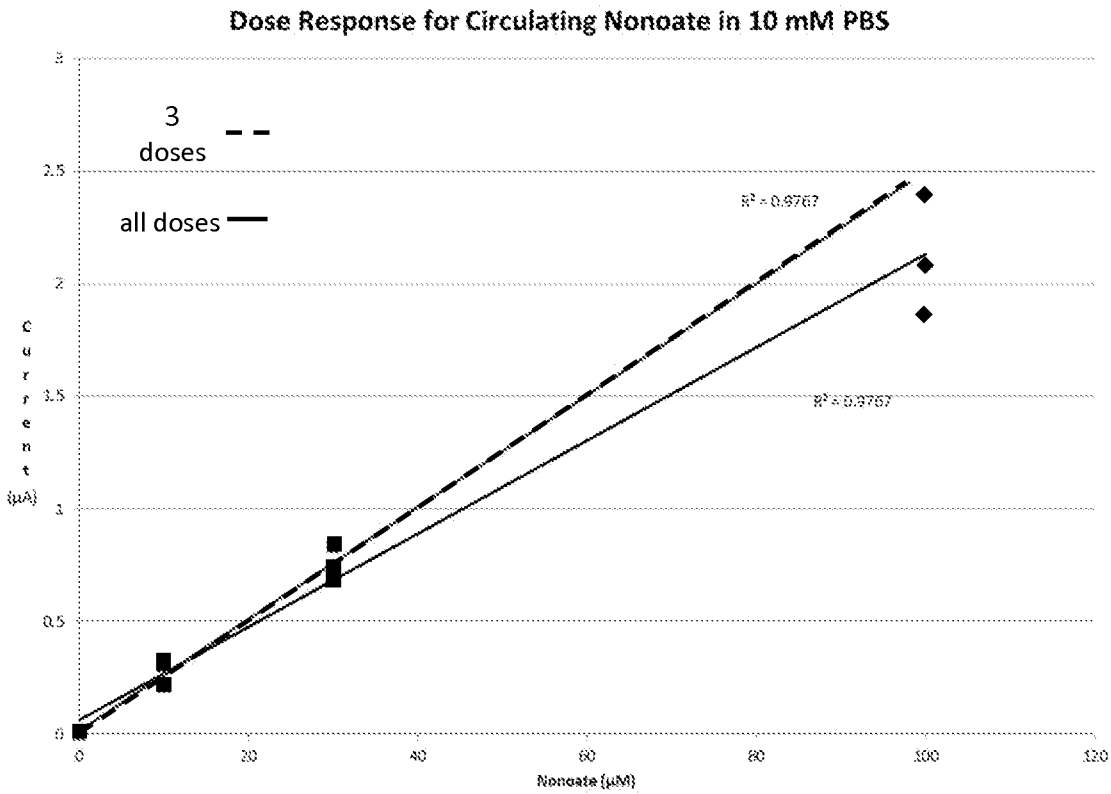
Dose Response for Circulating Nonoate in 10 mM PBS
3 doses
all doses
R^2 = 0.9767
R^2 = 0.9767
Current (μA)
Nonoate (μM)
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100
120

FIG. 31

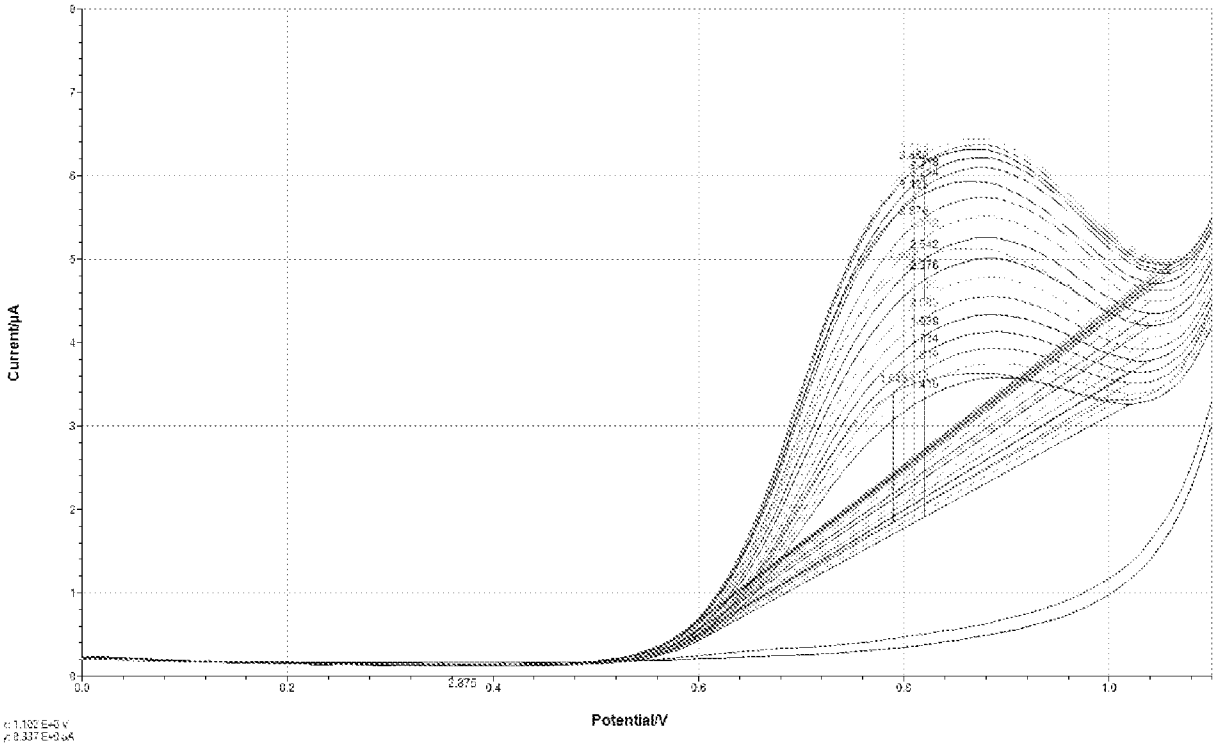

FIG. 32

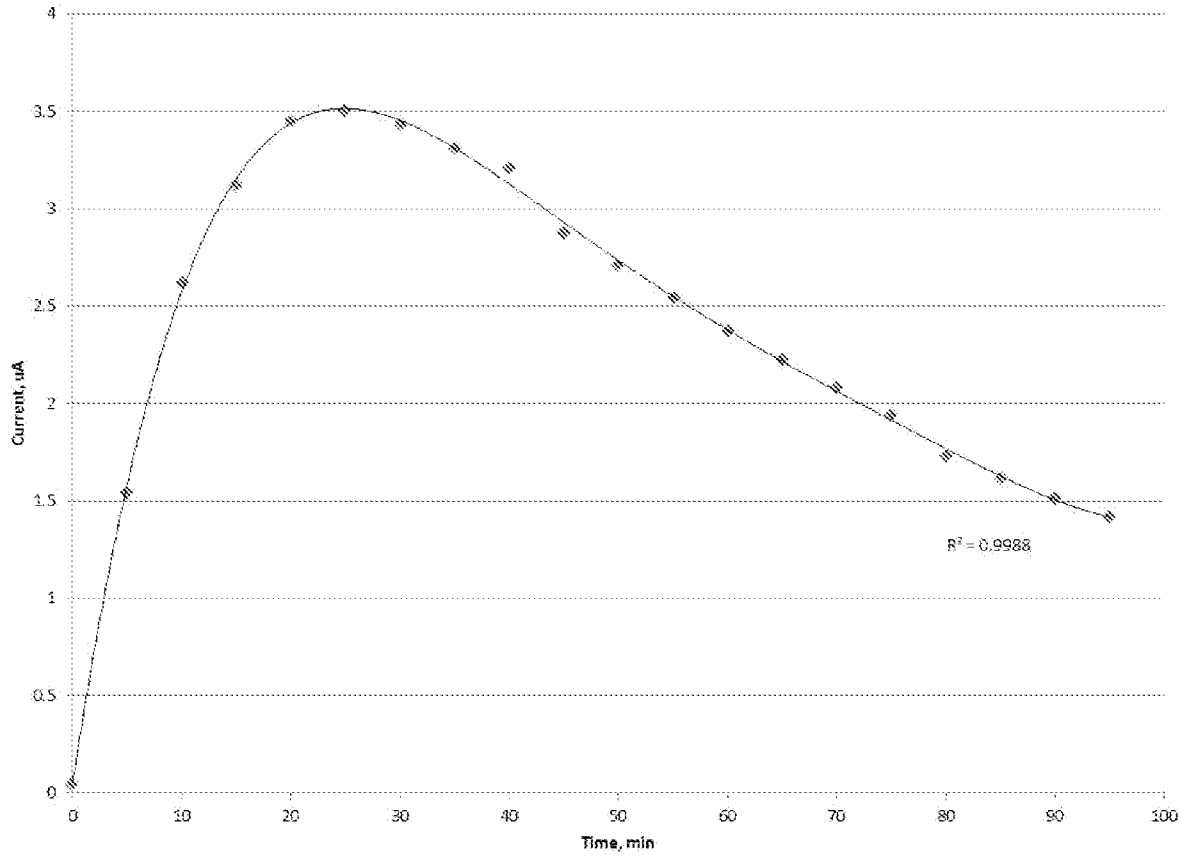
Current, uA
Time, min
$R^2 = 0.9988$
0
0.5
1
1.5
2
2.5
3
3.5
4
0
10
20
30
40
50
60
70
80
90
100

FIG. 33

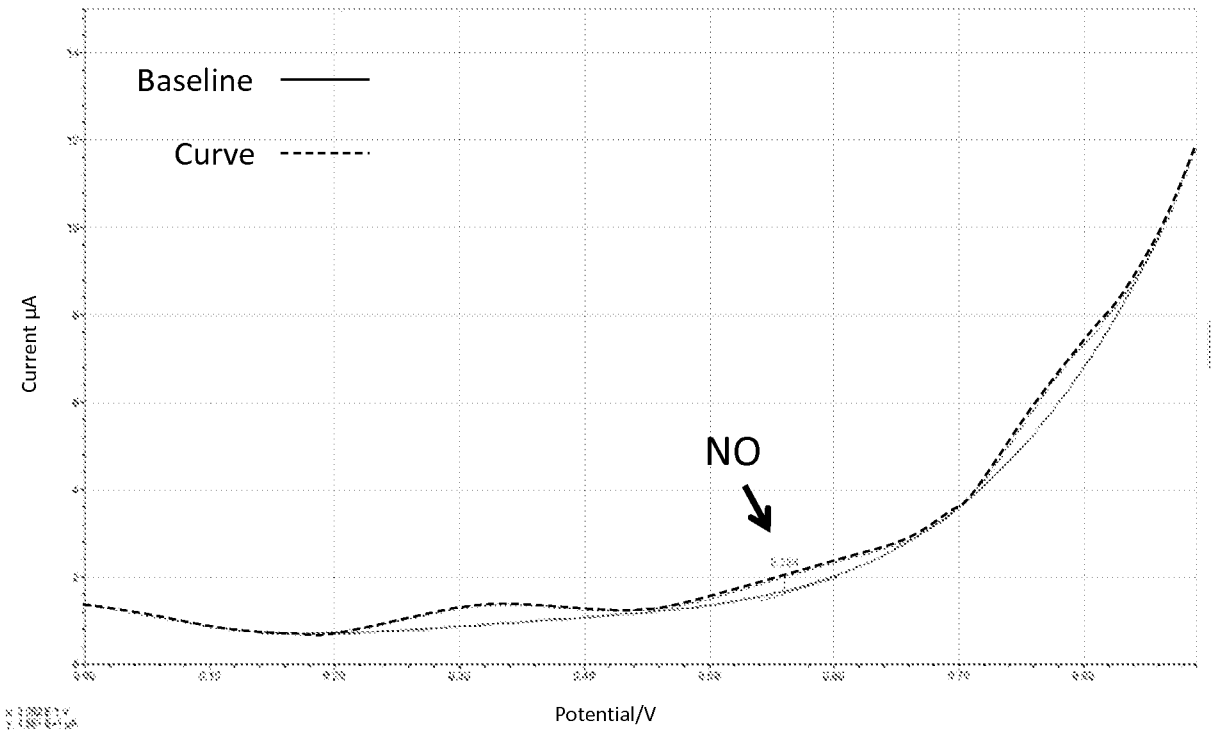

FIG. 34

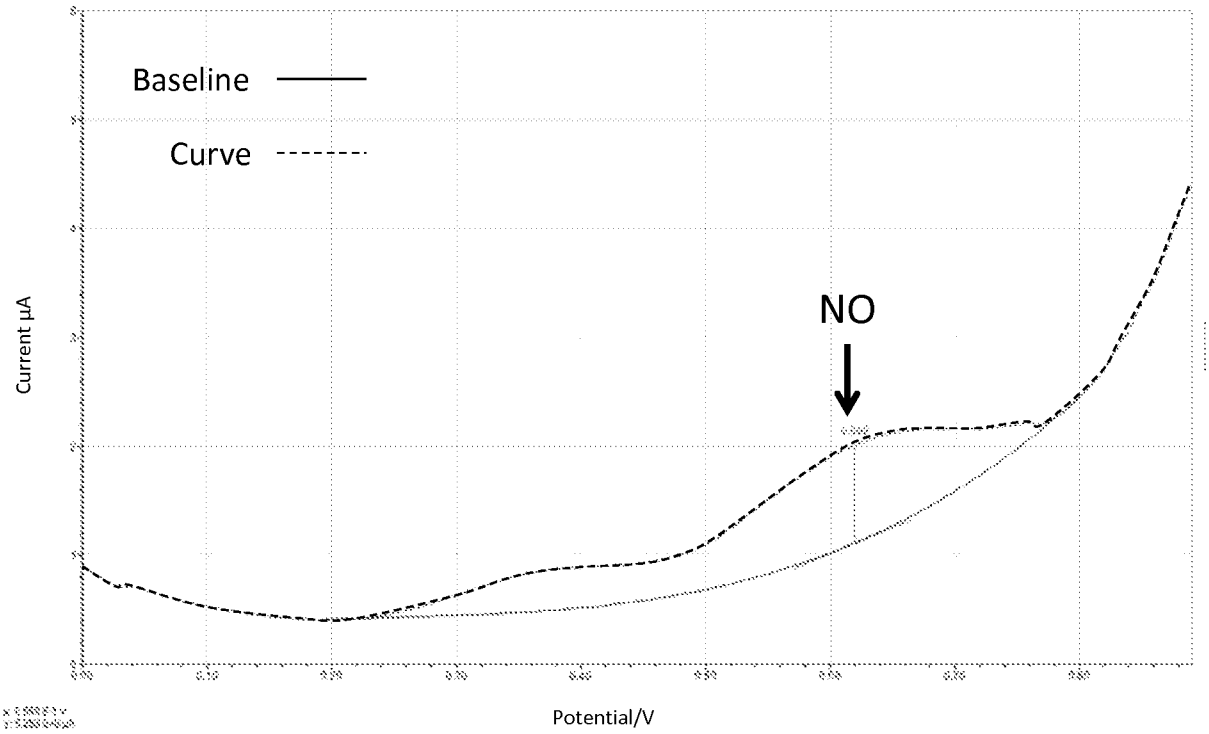

FIG. 35

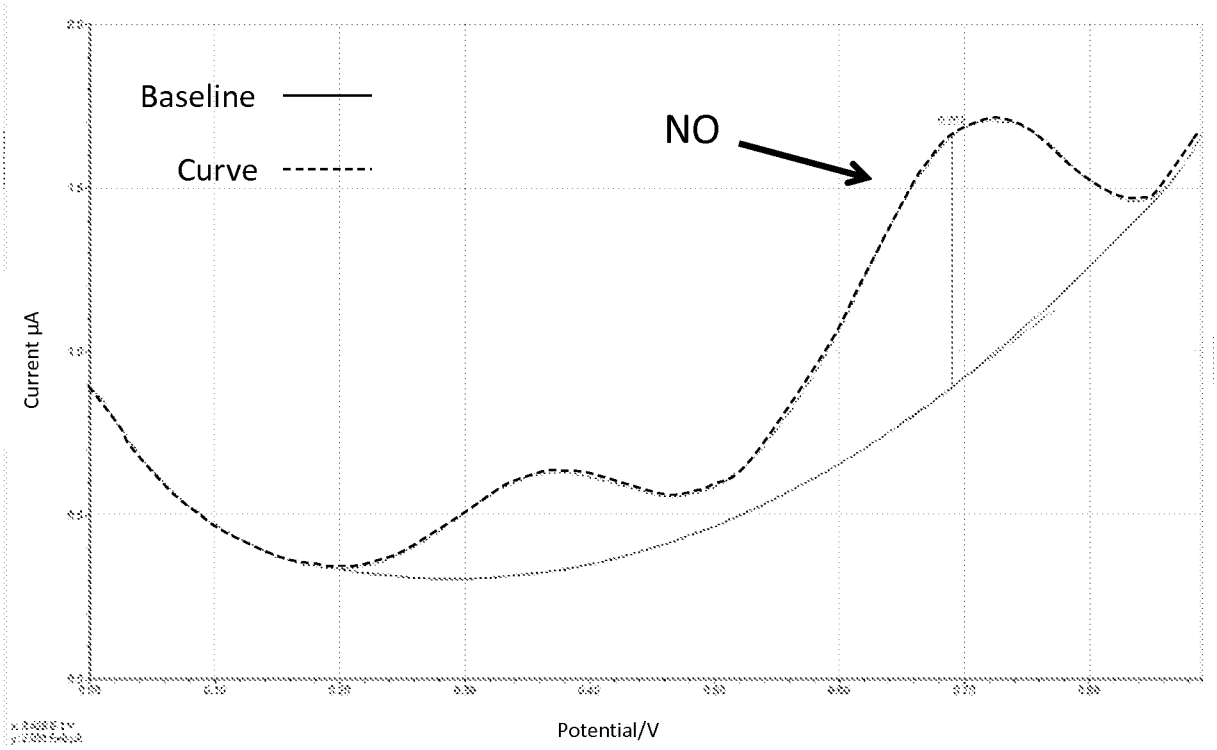

FIG. 36

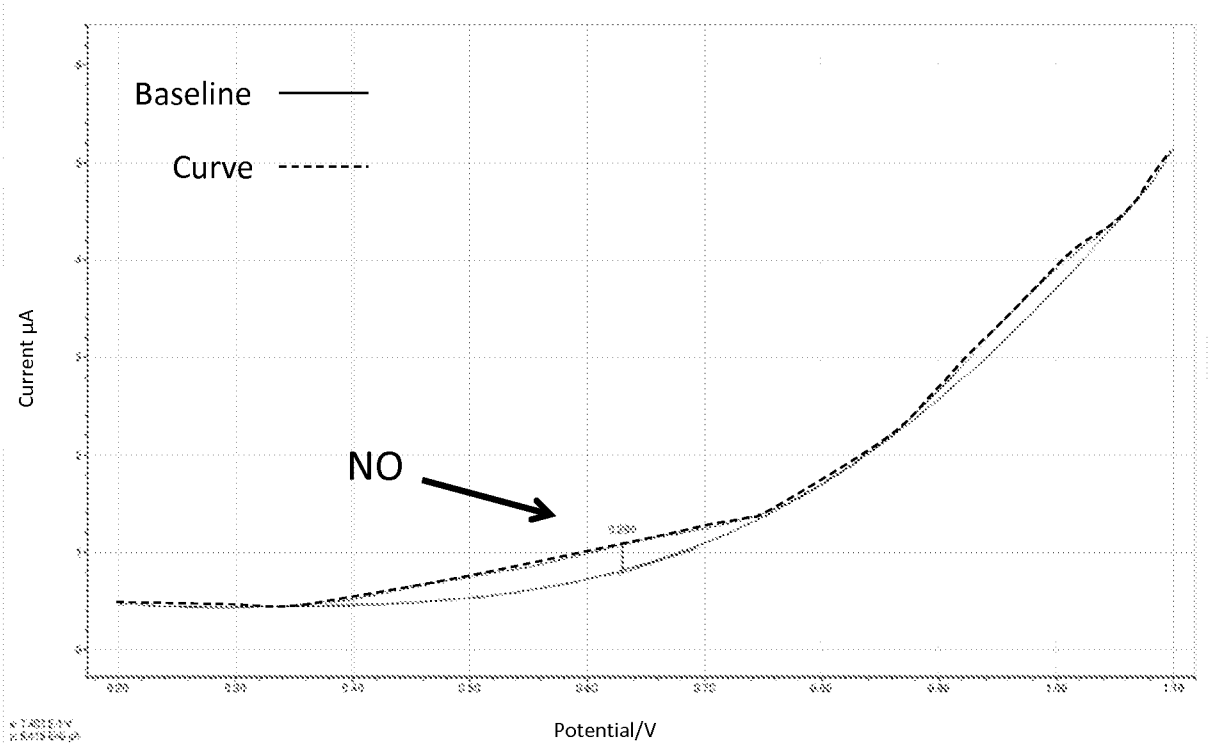

FIG. 37

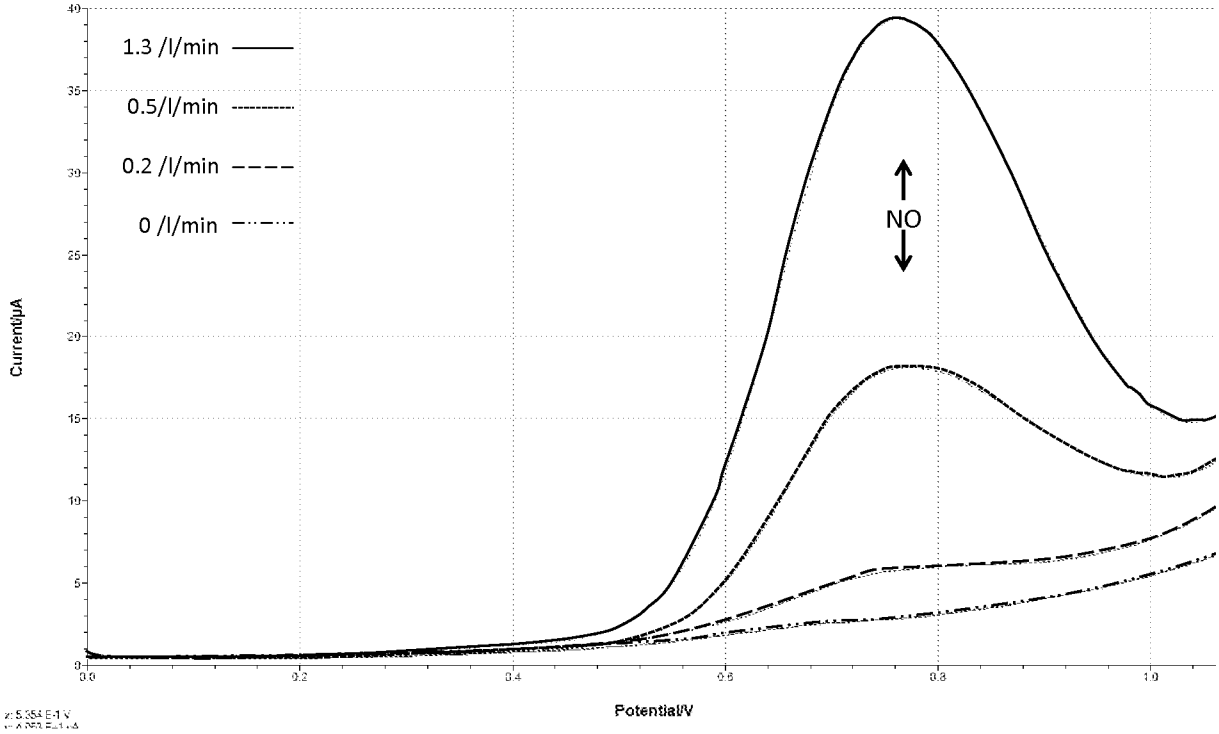
1.3 /l/min
0.5/l/min
0.2 /l/min
0 /l/min
NO
Current/μA
Potential/V
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
25
30
35
40

FIG. 38

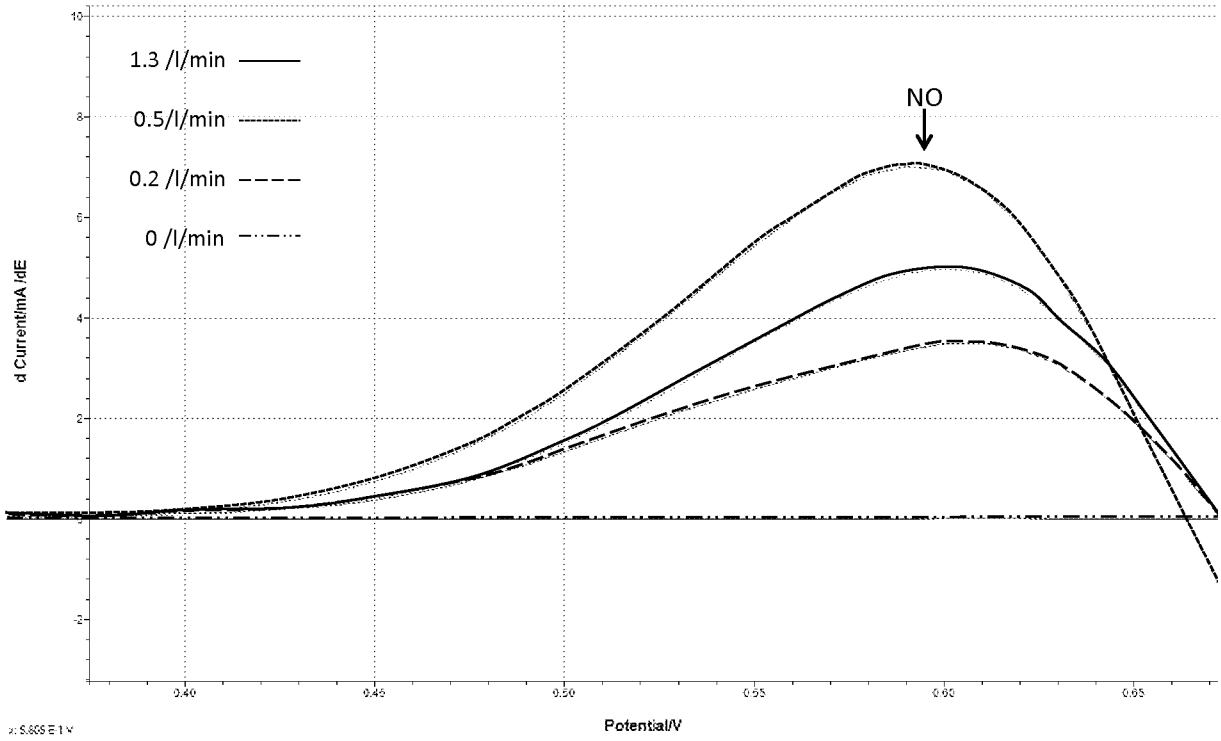
1.3 /l/min
0.5/l/min
0.2 /l/min
0 /l/min
NO
d Current/mA /dE
Potential/V
0.40
0.45
0.50
0.55
0.60
0.65

FIG. 39

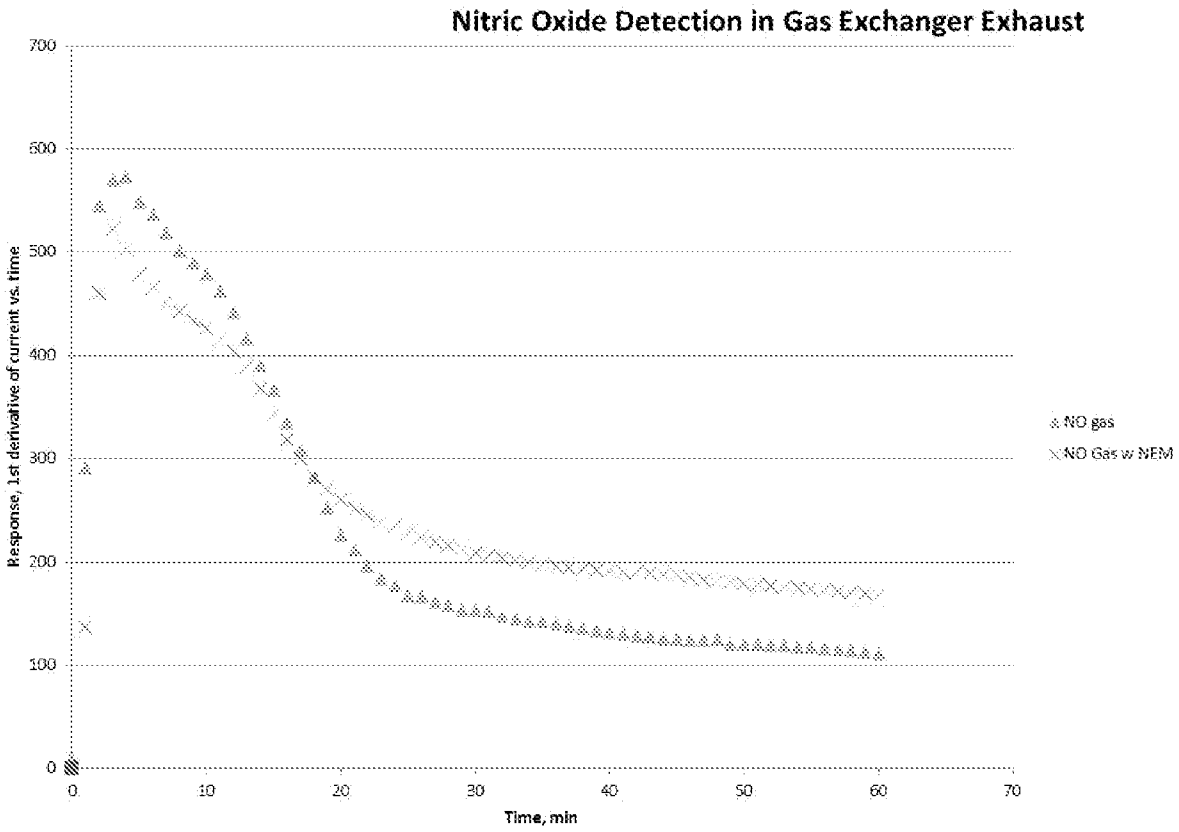
Nitric Oxide Detection in Gas Exchanger Exhaust
Response, 1st derivative of current vs. time
Time, min
NO gas
NO Gas w NEM
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70

FIG. 40

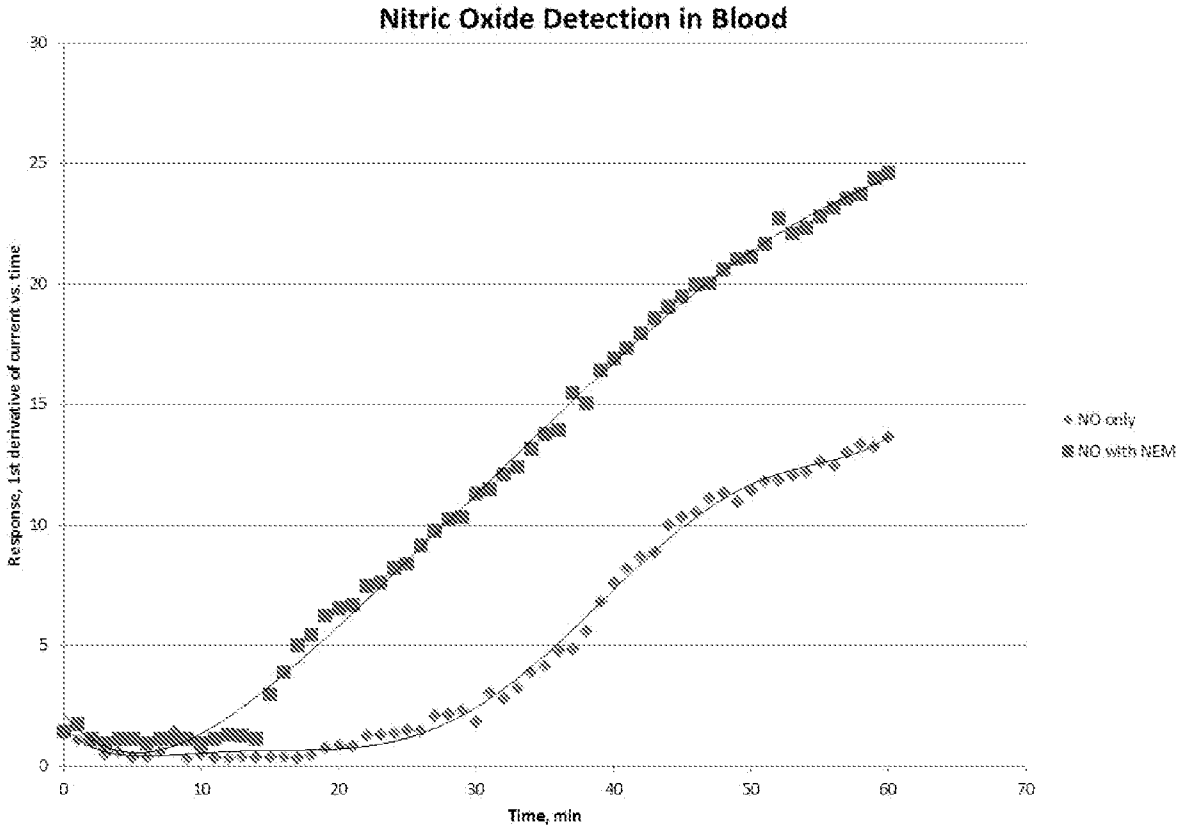
Nitric Oxide Detection in Blood
Response, 1st derivative of current vs. time
Time, min
NO only
NO with NEM
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70

FIG. 41

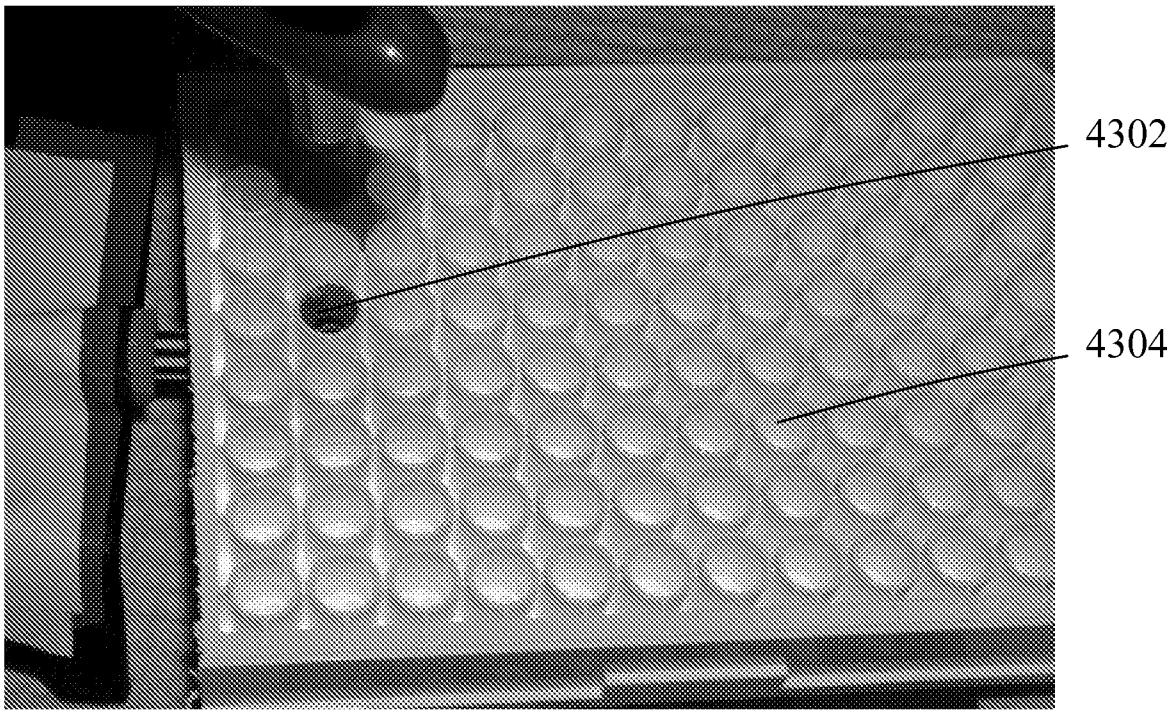

FIG. 42

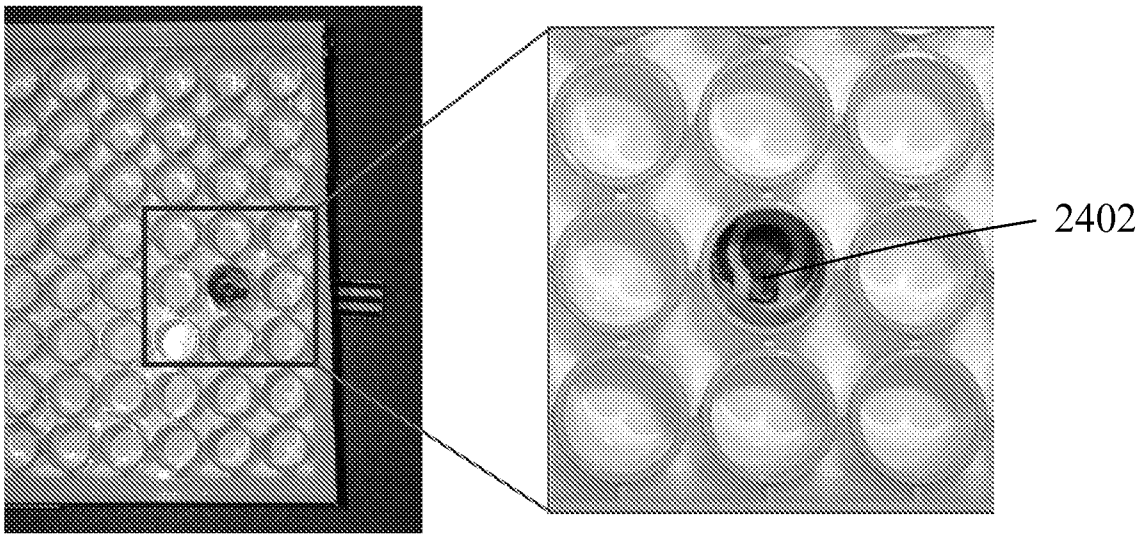

FIG. 43

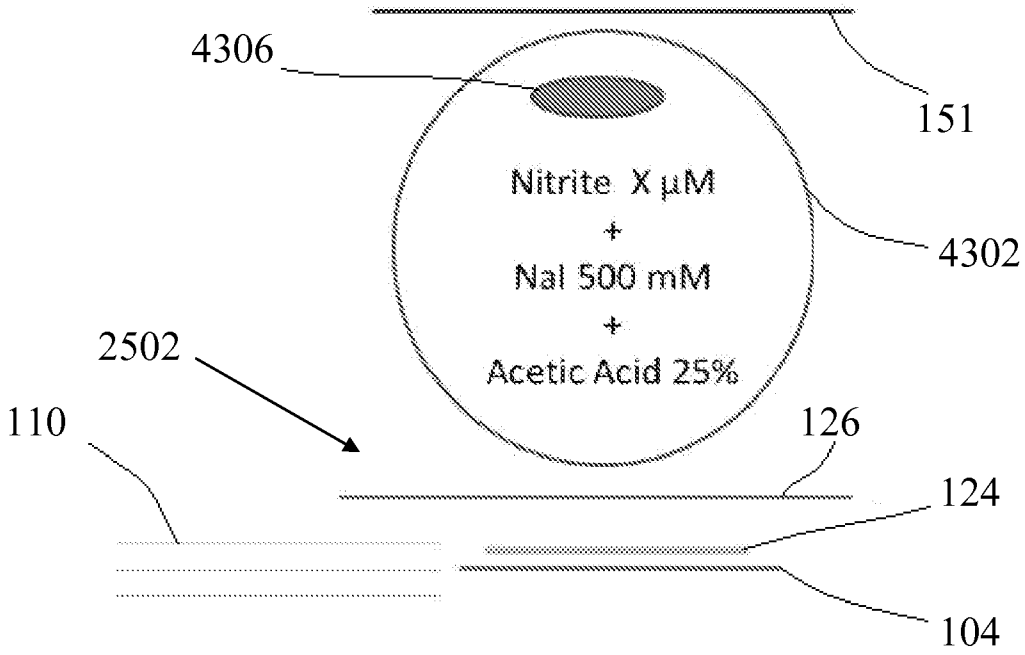
4306
151
Nitrite X µM
+
NaI 500 mM
+
Acetic Acid 25%
4302
2502
110
126
124
104

FIG. 44

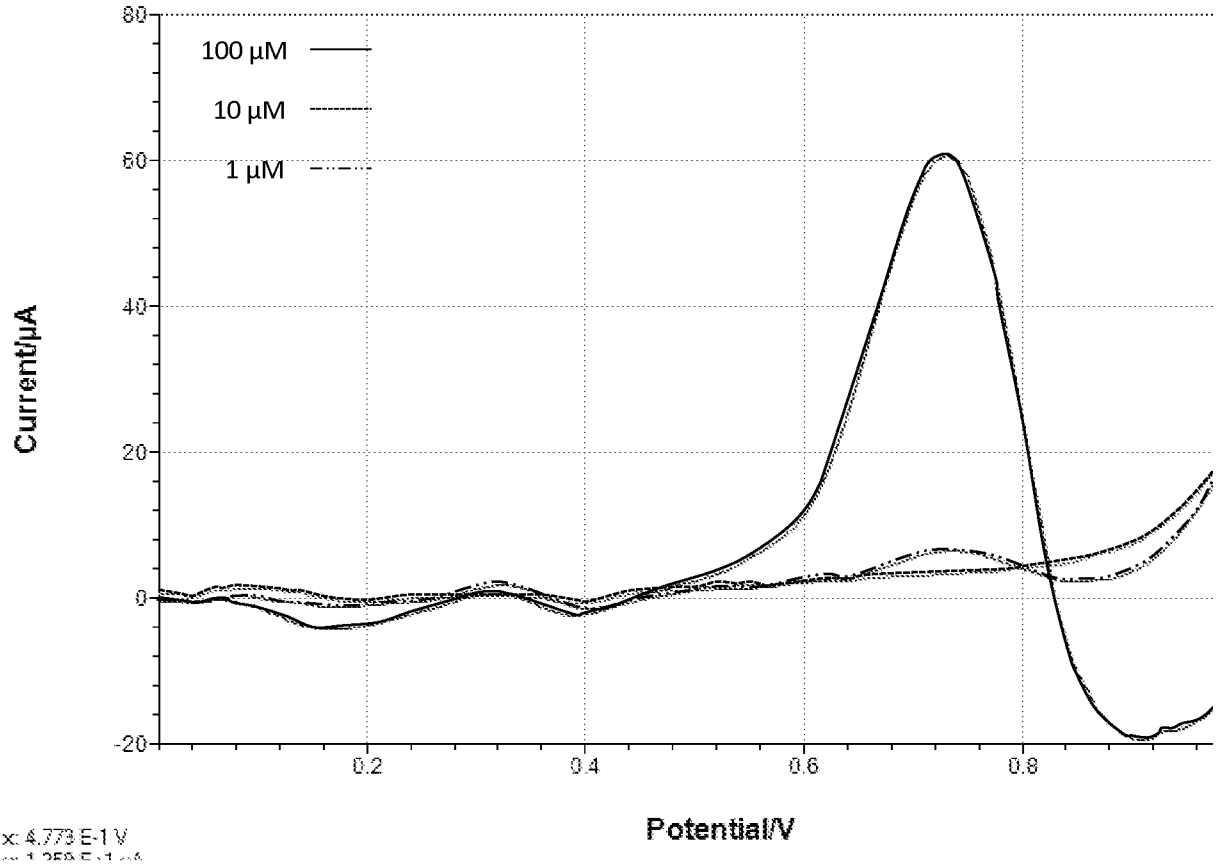
100 µM
10 µM
1 µM
80
60
40
20
0
-20
0.2
0.4
0.6
0.8
Current/µA
Potential/V
x: 4.773 E-1 V

FIG. 45

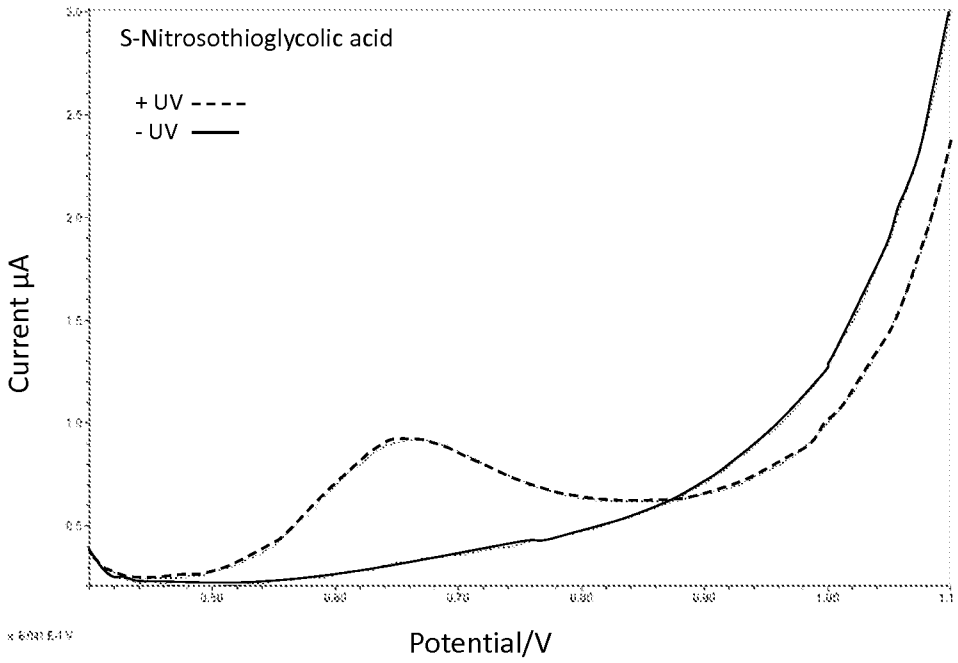

FIG. 46a

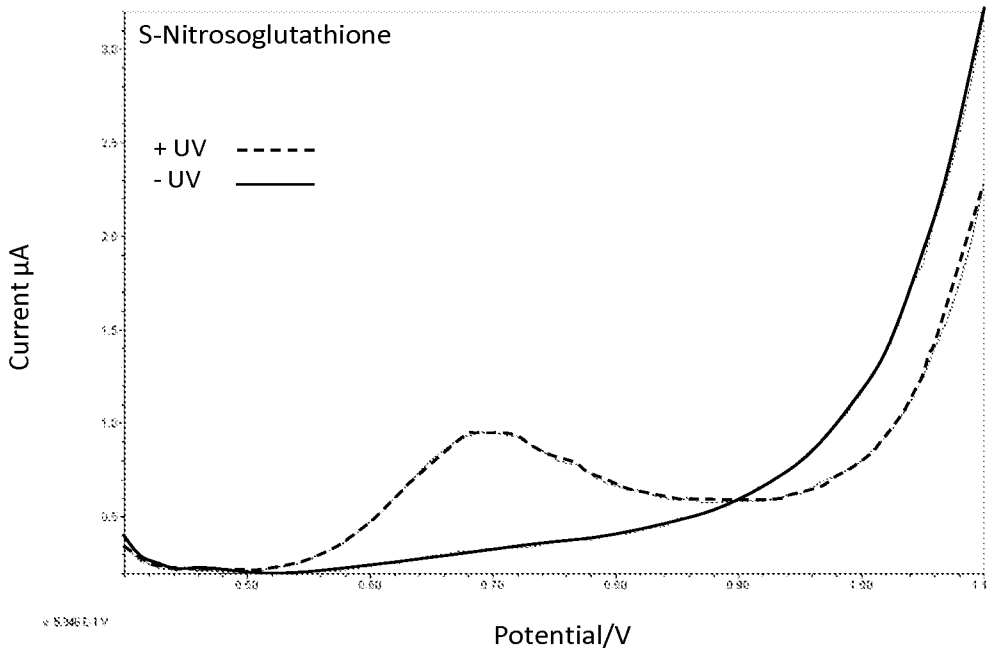

FIG. 46b

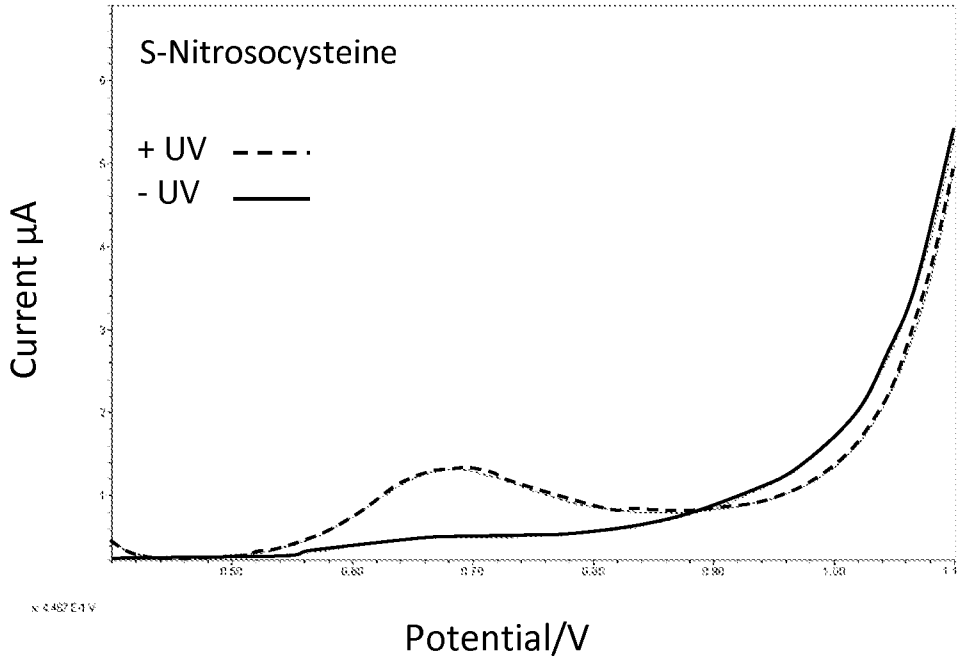

FIG. 46c

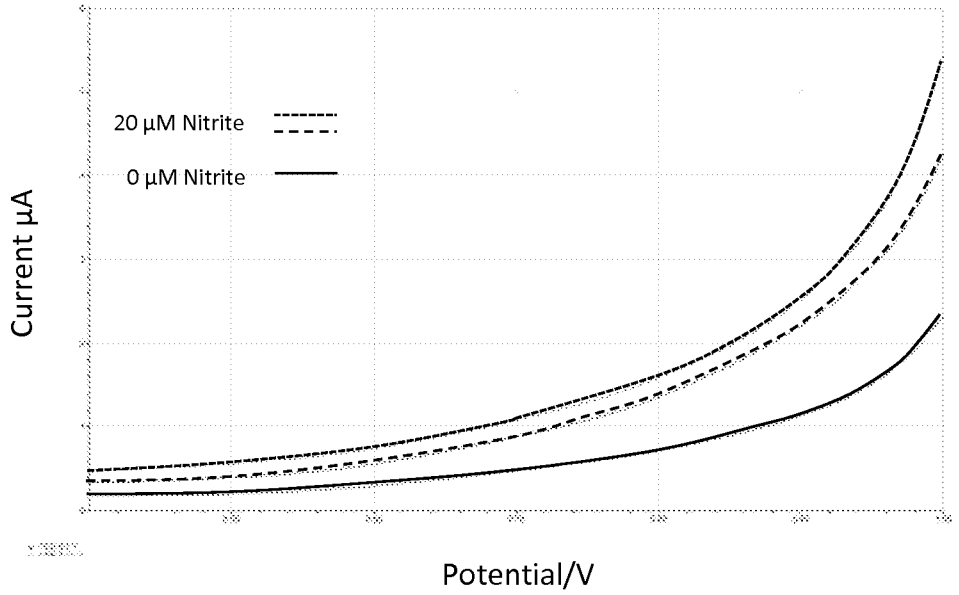

FIG.47a

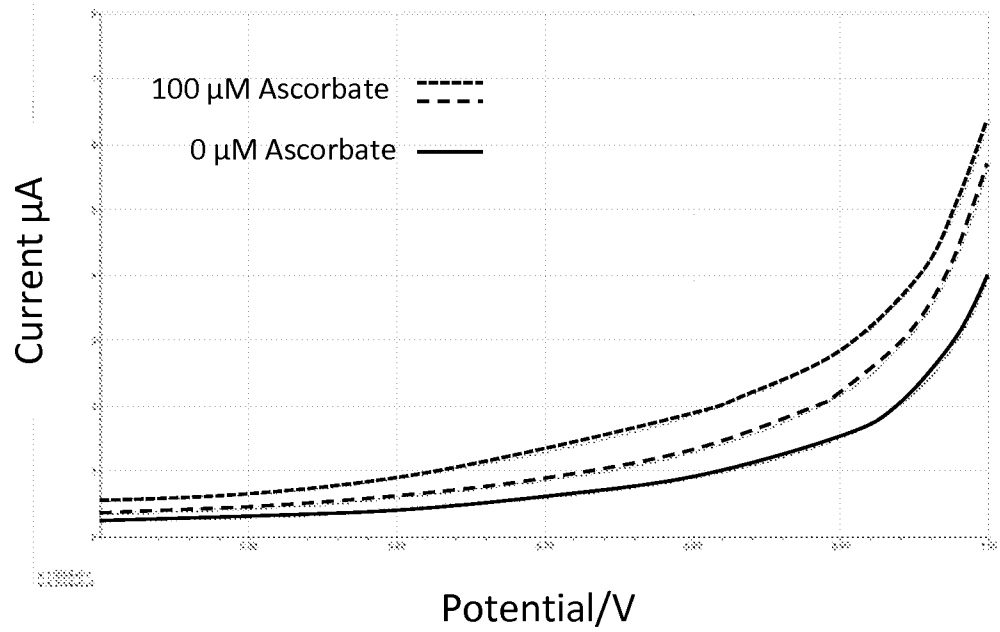

FIG. 47b

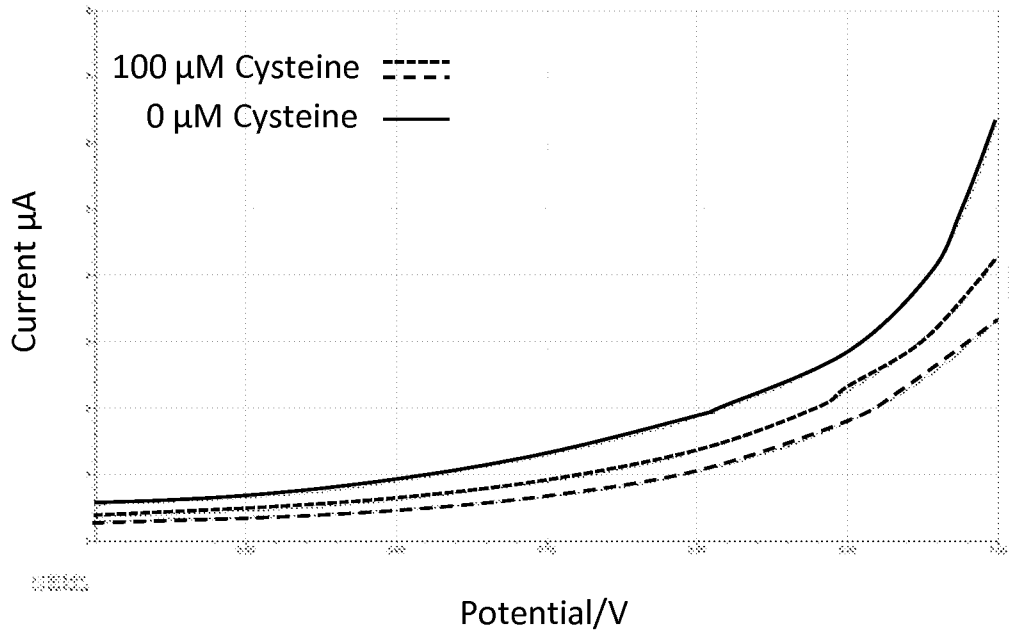

FIG. 47c

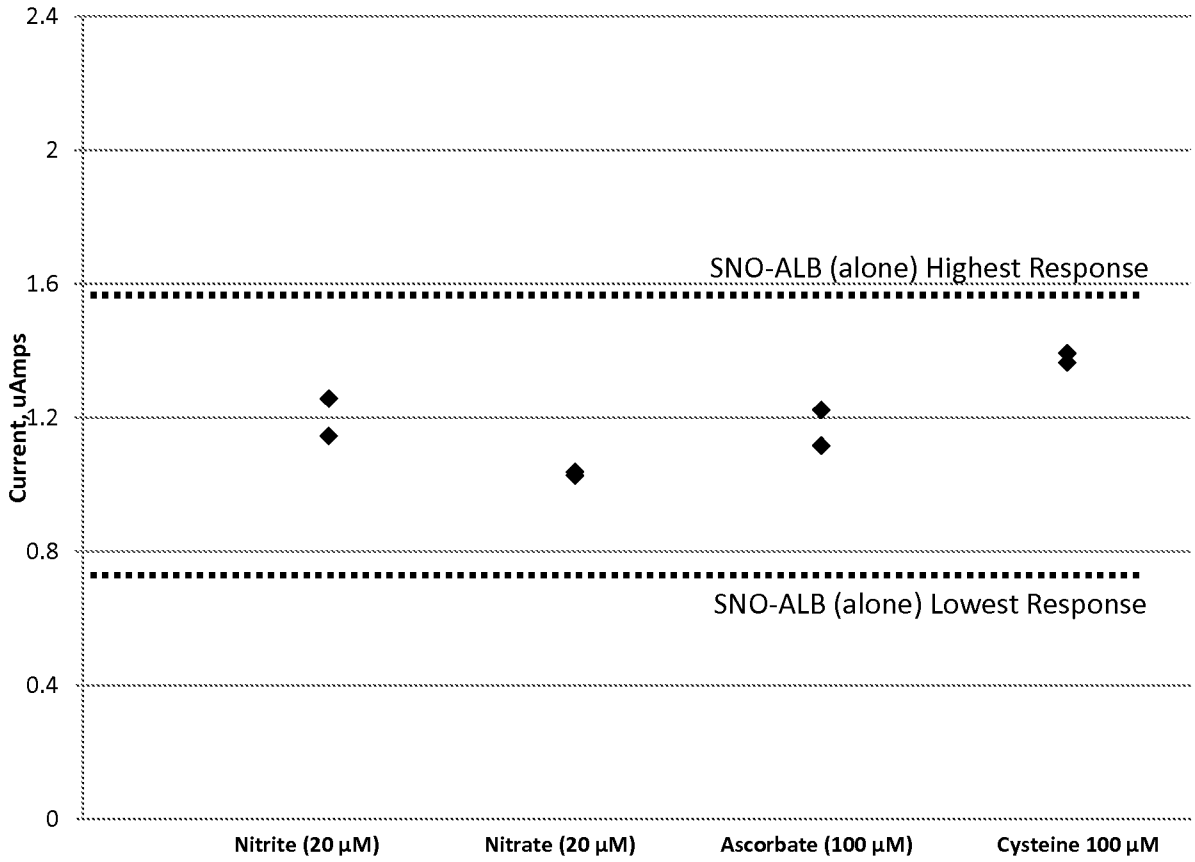
2.4
2
1.6
1.2
0.8
0.4
0
Current, uAmps
SNO-ALB (alone) Highest Response
SNO-ALB (alone) Lowest Response
Nitrite (20 µM)
Nitrate (20 µM)
Ascorbate (100 µM)
Cysteine 100 µM

FIG. 48

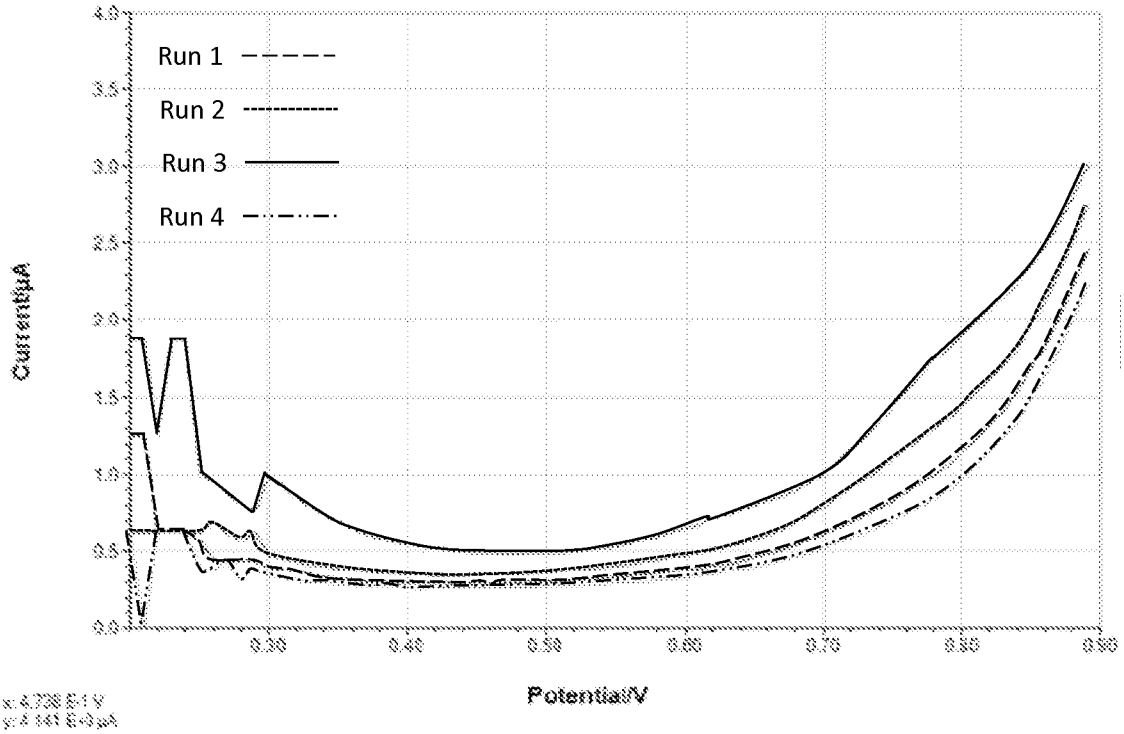

FIG. 49

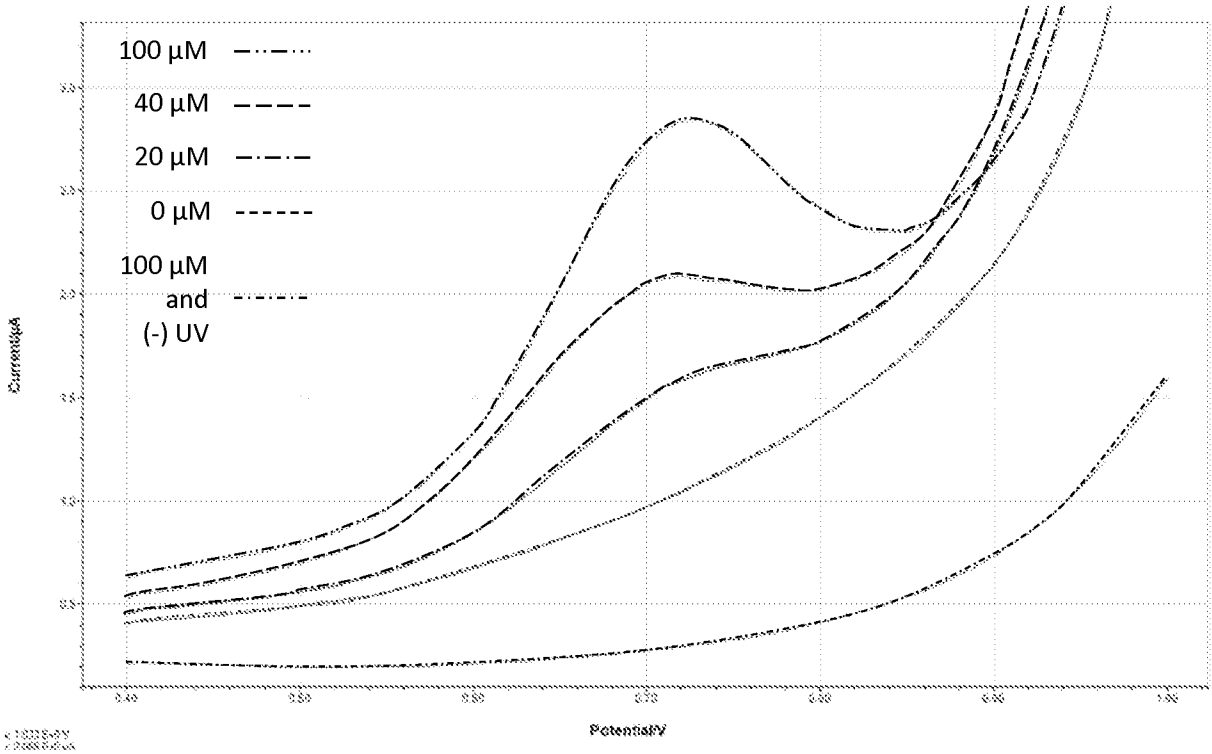

FIG. 50

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/031516

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/497 G01N33/543 G01N27/404
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 565 075 A (DAVIS BRIAN K [US] ET AL) 15 October 1996 (1996-10-15) the whole document abstract column 2, line 6 - line 45 column 3, line 24 - line 29 column 4, line 11 - line 14 column 4, line 48 - line 57; figure 1 column 6, line 10 - line 13 claims 1-3 ----- -/--	2,3, 9-15,76, 77,85-90

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2012	11/09/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gall, Anne-Laure

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/031516

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BEDIOUI FETHI ET AL: "Electrochemical nitric oxide sensors for biological samples: Principle, selected examples and applications", ELECTROANALYSIS, VHC PUBLISHERS, INC, US, vol. 15, no. 1, 1 January 2003 (2003-01-01), pages 5-18, XP002628478, ISSN: 1040-0397, DOI: 10.1002/ELAN.200390006 the whole document abstract page 11, left-hand column, paragraph 2 - right-hand column, paragraph 1; figure 4 table 2 paragraph bridging pages 13-14 page 16, right-hand column, paragraph 1 - page 17, left-hand column, paragraph 1 -----	2,3, 9-15,76, 77,85-90
Y	BENJAMIN J. PRIVETT ET AL: "Electrochemical nitric oxide sensors for physiological measurements", CHEMICAL SOCIETY REVIEWS, vol. 39, no. 6, 1 January 2010 (2010-01-01), pages 1925-1935, XP055028915, ISSN: 0306-0012, DOI: 10.1039/b701906h the whole document abstract page 1926, right-hand column, paragraph 2 - page 1927, right-hand column, paragraph 1 figure 1 page 1928, right-hand column table 2 page 1930, left-hand column, paragraph 2 page 1930, left-hand column, paragraph 3 - page 1931, right-hand column, paragraph 1 table 3 page 1932, left-hand column, last paragraph - page 1933, right-hand column, last paragraph page 1934, left-hand column, paragraph 1 ----- -/--	2,3, 9-15,76, 77,85-90

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/031516

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MISERERE S ET AL: "Biocompatible carbon-based screen-printed electrodes for the electrochemical detection of nitric oxide", ELECTROCHEMISTRY COMMUNICATIONS, ELSEVIER, AMSTERDAM, NL, vol. 8, no. 2, 1 February 2006 (2006-02-01), pages 238-244, XP028041420, ISSN: 1388-2481, DOI: 10.1016/J.ELECOM.2005.11.016 [retrieved on 2006-02-01]	1,4-8, 75,78-84
Y	the whole document abstract page 239, right-hand column, paragraph 2 - paragraph 3; figure 1 page 240, right-hand column, last paragraph - page 241, right-hand column, paragraph 2 page 241, right-hand column, paragraph 3 page 242, right-hand column, last paragraph - page 243, right-hand column, paragraph 1 page 244, left-hand column, last paragraph	2,3, 9-15,76, 77,85-90

X	ARAVINDALOCHANAN K ET AL: "Simulation and design of a nitric oxide sensor array for cell cultures", SENSORS, 2009 IEEE, IEEE, PISCATAWAY, NJ, USA, 25 October 2009 (2009-10-25), pages 325-328, XP031618557, ISBN: 978-1-4244-4548-6	1,4-8
Y	the whole document abstract page 327, right-hand column, last paragraph - left-hand column, last paragraph; figure 7	2,3, 9-15,76, 77,85-90

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/031516

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-15, 75-90

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/031516

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5565075 A	15-10-1996	AU 5720496 A	24-12-1996
		EP 0838030 A1	29-04-1998
		US 5565075 A	15-10-1996
		WO 9639626 A1	12-12-1996

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15, 75-90

   electrochemical sensors for the detection/quantification of nitric oxide (NO) characterized by a substrate, an electrode set and an ionomer coating on at least a portion of said electrode set and methods to manufacture said sensors;

   ---

2. claims: 16-52

   methods for measuring amounts of NO characterized by the use of a sensor comprising a gas permeable membrane, an absorbent pad and an electrode set;

   ---

3. claims: 53-59

   methods for measuring amounts of S-nitrosothiols (RSNO) characterized by the use of a stabilizing agent and by the use of a sensor comprising an absorbent pad and an electrode set;

   ---

4. claims: 60-63

   methods for measuring amounts of RSNO characterized by an anti-albumin immuno-affinity isolation step and by the use of a sensor comprising an absorbent pad and an electrode set;

   ---

5. claims: 64-74

   methods for measuring amounts of RSNO characterized by an anti-albumin immuno-affinity isolation step and by the use of a sensor comprising a gas permeable membrane and an electrode set.
